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Integrin $\alpha_3\beta_1$ inhibits directional migration and wound re-epithelialization in the skin

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ABSTRACT
Re-epithelialization after skin wounding requires both migration and hyperproliferation of keratinocytes. During migration over the provisional matrix, laminin-332 is deposited. To investigate the function of the laminin-332 binding integrin α3β1 in wound re-epithelialization, we generated Itgα3flox/flox; K14-Cre mice lacking the α3 subunit specifically in the basal layer of the epidermis. These mice are viable but display several skin defects including local inflammation, hair loss, basement membrane duplication and microblistering at the dermal-epidermal junction, whereas hemidesmosome assembly and keratinocyte differentiation are not impaired. Wound healing is faster in the absence of α3β1 while proliferation, the distribution of other integrins and the deposition of basement membrane proteins in the wound bed are unaltered. In vitro, cell spreading is rescued by increased surface expression of α6β1 in the absence of α3. The α3-deficient keratinocytes migrate with an increased velocity and persistence, whereas proliferation, growth factor signaling, hemidesmosome assembly, and laminin-332 deposition are normal. We suggest that α3β1 delays keratinocyte migration during wound re-epithelialization, by binding the laminin-332 that is newly deposited into the wound bed.

INTRODUCTION
The skin is composed of a layer of stratified squamous epithelium (the epidermis), and an underlying layer of connective tissue (the dermis), which are separated by a basement membrane (BM) consisting primarily of laminins and collagens (1). Attachment of basal epidermal keratinocytes to the BM is mediated by members of the integrin family. The integrin repertoire in basal keratinocytes is restricted to α2β1, α3β1 and α6β4, while de novo expression of integrins α5β1, αvβ5, αvβ6, and probably αvβ1, is induced upon wounding (2). Whereas α2β1 mediates attachment to collagens, adhesion to the main BM component laminin (Ln)-332 (previously named Ln-5, nicein, kalinin, or epiligrin) is established by the predominant epidermal integrins α3β1 and α6β4 (3-5). Integrin α3β1 links the extracellular matrix (ECM) to the actin cytoskeleton and is localized at the basolateral membrane. It is often found in clusters surrounding HDs (HDs), and in focal adhesions in cultured cells (6,7). In contrast, integrin α6β4 associates with the intermediate filament system, and its distribution is restricted to the basal surface of keratinocytes. There, it governs the assembly of hemidesmosomes (HDs), which consist further of the cytoskeletal linker proteins plectin and bullous pemphigoid (BP) antigen 230, and the transmembrane proteins BP180 and CD151 (8). Ablation in mice of the gene encoding α6 or β4 impairs HD formation and results in severe epidermal blistering and perinatal death, demonstrating the importance of α6β4 in the maintenance of skin integrity at the dermal-epidermal junction (DEJ; 9-11). Similarly, humans carrying a mutation in either of these genes or in the genes encoding the α3, β3, or γ2 chains of Ln-332 suffer from a skin blistering disease referred to as junctional epidermolysis bullosa (12-14).
Deletion of the gene encoding the integrin β1 subunit is lethal in the early embryo (15,16). Evidence for the function of β1 in skin stems from conditional knockout mouse models in which β1 ablation is restricted to the basal epidermal keratinocytes, resulting in skin blistering at the DEJ, a reduced number of HDs, failure of BM assembly, impaired invagination of hair follicles and eventual hair loss (17,18). This suggests that β1 integrins are required for hair growth, BM assembly, and HD formation. Furthermore, epidermal migration during wound healing is impaired in the absence of β1 (19). Since α2-null mice do not have any obvious skin defects, neither under steady-state conditions nor during wound healing (20,21), the wound healing defect caused by β1 deletion is possibly due to loss-of-function of α5β1 or αvβ1, whose expression is induced upon wounding, or loss-of-function of α3β1, which most likely explains the other features of the phenotype as well. Indeed, α3-null mice are born with a disorganized BM membrane and micob blistering at the DEJ, although the skin phenotype is much less severe than that observed in the absence of α6, β1, or β4 (22). However, these mice die neonatally as a consequence of defective kidney and lung organogenesis (23), complicating the study of α3β1 in adult skin and wound healing. In vitro evidence of the role of α3β1 in keratinocyte migration is controversial; whereas some studies have suggested that α3β1 promotes keratinocyte migration (24,25), the opposite has also been reported (26-29).

To address the role of α3β1 in adult skin homeostasis and wound healing, we have generated epidermis-specific α3 knockout mice by crossing Itga3^{fl/fl} mice with mice expressing Cre-recombinase under the control of the K14 promoter. The resulting Itga3^{fl/fl}; K14-Cre mice are viable but display several skin abnormalities including local inflammation, micro blistering at the DEJ, and BM duplication, whereas HD assembly and keratinocyte differentiation are normal. Skin wounds close faster in these animals whereas proliferation rates are similar, suggesting accelerated keratinocyte migration in the absence of α3. This is confirmed by in vitro observations; α3-deficient keratinocytes migrate with increased velocity and persistence compared to α3-expressing cells. Taken together, these results show that the integrin α3β1 is required for the maintenance of dermal-epidermal integrity but not for keratinocyte proliferation, differentiation, or HD formation. Furthermore, α3β1 delays keratinocyte migration during wound re-epithelialization, by binding the Ln-332 that is newly deposited into the wound bed.

RESULTS

Generation and characterization of epidermis-specific Itga3 knockout mice
Integrin α3-null mice die shortly after birth, displaying aberrant kidney and lung organogenesis and moderate skin blistering at the DEJ (22,23). To address the role of the α3 subunit in adult skin, specifically in adhesion and wound healing, we created epidermis-specific α3 knockout mice by crossing mice homozygous for a floxed
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Itga3 allele (Itga3<sup>lox/lox</sup>) (30) with mice expressing Cre-recombinase under the control of the K14 promoter (Figure S1). Deletion of the α3 subunit from the epidermis had no obvious effects on overall skin structure; the proliferative layer of the epidermis was typically 2-3 cell layers thick, and keratinization and HD assembly seemed to be normal (Figure 1A). However, microblistering at the DEJ was occasionally observed, which was associated with epidermal hyperthickening, and

Figure 1 Skin phenotype of Itga3<sup>lox/lox</sup>; K14-Cre mice. (A) Upper panel, cryosections from back-skin of neonatal Itga3<sup>lox/lox</sup> and Itga3<sup>lox/lox</sup>; K14-Cre mice were stained with antibodies directed against the α3 subunit and Ln-332. Bar, 100 μm. Middle panel, haematoxylin/eosin (H/E) stainings of back-skin of 4-month old Itga3<sup>lox/lox</sup> and Itga3<sup>lox/lox</sup>; K14-Cre mice. Bar, 200 μm. Lower panels, EM pictures and schematic representations of back-skin of 1-year old Itga3<sup>lox/lox</sup> and Itga3<sup>lox/lox</sup>; K14-Cre mice, showing the BM and HDs (indicated by arrowheads). Bar, 200 nm. (B) Top panel, H/E staining of back-skin of a 1-year old Itga3<sup>lox/lox</sup>; K14-Cre mouse. Arrowheads indicate microblisters at the DEJ. Bar, 500 μm. Lower panels, cryosections of a region containing microblisters were stained with antibodies against Ln-332 and β4. Arrowheads point toward blisters. Bar, 50 μm. (C) Ultrastructural analysis and schematic representation of back-skin of a 1-year old Itga3<sup>lox/lox</sup>; K14-Cre mouse, showing BM duplication. The 2 BMs are marked by the thick and dotted lines, and collagen fibers extending throughout the 2 BMs are indicated. Bar, 200 nm. (D) Regions of inflammation in a 4-month old Itga3<sup>lox/lox</sup>; K14-Cre mouse are denoted by arrows. Back skin sections were stained with an antibody against CD3. Arrows point to infiltrated lymphocytes. (E) Alopecia in a 1-year old Itga3<sup>lox/lox</sup>; K5-Cre mouse (right), as compared to an Itga3<sup>lox/lox</sup> mouse of the same age. BM, basement membrane; Col, collagen; D, dermis; E, epidermis.
the presence of an inflammatory infiltrate (Figure 1B). Ln-332 was distributed at both the roof and floor of the blisters, while localization of β4 was restricted to the roof (Figure 1B). Ultrastructural analysis revealed duplication of the BM (Figure 1C). Inflammation occurred frequently around 3-4 months after birth, especially at the ears and around the eyes (Figure 1D). The inflammatory infiltrate was mainly observed in regions where the epidermis was abnormally thick (Figure 1D). Around the same time, these mice developed alopecia (local hair loss), which progressed with age (Figure 1E). Differentiation in the epidermis was essentially normal, as assessed by staining of cryosections for keratins 10 and 14, filaggrin, and involucrin (Figure 2, and data not shown). Similar results were obtained when Itga3flx/flx mice were crossed with mice expressing Cre-recombinase under the control of the K5 promoter (data not shown). Summarizing, the targeted deletion of Itga3 in mouse epidermis causes several skin abnormalities associated with reduced adhesion of the epidermis to the dermis.

Wound closure is accelerated in α3-null skin due to increased migration but not proliferation
To investigate the role of integrin α3β1 in wound healing, 2 full-thickness excision wounds were inflicted on either side of the dorsal midline in 4-months old Itga3flx/flx and Itga3flx/flx; K14-Cre mice. Re-epithelialization of such wounds occurs by migration and hyperproliferation of keratinocytes from outside the wound bed over the dermis and granulation tissue (Figure 3A). In the tip of the advancing epidermis, integrin α3β1 is strongly upregulated and Ln-332 is deposited, whereas deposition of other ECM proteins such as Ln-511, Collagen (Col)-IV, and Nidogen (Nd) occurs behind the tip, to restore the damaged BM (Figure S2A). Upregulation and distribution of β1 in the epidermal tip appeared normal in Itga3flx/flx; K14-Cre mice, suggesting that another α-subunit binds β1

Figure 2 Deletion of integrin α3 in the epidermis does not inhibit keratinocyte differentiation. Skin cryosections from neonatal Itga3flx/flx and Itga3flx/flx; K14-Cre mice were stained with antibodies directed against keratin-14, filaggrin, or involucrin, and counterstained with Ln-332 and DAPI to visualize the BM and nuclei, respectively. Bar, 50 μm.
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Figure 3 Wound closure is accelerated in the absence of αβ1. Full-thickness wounds were generated on either side of the dorsal midline in Itga3\(^{flox/flox}\) or Itga3\(^{flox/flox}\); K14-Cre mice, and excised 3 or 7 days after injury. (A) Schematic representation of a wound. D, dermis; E, epidermis; Es, eschar; F, fat tissue; G, granulation tissue. (B) H/E stainings depicting wound closure 7 days after wounding in Itga3\(^{flox/flox}\) (top) and Itga3\(^{flox/flox}\); K14-Cre mice (bottom). Arrows mark the edges of the migrating epidermis. The number of closed wounds after 7 days of migration is represented in the bar graph. Depicted are the means ± SEM of at least 40 wounds pooled from 4 independent experiments (* p < 0.05). Bar, 500 \(\mu\)m. (C) The distance covered by the migrating epidermis (indicated by the dotted line) was quantified 3 days after wounding in Itga3\(^{flox/flox}\) (top) and Itga3\(^{flox/flox}\); K14-Cre mice (bottom). The graph indicates the means ± SEM of at least 35 wounds pooled from 3 experiments (* p < 0.05). Bar, 250 \(\mu\)m. (D) Proliferation in the re-epithelializing wounds was assessed by injecting BrdU 2 or 4 days after wounding, and determining the ratio of BrdU(+) cells over the total number of cells using ImageJ. Depicted are the means ± SEM of at least 30 images. Bar, 150 \(\mu\)m.
in the absence of \(\alpha_3\) in the leading keratinocytes (Figure S2B). Furthermore, the localization of integrins \(\alpha_5\), \(\alpha_6\), and \(\beta_4\) as well as the BM proteins Col-IV, Ln-511, Nd, and Ln-332 was comparable to that in \(\text{Itga3}^{\text{flox/flox}}\) mice, suggesting that the distribution of other integrins and the deposition of BM proteins are not affected by the deletion of \(\alpha_3\) (Figure S2B).

We next determined the rate of wound closure on H/E stained paraffin sections. Wound healing was not impaired in \(\text{Itga3}^{\text{flox/flox}}\); K14-Cre mice but was in fact accelerated compared to that in \(\text{Itga3}^{\text{flox/flox}}\) skin, as determined both by the number of wounds that had completely closed after a week, or by the extent of re-epithelialization at earlier time points (Figures 3B,C). To assess whether this was due to increased proliferation, BrdU was injected intraperitoneally at several timepoints after wounding, and skin sections were stained to detect BrdU incorporation. There was no significant difference in the number of proliferating cells in the advancing epidermis of \(\text{Itga3}^{\text{flox/flox}}\) versus \(\text{Itga3}^{\text{flox/flox}}\); K14-Cre mice, indicating that \(\alpha_3\) does not affect proliferation during wound healing (Figure 3D). Taken together, these results suggest that deletion of integrin \(\alpha_3\) in skin promotes wound healing by accelerating keratinocyte migration.

**In vitro adhesion to Ln-332 is mediated by \(\alpha_6\) integrins in the absence of \(\alpha_3\)**

To confirm the migration data in vitro and to investigate the underlying mechanism, we isolated keratinocytes from newborn \(\text{Itga3}^{\text{flox/flox}}\) mice and cultured them on Col-I (3 \(\mu\)g/ml). Several spontaneously immortalized clones were obtained, which we named mouse keratinocytes (MK)\(\alpha_3(+)\). The clones were unable to grow in Ca\(^{2+}\)-rich medium and did not give rise to tumors when injected subcutaneously into nude mice (10\(^7\) cells/injection, 8 injections in 2 independent experiments), indicating that they are not transformed. To generate \(\alpha_3\)-null cells MK\(\alpha_3(-)\), \(\alpha_3\) was efficiently deleted by adenoviral delivery of Cre-recombinase (Figures 4A, S3A), thus the \(\alpha_3\)-knockout cells were derived directly from the same clones. There was no significant difference in proliferation rates between MK\(\alpha_3(+)\) and MK\(\alpha_3(-)\) cells (Figure S3B). Moreover, both MK\(\alpha_3(+)\) and MK\(\alpha_3(-)\) cells responded similarly to transfer to Ca\(^{2+}\)-rich medium, by initiating the formation of cell-cell contacts such as adherens junctions and tight junctions, as suggested by zonula occludens (ZO)-1, occludin, \(\beta\)-catenin, and E-cadherin stainings (Figure S3C), and the assembly of HD-like structures (Figure S3D). These results are in line with the in vivo observations and suggest that the integrin \(\alpha_3\beta_1\) is not per se required for the formation of cell-cell contacts, HD assembly, or proliferation. To determine whether \(\alpha_3\) deletion affected the expression of other integrins, we determined integrin cell surface levels by flow cytometry. Expression of \(\beta_1\) on the cell surface was downregulated in the knockout cells whereas the expression of \(\alpha_5\), \(\alpha_v\), \(\alpha_2\), \(\alpha_6\), and \(\beta_4\) was not significantly different (Figure 4B). To investigate whether cell adhesion was compromised, we performed adhesion assays on Col-I or Ln-332. Adhesion of MK\(\alpha_3(-)\) cells to Ln-332 was slightly reduced (~70% of control cells) but decreased dramatically in the presence of the \(\alpha_6\)-blocking antibody GoH3, indicating that adhesion
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to Ln-332 in the absence of α3 is mediated by α6 integrins (Figure 4C). Adhesion to Col-1 was similar for MKα3(+) and MKα3(-) cells, and was not blocked by GoH3 in either cell line, as expected (Figure 4C). Immunoprecipitation experiments revealed increased association of α6 with β1 in the knockout cells, whereas α6β1 levels in MKα3(+) cells were hardly detectable (Figure 4D). Together, these data show that keratinocyte adhesion to Ln-332 is rescued by α6 integrins, and that surface expression of integrin α6β1 is upregulated in the absence of α3.

Cell spreading on endogenous Ln-332 is mediated by α6β1 in the absence of α3

Next, we analyzed whether cell spreading was affected by the deletion of α3. Consistent with the adhesion data, cell spreading on Ln-332 was slightly reduced in MKα3(-) cells compared to control cells,
and addition of the α6-blocking antibody GoH3 decreased cell spreading significantly in the MKα3(-) but not MKα3(+) cells (Figure 5A). Surprisingly, the same results were obtained on Col-I, suggesting that cell spreading of MKα3(-) cells on Col-I is also mediated by α6 integrins. We assume that Col-I is used for initial adhesion and spreading but that spreading on the patch of Ln-332 that is deposited over time underneath the cell is maintained by Ln-332-binding integrins, i.e. α3β1 in the control cells and α6β1 in the absence of α3. To exclude that Ln-332 deposition itself was affected by the loss of α3, we next compared Ln-332 deposition by Western blotting and immunofluorescence. Ln-332 deposition by knockout cells was not impaired over time (Figure 6A), and the pattern of deposition was similar to that of MKα3(+) cells (Figure 6B). However, trails of Ln-332 that were left behind by migrating MKα3(-) cells were strikingly longer, suggesting increased motility and migration in the absence of α3 (Figure 6C).

**Figure 5 Integrin α6β1 mediates cell spreading on endogenous Ln-332 in the absence of α3β1.** MKα3(+) and MKα3(-) cells were seeded on Ln-332 (A) or Col-1 (B), allowed to spread, and then incubated with α6-blocking antibody GoH3 (10 μg/ml). After 3 hrs, the number of spread cells was scored and expressed as a percentage of the total number of cells. In each independent experiment, approximately 500 cells were counted per condition. The graphs depict the averages of 3 experiments (* p < 0.05, *** p < 0.0005). Bar, 50 μm.
are not due to adaptation in culture, we also performed adhesion and cell spreading assays in primary, non-immortalized keratinocytes isolated from neonatal \textit{Itga3}^{\text{flox/flox}} and \textit{Itga3}^{\text{flox/flox}; K14-Cre} mice. These assays yielded essentially the same results (Figure S4). In conclusion, these data show that the deletion of \(\alpha 3\) in keratinocytes does not affect the deposition of endogenous Ln-332, and that cell spreading over endogenous Ln-332 is maintained by upregulation of integrin \(\alpha 6\beta 1\).

**Deletion of \(\alpha 3\) enhances velocity and directionality of keratinocyte migration**

We next mimicked wound healing in an \textit{in vitro} assay. Upon wounding, quiescent keratinocytes become activated by the release of growth factors such as epidermal growth factor (EGF), inducing hyper-proliferation and migration over exposed dermal collagens. Therefore, MK\(\alpha 3(+)\) and MK\(\alpha 3(-)\) cells were grown to confluence on Col-I, deprived of supplements and growth factors overnight, and then scratched with the tip of a pipette, after which they were allowed to migrate into the artificial wound in the presence of EGF (5 ng/ml). Proliferation was inhibited with mitomycin C. Consistent with the \textit{in vivo} observations, the MK\(\alpha 3(-)\) cells migrated significantly faster into the denuded area than the MK\(\alpha 3(+)\) cells (Figure 7A). This was probably not due to altered growth factor signaling, as stimulation with EGF after growth factor depletion induced similar activation of the ERK pathway (Figure 7B). To rule

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**Figure 6 Ln-332 synthesis and deposition are not affected by the deletion of integrin \(\alpha 3\).** (A) MK\(\alpha 3(+)\) and MK\(\alpha 3(-)\) cells were seeded on Col-I and detached with EDTA at the indicated time points thereafter. The ECM was then dissolved in sample buffer, subjected to SDS-PAGE, and the \(\gamma 2\) chain of Ln-332 was detected by Western blotting. (B) Immunofluorescence images demonstrating patches of deposited Ln-332 in spread MK\(\alpha 3(+)\) cells (left panel) and MK\(\alpha 3(-)\) cells (right panel). Bar, 10 \(\mu m\). (C) Low-magnification immunofluorescence images demonstrating Ln-332 trails left behind by MK\(\alpha 3(+)\) cells (left), and MK\(\alpha 3(-)\) cells (right) migrating over Col-I. Bar, 10 \(\mu m\).
Figure 7 Loss of $\alpha_3$ enhances directionality and velocity of keratinocyte migration. (A) Confluent MK$\alpha_3$(+) and MK$\alpha_3$(-) were deprived of growth factors, incubated for 2 hrs with mitomycin C (10 $\mu$g/ml), and scratched with the tip of a pipette prior to EGF stimulation. The black bars indicate the wound edges at $t = 0$. Scale bar, 100 $\mu$m. Wound areas were determined using Image J, and the ratio of the wound area after overnight migration over the wound area at $t = 0$ was calculated and expressed in the bar graph. Values shown represent the means ± SEM of 3 independent experiments ($** p < 0.0005$). (B) EGF-induced phosphorylation of ERK-1/2 was monitored by Western blotting in lysates of MK$\alpha_3$(+) and MK$\alpha_3$(-) cells, that were deprived of growth factors prior to EGF stimulation for the indicated time points. (C) Cells were sparsely seeded on Col-I and monitored in time-lapse recordings during 16 hrs. An image was captured every 5 min. Cell tracks were then determined using ImageJ. The migration plots indicate tracks from 10 individual cells out of 4 independent experiments. To quantify average velocity and directionality ($D/T$ ratio), data from 4 independent experiments were pooled. The graphs represent the means ± SEM from ~50 cells ($p < 0.05$, $** p < 0.005$, $*** p < 0.0005$). To determine stable polarization, cells were sparsely seeded on Col-I and monitored by time-lapse recordings. Images were captured every 5 min. Cells were considered stably polarized when maintaining a leading lamellipodium during 1 hr. The number of polarized cells was counted and expressed as a percentage of the total number of cells. The graph shows the means ± SEM from 250 cells pooled from 4 independent experiments ($p < 0.05$). (D) Cells were sparsely seeded on Ln-332 or Col-I, serum-starved, and then stimulated with 5 ng/ml EGF at the indicated time points. An image was captured every 5 min. Cell tracks were determined using Matlab (Mathworks), and average velocity was plotted over time. The graphs depict the average velocity of ~50 cells from a representative experiment.
out that the observed effects were due to differences in cell-cell adhesion, we next assessed single-cell migration on Col-I by time-lapse video microscopy. Consistent with the observed differences in length of the Ln-332 trails (Figure 6C), the migration tracks of MKα3(-) cells were clearly longer than those of MKα3(+) cells within the same time-frames (Figure 7C). Quantification of the average velocity confirmed that MKα3(-) cells migrated faster (~80 μm/h vs ~60 μm/h). To analyze the persistence of migration, the direct distance from start to end point (D) was divided by the total track distance (T). The resulting D/T ratio was almost 2-fold higher in α3(-) cells, indicating that not only migration speed but also the mode of migration is modulated by integrin α3: whereas MKα3(+) cells moved randomly and in a ‘back-and-forth’ fashion, deletion of α3 stimulated cell migration in a more persistent manner (Figure 7C). In line with these observations, a significantly larger fraction of the MKα3(-) cells was stably polarized throughout time, as determined by the adoption of a fan-shaped morphology in time-lapse movies (Figure 7C). The effect of EGF was also investigated in single-cell migration assays. The EGF-induced increase in migration velocity followed comparable kinetics in the absence and the presence of α3. However, the average velocity of migration was always higher in MKα3(-) cells, which was observed both on Col-1 and on Ln-332 (Figure 7D).

To verify that the observed effects were indeed due to the absence of α3, MKα3(-) cells were reconstituted with the gene encoding human α3A (hα3A) by retroviral transduction followed by FACS sorting, generating a cell line which we designated MKα3(R) (Figure 8A). Addition of GoH3 to MKα3(R) monolayers did not induce cell detachment, neither on Col-1 nor on Ln-332, which confirms that α6β1 was no longer required to maintain cell spreading as in α3(-) cells (Figures 8B,C). As expected, MKα3(R) cells migrated more slowly in scratch assays, although the rate of scratch closure was higher than in MKα3(+) cells, probably because the expression of hα3A was much lower than the expression of endogenous α3 (Figure 8D). Altogether, these data are consistent with the results of the in vivo wound healing assays, and confirm that the loss of α3 promotes keratinocyte migration.

DISCUSSION

Here, we show that loss of α3β1 from the epidermis causes skin abnormalities, including microblistering at the DEJ, BM duplication, and progressive hair loss. Although the microblistering suggests that α3β1 is only a minor contributor to adhesion, fragile and inflamed skin areas were nevertheless frequently observed. We assume that mechanical trauma, e.g. as applied during cleansing, causes repeated dissociation of the epidermis from the dermis because of reduced adhesion strength. BM duplication is either a compensation mechanism, or a result of rupture within the plane of the BM in regions where blistering occurs. The relative mildness of the blisters is probably explained by rescue of adhesion by...
Figure 8 The phenotype of MKα3(-) cells is reversed by reconstitution with human α3A. (A) MKα3(-) cells were transduced with the gene encoding for hα3A, and cell surface expression was verified by FACS analysis using monoclonal antiserum J143 against hα3A. Negative control (secondary antibody only) is indicated by the black line. (B) MKα3(R) cells were seeded on Ln-332 (left panel) or Col-I (right panel), allowed to spread, and then incubated for 3 hrs with GoH3 (10 μg/ml). Bar, 20 μm. (C) The number of spread cells was scored and expressed as a percentage of the total number of cells. In each independent experiment, approximately 500 cells per condition were counted. The graphs depict the averages of 3 experiments (** p < 0.0005). (D) Lysates of MKα3(+), MKα3(-), and MKα3(R) cells were analyzed by SDS-PAGE, and expression of integrin α3 was determined by Western blotting using mAb 29A3 recognizing both human and murine α3 (L = light chain). Confluent MKα3(+), MKα3(-), and MKα3(R) cells were deprived of growth factors, incubated for 2 hrs with mitomycin C (10 μg/ml), and scratched with the tip of a pipette prior to EGF stimulation. Wound areas were determined using Image J, and the ratio of the wound area after overnight migration over the wound area at t = 0 was calculated and expressed in a bar graph. Values shown represent the means ± SEM of 3 independent experiments (* p < 0.05, *** p < 0.0005).
α6-containing integrins, as HD assembly is normal in these mice. The progressive hair loss is reminiscent of mice deficient for Ln-511, a high-affinity ligand for α3β1 and the major Ln isoform expressed in hair follicles (31). Several abnormalities in hair follicle morphology have previously been described in the absence of α3, in transplantation studies grafting skin from newborn α3-deficient mice onto wild-type recipients (32). The observed phenotype was partly due to aberrant organization of the actin cytoskeleton. We are currently investigating the cause of hair loss in our system.

β1 integrins are required for keratinocyte migration in vitro and during wound healing (19). Since deletion of α2 has no effect on wound re-epithelialization (20), we expected α3β1 to be essential for keratinocyte migration. Surprisingly, wounds heal faster in Itga3<sup>flox/flox</sup>; K14-Cre mice than in Itga3<sup>flox/flox</sup> mice, suggesting an inhibitory effect of α3 on wound healing. Differences in the distribution of integrins during re-epithelialization were not detected, suggesting that the loss of α3 does not affect other integrins. A similar conclusion was reached previously when analyzing the skin of neonatal α3-null animals under steady-state conditions (28). This suggests that α3β1 inhibits or at least delays keratinocyte migration during wound healing. Possibly, the β1-integrin that drives epidermal migration is α5β1, αvβ1, or a combination of these. Integrins α5β1 and αvβ1 are de novo expressed upon wounding (33-35), and bind fibronectin (FN) that is deposited in the provisional matrix. Since deletion of α5 is embryonically lethal (36), mice with a specific deletion of α5 in the epidermis should shed light on this matter.

To confirm our observations in vitro, we isolated keratinocytes from newborn Itga3<sup>flox/flox</sup> mice and deleted Itga3, thus generating cells identical to those of the original clones except for the presence of α3. Although a role of α3β1 in the establishment of cell-cell contacts has been suggested (37,38), we found no obvious defects in the localization of tight junction or adherens junction proteins in knockout cells. Adhesion to Ln-332, but not to Col-1, was somewhat compromised whereas cell spreading was slightly reduced on both matrices. Both adhesion on Ln-322 and cell spreading over exogenous Ln-332 or Col-I were mediated by α6 integrins in the knockout cells. Increased surface expression of α6β1, which also binds Ln-332 (39), probably rescues cell spreading both on exogenous Ln-332 as well as on endogenous Ln-332 that is deposited on top of an exogenous substrate such as Col-I. Previously, α3β1 has been implicated in Ln-332 deposition via Tiam1-Rac signaling (40). From the results presented here, it is obvious that α3β1 is not necessary for Ln-332 deposition. Likely, there are more integrins that can create a platform for Rac activity and thus, in this case, for Ln-332 deposition. Indeed, Rac activity levels were the same in the presence or absence of α3 (data not shown). Whereas in vitro adhesion and cell spreading was rescued by increased surface expression of α6β1, we only detected very low amounts of α6β1 in vivo in the epidermis of both Itga3<sup>flox/flox</sup>; K14-Cre and Itga3<sup>flox/flox</sup> animals, and there was no upregulation in the absence of α3 (data not shown).

Keratinocyte migration was investigated in more detail in vitro. Whereas MKα3(+) keratinocytes moved in a
random fashion, mainly in circular and ‘back and forth’ patterns, α3(-) cells migrated faster and with higher directional persistence. This is a direct consequence of the loss of α3β1, which would implicate that α3β1 under normal conditions retards keratinocyte migration, or an effect of the upregulation of α6β1, suggesting that α6β1 specifically enhances the velocity and directionality of migration. Alternatively, α6β4 could drive migration in the absence of α3, as several studies have implicated this integrin in migration (41). However, this is probably not the case, because 1) we did not observe a difference in cell surface levels of α6β4 in the absence or the presence of α3, 2) we did not detect a redistribution of α6β4 from HDs into migration-associated structures, and 3) the enhanced migration of the α3-null cells is not only observed on Ln-332 but also on Col-1, which is not a substrate for α6β4. The phenotype can probably also not be attributed to the upregulation of α6β1, since increased migration velocity was also reported in keratinocytes derived from α3-null mice, in which α6β1 was not upregulated (28,29). Similarly, inhibiting α3 function with blocking antibodies in human keratinocytes increased migration, without affecting the levels of other integrins (26,27). In addition, affinity of α3β1 for Ln-332 is higher than of α6β1 (39,42). This is reflected in the reduced adhesion and cell spreading of the MKα3(-) cells, and indicates that MKα3(+) cells are more tightly ligated to Ln-332 than knock-out cells. Finally, enhanced migration is observed not only on Ln-332, but also on Col-1, which is not a substrate for α6β1. Integrin binding to deposits of endogenously produced Ln-332 can inhibit migration, but cannot drive migration on an exogenous substrate. This is underlined by the fact that Ln-332 is always observed in the rear of migrating cells, and not in the front. We therefore conclude that the enhanced motility is mainly a direct effect of the loss of inhibition by α3β1, which is supported by previous reports suggesting that α3β1 inhibits keratinocyte migration on FN, Col-I, or Col-IV (26-29), caused by ligation to endogenous Ln-332 deposits (27,43,44). Consistent with this idea is the hypermotility observed in keratinocytes isolated from an epidermolysis bullosa patient that do not express the γ2 chain of Ln-332 (45), which is reversed by the restoration of Ln-332 expression (46). However, the exact effect of the α3β1-Ln-332 interaction on keratinocyte migration is controversial, as promotion of migration by this interaction was also reported for normal human keratinocytes (24,25). Similarly, human keratinocytes defective in Ln-332 expression due to a deletion in the LAMB3 gene were found to migrate with decreased velocity and directional persistence (47). The apparent paradox is most likely explained by differences in the matrices used; on a matrix that consists exclusively of Ln-332, α3β1 supports polarization, cell spreading and migration (48), whereas on a different ligand or a complex matrix offering various ECM components, as in vivo when keratinocytes migrate over a provisionally formed matrix, migration is driven by other integrins while α3β1 maintains adhesion to endogenous Ln-332 deposits at the rear of the cell. Interaction with these deposits regulates cell polarization (49), and is crucial to maintain adhesion.
during migration. This is illustrated by the observation that in the MKα3(-) cells, GoH3 induced detachment not only of sessile but also of migrating cells (data not shown). Thus, keratinocytes require both a motogenic factor at the cell front and adhesion to Ln-332 deposits at the rear in order to polarize and maintain adhesion during migration.

**MATERIALS AND METHODS**

**Generation of Itga3 conditional knockout mice**

A genomic fragment of 15 kb encompassing exons 1-3 of Itga3 was isolated from a Lambda-FixII SV129 library and sub-cloned into plasmid vector pBS-SK+. A single loxP site and a loxP-PGKneo'-PGKtk-loxP (floxed neo/tk cassette) were inserted into HpaI and BamHI, respectively. The targeting construct (excised from the plasmid with NotI and Swal) was electroporated into 129/Ola-derived embryonic stem cells. Colonies resistant to geneticin (G418) were screened for the desired homologous recombination by Southern blotting. The floxed neo-tk cassette was deleted by transient transfection of Cre-expression plasmid pOG231. A recombinant clone harboring the conditional Itga3 allele was injected into mouse C57BL/6 blastocysts, which were transferred to mothers of the same strain. The chimeric male offspring was mated with FVB/N females. Agouti coat-colored offspring were screened for presence of the conditional allele by genotyping tail DNA with primers P1 (GAACAACATCTGCTGGAGGTTC) and -Cre5 (GCACGTTCACCGGCATCAAC). Removal of exon 1 by Cre-mediated recombination was confirmed by PCR using primers P1 and P3 (CAACAGCATGCTGTAGCA). All animal experiments were carried out with approval from the relevant institutional animal ethics committees.

**Establishment of keratinocyte cell lines and cell culture**

Isolation of primary keratinocytes from neonatal Itga3^fl/fl^ mice and Cre-mediated deletion of the Itga3^fl/fl^ allele was performed essentially as described previously (50). For all experiments, 3 clones were used with similar results. Throughout this manuscript, results obtained with 1 clone (K3) are presented. Retroviral delivery of the human α3A subunit cloned into pLZRS-MS-IRES-Zeo/pBR vector was established according to previously described protocols (51). Cells were cultured at 37°C and 5% CO2 in keratinocyte serum-free medium (K-SFM; Gibco BRL) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml EGF, 100 U/ml penicillin and 100 U/ml streptomycin. To induce differentiation, keratinocytes were maintained for up to 48 hrs in DMEM (Gibco BRL) containing 10% FCS and 100 U/ml penicillin/streptomycin.
cin. Rac-11P cells (52) and NIH3T3 cells were cultured in DMEM with 10% FCS and antibiotics.

**Antibodies and other materials**

Mouse mAbs used in this study were directed against: α-catenin, β-catenin, p120 catenin, and E-cadherin (Transduction laboratories, Lexington, KY), BrdU (Bu20a from DAKO Corp., Carpinteria, CA), integrin α3A (29A3) (53), human integrin α3A (J143) (54), integrin β1 (U19, from U. Mayer, University of Manchester, Manchester, UK) and pan-actin (Chemicon international). Rat mAbs were: GoH3 against α6 (55), 4G6 against Ln-511 from L. Sorokin (University of Muenster, Muenster, Germany), 346-11A against β4 from S.J. Kennel (Oak Ridge Laboratories, Oak Ridge, TN), and BMA5 against α5 and MB1.2 against β1, both from B.M.C. Chan (University of Ontario, Ontario, Canada). Hamster mAbs against integrins α2 (HMα2) and αv (H9.2B8) were from PharMingen (San Diego, CA), and goat mAb against integrin α3A was from R&D systems. Rabbit polyclonal antibodies were directed against ZO-1 and occludin (Zymed laboratories), BP180 (mo-NC16a) from L. Bruckner-Tuderman (University of Freiburg, Freiburg, Germany), Col-IV from E. Engvall (The Burnham Institute, La Jolla, CA), Ln-332 and Nd from T. Sasaki (Shriners Hospital for Children Research Center, Portland, OR), CD3 from Thermo Fisher Scientific Inc. (Fremont, California), and filaggrin, involucrin and keratin-5, -6, -10, and -14 from Covance Research Products Inc. Texas Red-, TRITC- and FITC-conjugated secondary antibodies, phalloidin and DAPI were from Molecular Probes (Eugene, OR), HRP-conjugated secondary antibodies were from Amersham, BrdU was from Sigma-Aldrich (Steinheim, Germany), and Col-I was from Vitrogen (Nutacon, Leimuiden, The Netherlands).

**Preparation of ECM matrices**

Culture dishes were coated with Col-1 (3 μg/ml) or 2% BSA for 30 min at 37°C. Ln-332-containing matrix was prepared by growing Rac-11P cells to confluency, prior to overnight detachment with 10 mM EDTA at 4°C. The plates were then washed twice with PBS, blocked with 2% BSA for 1 hr at 37°C, and washed twice with PBS before use.

**In vivo wound healing and proliferation experiments**

Wound healing experiments were conducted as previously described (40). Sections were photographed on an Axiovert S100 widefield system equipped with an Axiocam CCD camera (Zeiss). Wound closure was determined by counting the number of closed wounds 7 days after wounding, and expressing the number of closed wounds as a percentage of the total number of wounds. Alternatively, the length of the neo-epidermis was determined 3 days after wounding using ImageJ. The graphs depict the average values of approximately 40 wounds pooled from 3-4 independent experiments. To analyze proliferation, BrdU was injected intra-peritoneally (50 μg/g body weight) at 2 or 4 days after wounding, 3 hrs before sacrifice. The number of BrdU(+) cells was quantified from sections using ImageJ and expressed as a percentage of the total number of cells. At least 30 sections per condition were analyzed.
Immunoprecipitations and Western blotting
Immunoprecipitation of integrins was performed as described previously (51). Total cell lysates were prepared in SDS sample buffer, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and analyzed by Western blotting followed by ECL using the SuperSignal system (Pierce Chemical Co.).

Ultrastructural analysis, immunofluorescence microscopy, and flow cytometry
Electron microscopy on mouse skin and immunofluorescence labeling of cryosections and cells on coverslips were carried out as previously reported (50). Images were acquired at RT with a confocal Leica TCS NT or AOBS microscope using 20x (NA 0.7) dry, 40x (NA 1.25) oil, and 63x (NA 1.32) oil objectives (Leica) and AxioVision 4 software (Carl Zeiss MicroImaging, Inc.). Pictures were processed and cell debris was masked using Photoshop 7.0 and ImageJ. Flow cytometry and cell sorting were performed as previously described (51).

Adhesion assays
Subconfluent cells were trypsinized, resuspended in K-SFM with or without GoH3 (10 μg/ml), and then seeded in 96-well plates coated with BSA, Col-I, or Ln-332 at a density of 3x10⁴ cells per well. After 30 min at 37°C, nonadherent cells were washed away with PBS. The adherent cells were fixed in 4% PFA, washed twice with H₂O, stained for 10 min with crystal violet, washed twice with H₂O, and then lysed in 2% SDS. Absorbance was measured at 490 nm on a microplate reader. Background values (binding to BSA) were subtracted from all other values, and the number of adherent MKα3(+) cells was set to 100%.

Cell spreading assays
Cells were seeded in K-SFM on 24-well plates coated with Col-I or Ln-332 and cell spreading was allowed for 5 hrs, after which the cells were maintained in the absence or presence of GoH3 (10 μg/ml) for an additional 3 hrs. Cells were then photographed on a Widefield CCD system using 10x and 20x dry lens objectives (Carl Zeiss MicroImaging, Inc.) and images were processed using Photoshop 7.0. The number of spread cells was counted and expressed as a percentage of the total number of cells. Values shown represent the averages of 3 experiments. In each experiment, approximately 500 cells were analyzed for each condition.

Single-cell migration, scratch assays, and polarization assays
For scratch assays, cells were grown to confluency and starved overnight. Mitomycin C (Nycomed Inc., Breda, The Netherlands; 10 μg/ml) was added 2 hrs prior to scratching with a yellow pipette tip. After 2 washings with K-SFM, cells were stimulated with 5 ng/ml EGF. To analyze single-cell migration and polarization, cells were seeded sparsely on Col-I or Ln-332 in K-SFM with or without supplements, covered with mineral oil, and EGF (5 ng/ml) was added when appropriate at the indicated time-point after seeding. Phase/contrast images were captured every 5 min at 37°C and 5% CO₂ on a Widefield CCD system using a 10x dry lens objective (Carl Zeiss MicroImaging, Inc.). Images were processed using
Photoshop 7.0, and migration tracks or scratch areas were analyzed using ImageJ or MatLab (Mathworks). Scratch closure is represented as the ratio of the wound area after overnight migration over the wound area at $t = 0$. Values shown represent the means ± SEM of 3 independent experiments. The number of polarized cells was counted from ~250 cells pooled from 4 independent experiments, and expressed as a percentage of the total number of cells. Cells were considered stably polarized when they maintained a leading lamellipodium for at least 1 hr. Average velocity and persistence of single-cell migration were calculated from ~50 cells per experiment using ImageJ or Matlab, and the graphs represent either the averages ± SEM pooled from 4 independent experiments, or the averages of 1 representative experiment as indicated.

**Statistical analysis**

Data were analyzed using a homoscedastic 2-tailed $t$ test, and $p<0.05$ was considered statistically significant.

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**ABBREVIATIONS**

BM, basement membrane; BP, bullous pemphigoid; Col-I, collagen-I; Col-IV, collagen-IV; DEJ, dermal-epidermal junction; EGF, epidermal growth factor; FN, Fibronectin; HD, hemidesmosome; H/E, haematoxylin/eosin; K-SFM, keratinocyte serum-free medium; Ln-332, laminin-332; Ln-511, laminin-511; MK, mouse keratinocytes; Nd, nidogen; ZO-1, zonula occludens-1

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