Integrin α3β1 inhibits directional migration and wound re-epithelialization in the skin

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Summary

Re-epithelialization after skin wounding requires both migration and hyperproliferation of keratinocytes. Laminin-332 is deposited during migration over the provisional matrix. To investigate the function of the laminin-332 binding integrin α3β1 in wound re-epithelialization, we generated *Itga3*$^{flox/flox}$; 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 slightly faster in the absence of integrin α3β1, whereas 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 integrin in the absence of integrin α3. The α3-deficient keratinocytes migrate with an increased velocity and persistence, whereas proliferation, growth factor signaling, hemidesmosome assembly, and laminin-332 deposition appeared to be normal. We suggest that integrin α3β1 delays keratinocyte migration during wound re-epithelialization, by binding to the laminin-332 that is newly deposited on the wound bed.

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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 consisting primarily of laminins and collagens (for a review, see Burgeson and Christiano, 1997). Attachment of basal epidermal keratinocytes to the basement membrane is mediated by members of the integrin family. The integrin repertoire in basal keratinocytes is restricted to integrins α2β1, α3β1, α9β1 and α6β4, whereas de novo expression of additional integrins, for example, integrins α5β1, αvβ1, αvβ5 and αvβ6 is induced upon wounding (reviewed by Watt, 2002). Although integrin α2β1 mediates attachment to collagens, adhesion to the main basement membrane component laminin-332 (Ln-332; previously named laminin-5, nicein, kalinin or epiligrin) is established by the predominant epidermal integrins α3β1 and α6β4 (Carter et al., 1991; Rouselle et al., 1991; Aumailley and Rouselle, 1999). Integrin α3β1 links the ECM to the actin cytoskeleton and is localized at the basolateral membrane. It is often found in clusters surrounding hemidesmosomes, and in focal contacts in culture (Carter et al., 1990a; DiPersio et al., 1995). By 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, which further consist of the cytoskeletal linker proteins plectin and bullous pemphigoid (BP) antigen 230, and the transmembrane proteins BP180 and CD151 (reviewed by Borradori and Sonnenberg, 1999). Ablation in mice of the gene encoding integrin α6 or integrin β4 impairs hemidesmosome formation and results in severe epidermal blistering and perinatal death, demonstrating the importance of α6β4 integrin in the maintenance of skin integrity at the dermal-epidermal junction (DEJ) (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). 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 called junctional epidermolysis bullosa (Niessen et al., 1996; Ashton et al., 2001; Castiglia et al., 2001).

Deletion of the gene encoding the integrin β1 subunit causes early embryonic lethality (Fassler and Meyer, 1995; Stephens et al., 1995). Evidence for the function of β1 integrin in skin stems from conditional knockout mouse models in which the ablation of integrin β1 is restricted to the basal epidermal keratinocytes, resulting in skin blistering at the DEJ, a reduced number of hemidesmosomes, failure of basement membrane assembly, impaired invagination of hair follicles and eventual hair loss (Raghavan et al., 2000; Brakebusch et al., 2000). This suggests that β1 integrins are required for hair growth, basement membrane assembly and hemidesmosome formation. Furthermore, epidermal migration during wound healing is impaired in the absence of β1 integrin (Grose et al., 2002). Since there are no obvious skin defects in α2-null mice, either in the structure of the skin or in wound healing (Chen et al., 2002; Zweers et al., 2006), the wound-healing defect caused by the deletion of β1 integrin is possibly due to a loss of function of α5β1, α9β1, αvβ1, or combinations of two or three of these integrins, or a loss of function of α3β1 integrin, which is the most likely explanation for the other features of the phenotype

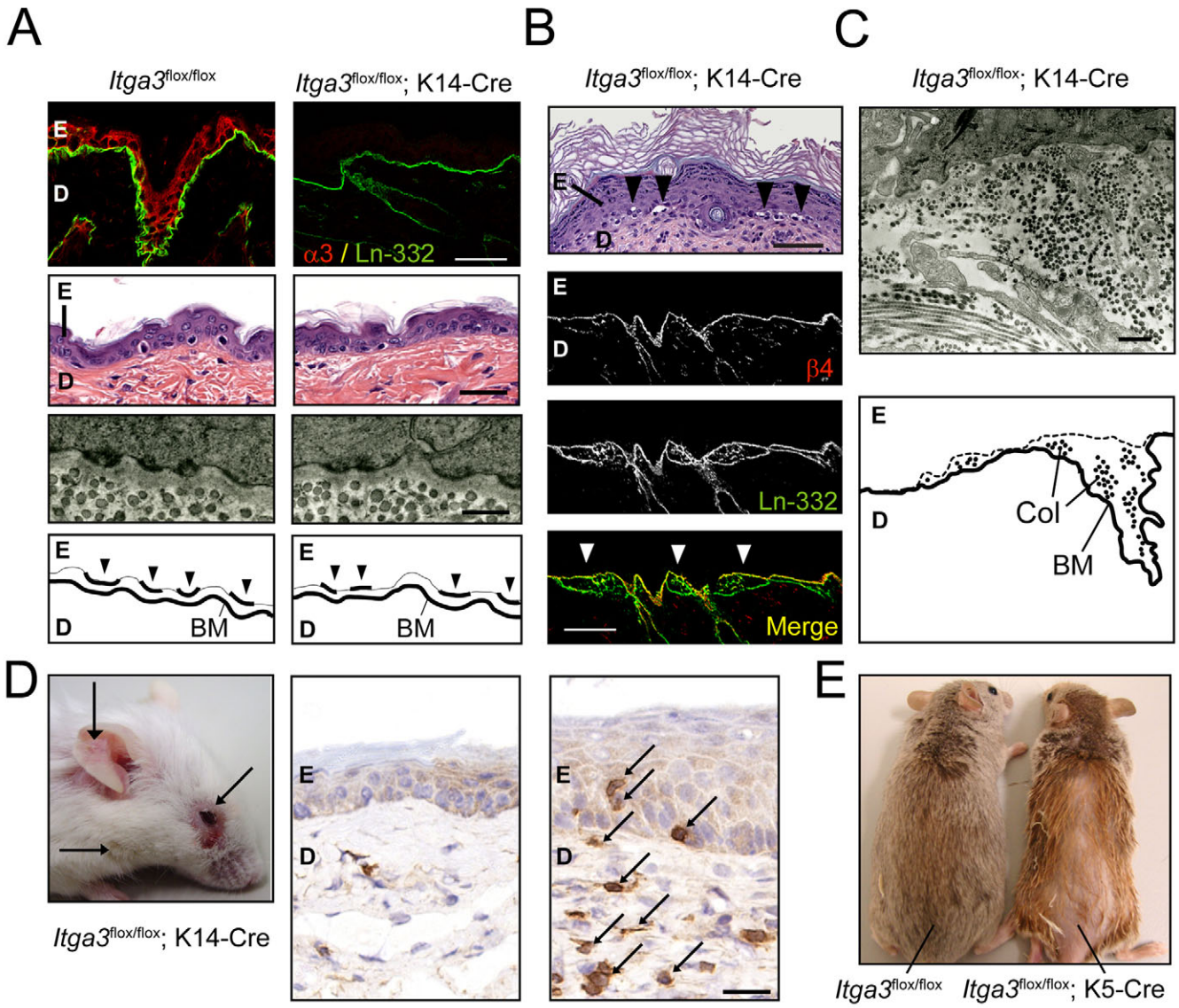

Fig. 1. Skin phenotype of *Itga3*$^{flox/flox}$; K14-Cre mice. (A) Cryosections from back skin of neonatal *Itga3*$^{flox/flox}$ and *Itga3*$^{flox/flox}$; K14-Cre mice were stained with antibodies directed against the α3 subunit and Ln-332 (top row). Scale bar: 100 µm. H&E-stained section showing the structure of the epidermis and the dermis from back skin of 4-month-old *Itga3*$^{flox/flox}$ and *Itga3*$^{flox/flox}$; K14-Cre mice are shown in second row from top. Scale bar, 200 µm. Bottom two rows are EM images and schematic representations of back skin of 1-year-old *Itga3*$^{flox/flox}$ and *Itga3*$^{flox/flox}$; K14-Cre mice, showing the basement membrane and hemidesmosomes (indicated by arrowheads). Scale bar: 200 nm. (B) H&E staining of back skin of a 1-year old *Itga3*$^{flox/flox}$; K14-Cre mouse (top). Arrowheads indicate microblisters at the DEJ. Scale bar: 500 µm. Lower panels are cryosections of a region containing microblisters stained with antibodies against Ln-332 and β4. Arrowheads indicate blisters. Scale bar: 50 µm. (C) Ultrastructural analysis and schematic representation of back skin of a 1-year-old *Itga3*$^{flox/flox}$; K14-Cre mouse, showing basement membrane duplication. The two basement membranes are marked with solid and dashed lines, and collagen fibers extending throughout the two basement membranes are indicated. Scale bar: 200 nm. (D) Regions of inflammation in a 4-month-old *Itga3*$^{flox/flox}$; K14-Cre mouse are denoted by arrows. Back skin sections were stained with an antibody against CD3. Arrows indicate infiltrated lymphocytes. Scale bar in the third panel: 200 nm. (E) Alopecia in a 1-year-old *Itga3*$^{flox/flox}$; K5-Cre mouse (right), compared with an *Itga3*$^{flox/flox}$ mouse of the same age. BM, basement membrane; Col, collagen; D, dermis; E, epidermis.

as well. Indeed, α3-null mice are born with a disorganized basement membrane and microblistering at the DEJ, although the skin phenotype is much less severe than that observed in the absence of integrin α6, β1 or β4 (DiPersio et al., 1997). However, these mice die during the neonatal period as a consequence of defective kidney and lung organogenesis (Kreidberg et al., 1996), complicating the study of the function of α3β1 integrin in the adult skin and wound healing. In vitro evidence of the role of α3β1 integrin in keratinocyte migration is controversial; although some studies have suggested that integrin α3β1 promotes keratinocyte migration (Zhang and Kramer, 1996; Nguyen et al., 2000), the opposite has also been reported (Kim et al., 1992; O'Toole et al., 1997; Hodivala-Dilke et al., 1998; DeHart et al., 2003).

To address the role of α3β1 integrin in adult skin homeostasis and wound healing, we generated epidermis-specific α3-knockout mice by crossing *Itga3*$^{flox/flox}$ mice with mice expressing Cre recombinase under the control of the K14 promoter. The resulting *Itga3*$^{flox/flox}$; K14-Cre mice are viable but display several skin abnormalities including local inflammation, microblistering at the DEJ and basement membrane duplication, whereas hemidesmosome

assembly and keratinocyte differentiation are normal. Skin wounds close faster in these animals, whereas proliferation rates of keratinocytes are similar, suggesting that the migration of keratinocytes is accelerated when the α3 subunit is absent. This is confirmed by in vitro observations; α3-deficient keratinocytes migrate with increased velocity and persistence compared with α3-expressing cells. Taken together, these results show that the integrin α3β1 is required for the maintenance of dermal-epidermal integrity but not for the proliferation and the differentiation of keratinocytes, or for hemidesmosome formation. Furthermore, α3β1 delays keratinocyte migration during wound re-epithelialization, by binding to the Ln-332 that is newly deposited on the wound bed.

Results

Generation and characterization of epidermis-specific *Itga3*-knockout mice

Integrin-α3-null mice die shortly after birth, displaying disturbed kidney and lung organogenesis and moderate skin blistering at the DEJ (Kreidberg et al., 1996; DiPersio et al., 1997). To address the function of the α3 subunit in adult skin, specifically in adhesion and wound healing, we generated epidermis-specific α3-knockout mice by crossing mice homozygous for a floxed *Itga3* allele (*Itga3*$^{flox/flox}$) (Sachs et al., 2006) with mice expressing Cre recombinase under the control of the K14 promoter (Huelsken et al., 2001) (supplementary material Fig. S1 and Materials and Methods). Fig. 1A shows that the deletion of the α3 subunit in the epidermis of 4-month-old *Itga3*$^{flox/flox}$; K14-Cre mice is efficient. Haematoxylin and eosin (H&E) staining and electron microscopy images of skin sections revealed no obvious effects of the deletion of integrin α3 on overall skin structure; the epidermis was typically 2-3 cell layers thick, and keratinization and hemidesmosome assembly seemed to be normal (Fig. 1A). However, microblistering at the DEJ was occasionally observed, which was associated with thickening of the epidermis and the presence of an inflammatory infiltrate (Fig. 1B). Ln-332 was distributed at both the roof and floor of the blisters, whereas integrin β4 was restricted to the roof (Fig. 1B). Ultrastructural analysis revealed a duplication of the basement membrane in the dermis along the entire blister (Fig. 1C). Inflammation occurred frequently around 3 to 4 months after birth, especially at the ears and around the eyes (Fig. 1D). The inflammatory infiltrate was mainly observed in regions where the epidermis was abnormally thick (Fig. 1D). Around the same time, these mice developed alopecia (local hair loss), which progressed with age (Fig. 1E). Differentiation in the epidermis was essentially normal, as assessed by staining of cryosections for keratin 14, filaggrin, and involucrin (Fig. 2). Similar results were obtained when *Itga3*$^{flox/flox}$ mice were crossed with mice expressing the Cre recombinase under the control of the K5 promoter (data not shown). In summary, the targeted deletion of *Itga3* in the mouse epidermis causes several skin abnormalities associated with a reduced adhesion of the epidermis to the dermis.

Wound closure is accelerated in integrin-α3-null skin as a result of increased migration but not increased proliferation

To investigate the role of the integrin α3β1 in wound healing, two full-thickness excision wounds were inflicted on either side of the dorsal midline in 4-month- old *Itga3*$^{flox/flox}$ and *Itga3*$^{flox/flox}$; K14-Cre mice. Re-epithelialization of such wounds occurs by the migration and hyperproliferation of keratinocytes from outside the wound bed over the dermis and granulation tissue (represented schematically in Fig. 3A). In the tip of the advancing epidermis, α3β1 is strongly upregulated and Ln-332 is deposited, whereas deposition of other ECM proteins such as Ln-511, Collagen type IV (Col-IV) and Nidogen (Nd) are deposited at some distance from the tip, to restore the damaged basement membrane (supplementary material Fig. S2A). Increased expression and distribution of β1 in the tip of the migrating epithelial sheet appeared to be normal in *Itga3*$^{flox/flox}$; K14-Cre mice, suggesting that another α subunit binds β1 in the absence of α3 integrin in the leading keratinocytes (supplementary material Fig. S2B). Furthermore, the localization of the integrin subunits α5, α6 and β4 as well as the basement membrane proteins Col-IV, Ln-511, Nd and Ln-332 was comparable with that in *Itga3*$^{flox/flox}$ mice suggesting that the distribution of other integrins and the deposition of basement membrane proteins are not affected by the deletion of α3 integrin (supplementary material Fig. S2B). We next determined the rate of wound closure on H&E-stained paraffin sections. Wound healing was not impaired in *Itga3*$^{flox/flox}$; K14-Cre mice but was in fact faster than in *Itga3*$^{flox/flox}$ skin, as determined both by the number of wounds that had completely closed after a week, and by the extent of re-epithelialization at earlier time points (Fig. 3B,C). To assess whether this was due to increased proliferation, BrdU was injected intraperitoneally at several time points 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 *Itga3*$^{flox/flox}$ versus *Itga3*$^{flox/flox}$; K14-Cre mice, indicating that α3 does not affect proliferation during wound healing (Fig. 3D). Taken together, these results suggest that deletion of α3 integrin in the skin promotes wound healing by accelerating keratinocyte migration.

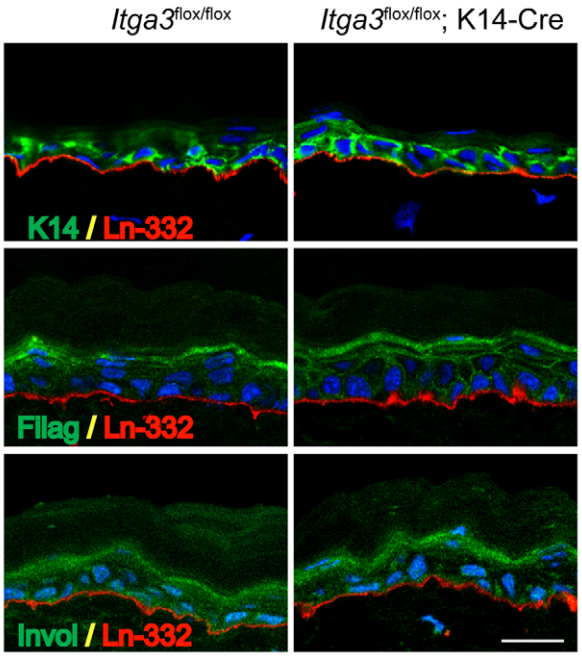

Fig. 2. Deletion of integrin α3 in the epidermis does not inhibit keratinocyte differentiation. Skin cryosections from neonatal *Itga3*$^{flox/flox}$ and *Itga3*$^{flox/flox}$; K14-Cre mice were stained with antibodies directed against keratin 14, filaggrin or involucrin, and counterstained with Ln-332 and DAPI to visualize the basement membrane and nuclei, respectively. Scale bar: 50 μm.

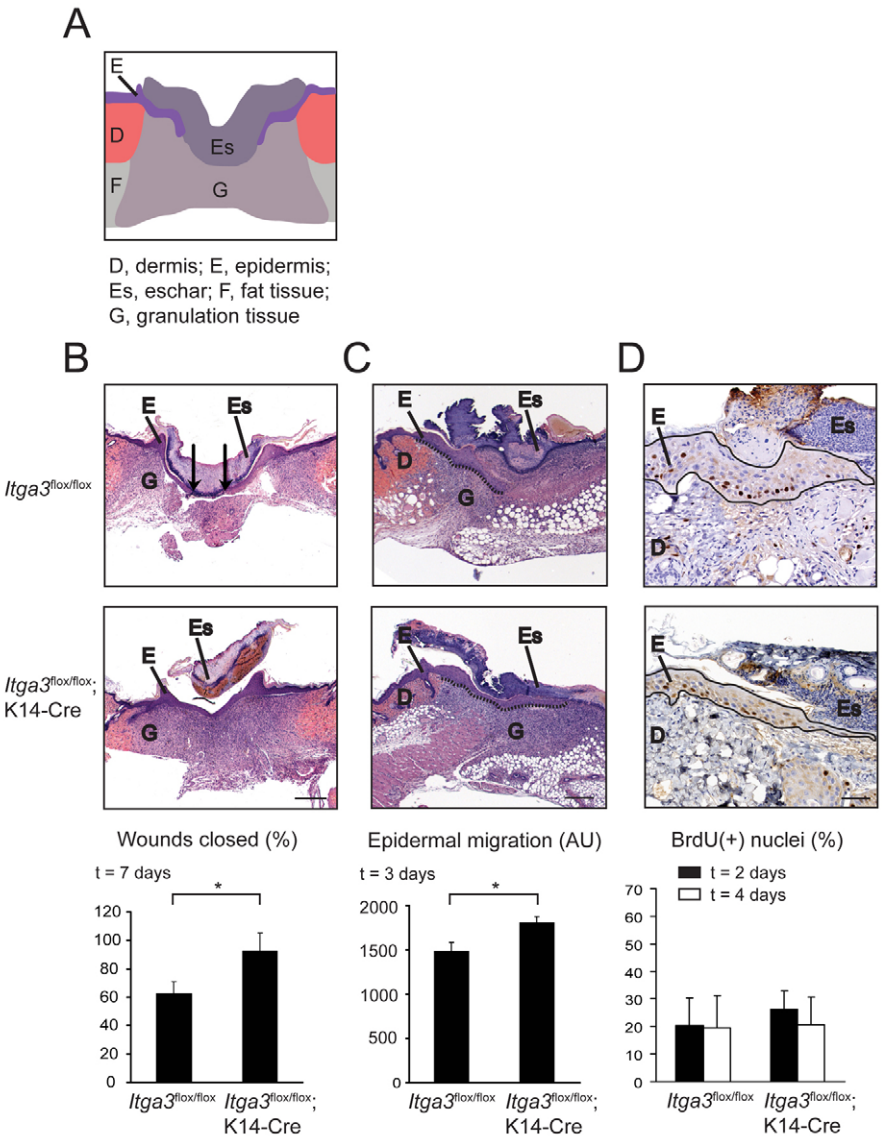

Fig. 3. Wound closure is accelerated in the absence of α3β1 integrin. Full-thickness wounds were generated on either side of the dorsal midline in *Itga3*$^{flox/flox}$ or *Itga3*$^{flox/flox}$; K14-Cre mice, and excised 3 or 7 days after injury. (A) Schematic representation of a wound. D, dermis; E, epidermis; Es, eschar; F, fat tissue; G, granulation tissue. (B) H&E-stained sections depicting wound closure 7 days after wounding in *Itga3*$^{flox/flox}$ (top) and *Itga3*$^{flox/flox}$; K14-Cre mice (bottom). Arrows indicate the edges of the migrating epithelium. The number of closed wounds after 7 days of migration is represented in the bar graph. Depicted are the means ± s.e.m. of at least 40 wounds pooled from four independent experiments (*P<0.05). Scale bar, 500 µm. (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 ± s.e.m. of at least 35 wounds pooled from three experiments (*P<0.05). Scale bar, 250 µm. (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 ± s.e.m. of at least 30 images. Scale bar: 150 µm.

In vitro adhesion to Ln-332 is mediated by α6 integrins when the α3 subunit is absent

To confirm the migration data in vitro and to investigate the underlying mechanism, we isolated keratinocytes from newborn *Itga3*$^{flox/flox}$ mice and cultured them on Collagen type I (Col-I; 3 µg/ml). Several spontaneously immortalized clones were obtained, which we named mouse keratinocytes (MK)α3$^+$. 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, eight injections in two independent experiments), indicating that they are not transformed. To generate the MKα3$^-$ (α3-null cells), the α3 subunit was deleted by adenoviral delivery of Cre recombinase (Fig. 4A and supplementary material Fig. S3A), thus the α3-knockout cells were derived directly from the same clones. There was no significant difference in proliferation rates between MKα3$^+$ and MKα3$^-$ cells (supplementary material Fig. S3B). Moreover, both MKα3$^+$ and MKα3$^-$ cells responded in a similar fashion to transfer to Ca^{2+}-rich medium, initiating the formation of cell-cell contacts such as adherens junctions and tight junctions, as suggested by zonula occludens (ZO)-1, occludin, β-catenin and E-cadherin staining (supplementary material Fig. S3C), and the assembly of hemidesmosome-like structures (supplementary material Fig. S3D). These results are in line with the in vivo observations and show that the integrin α3β1 is not essential for the formation of cell-cell contacts, hemidesmosome assembly or proliferation. To investigate whether the deletion of the α3 subunit affected the expression of other integrins, we determined the level of integrins at the cell surface by flow cytometry. Expression of β1 integrin on the cell surface was downregulated in the knockout cells whereas the expression of α2, α5, α6, αv and β4 subunits was not significantly different (Fig. 4B). To investigate whether cell adhesion was compromised, we performed adhesion assays on Col-1 or Ln-332. Adhesion of MKα3$^-$ cells to Ln-332 was slightly reduced (~70% of that of control cells) but decreased dramatically in the presence of the α6-blocking antibody GoH3, indicating that adhesion to Ln-332 in the absence of α3 is mediated by α6 integrins (Fig. 4C). Adhesion of MKα3$^+$ and MKα3$^-$ cells to Col-1 was similar and was not blocked by GoH3, as expected (Fig. 4C). Immunoprecipitation experiments revealed that the association of integrin α6 with integrin β1 was increased in the knockout cells, whereas integrin α6β1 was hardly detectable

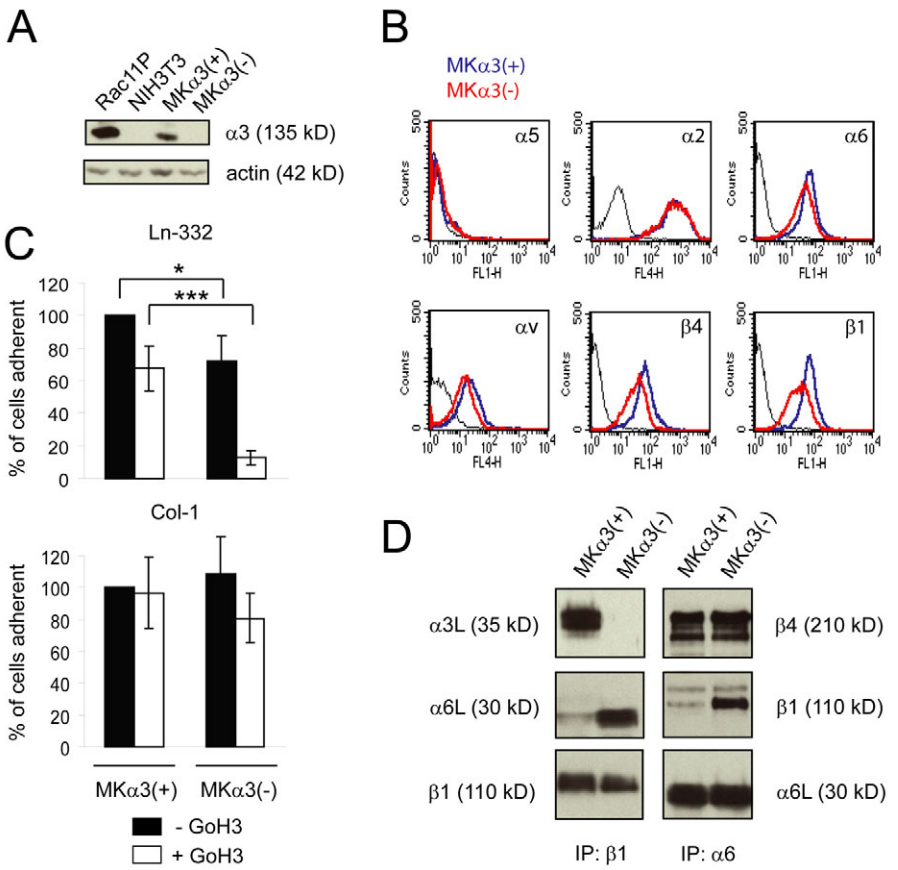

Fig. 4. In vitro adhesion to Ln-332 is rescued by α6 integrins in the absence of α3β1 integrin. Keratinocytes were isolated from newborn *Itga3*$^{flox/flox}$ mice and designated MKα3^{+}. MKα3^{-} cells were then obtained by in vitro deletion of *Itga3*. (A) Immunoblot depicting the expression of α3 in MKα3^{+} and MKα3^{-} cells. Murine mammary cell line Rac-11P and murine fibroblast cell line NIH3T3 were included as a positive and negative control, respectively. (B) Cell surface expression of integrin subunits α5, α2, α6, αv, β4 and β1 in MKα3^{+} and MKα3^{-} was determined by FACS analysis. The negative control (only secondary antibody) is indicated by the black graph. (C) MKα3^{+} and MKα3^{-} cells were seeded onto Col-1 or Ln-332 in K-SFM without supplements in the absence or the presence of the α6-blocking antibody GoH3 (10 μg/ml). After 30 minutes, non-adherent cells were washed away. Values shown represent the average percentages of adherent cells from three experiments performed in triplicate (*P<0.05, ***P<0.0005). (D) Immunoprecipitation of integrin subunits α6 and β1 was performed on lysates of MKα3^{+} and MKα3^{-} cells. The precipitates were resolved by SDS-PAGE, and analyzed for the presence of the integrins β1 and β4, and the light chains (L) of α3 and α6 by western blotting.

in MKα3^{+} cells (Fig. 4D). Together, these data show that keratinocyte adhesion to Ln-332 is rescued by α6 integrins, and that surface expression of α6β1 integrin is upregulated when α3 integrin is absent.

Cell spreading on endogenous Ln-332 is mediated by α6β1 integrin when α3 integrin is absent

Next, we analyzed whether cell spreading is affected by the deletion of α3 integrin. Consistent with the adhesion data, cell spreading on Ln-332 of MKα3^{-} cells was slightly reduced compared with that of control cells, and addition of the α6-blocking antibody GoH3 decreased cell spreading significantly of the MKα3^{-} but not that of the MKα3^{+} cells (Fig. 5A). Surprisingly, the same results were obtained on Col-I (Fig. 5A), suggesting that cell spreading of MKα3^{-} cells on Col-I is also mediated by α6 integrins. We assume that the initial adhesion and spreading occurs on Col-I but that spreading on the patches of Ln-332 that are deposited over time underneath the cells is then maintained by Ln-332-binding integrins: α3β1 integrin in the control cells and α6β1 integrin when α3 is absent. To exclude that Ln-332 deposition itself was affected by the loss of α3 integrin, we next compared Ln-332 deposition by western blotting and immunofluorescence. Ln-332 deposition by knockout cells was not impaired over time (Fig. 6A), and the pattern of deposition was similar to that of MKα3^{+} cells (Fig. 6B). However, trails of Ln-332 that were left behind by migrating MKα3^{-} cells were strikingly longer, suggesting that motility and migration are increased when α3 integrin is absent (Fig. 6C). To verify that the observed effects are not due to adaptation in culture, we also performed adhesion and cell spreading assays with primary, non-immortalized keratinocytes isolated from neonatal *Itga3*$^{flox/flox}$ and *Itga3*$^{flox/flox}$; K14-Cre mice. These assays yielded essentially the same results (supplementary material Fig. S4). In conclusion, these data show that the deletion of α3 in keratinocytes does not affect the deposition of endogenous Ln-332, and that cell spreading over endogenous Ln-332 is maintained by upregulation of the integrin α6β1.

Deletion of α3 integrin enhances the velocity and directionality of keratinocyte migration

We next wished to mimic wound healing in an in vitro assay. Upon wounding, quiescent keratinocytes become activated by the release of growth factors such as EGF, inducing migration and hyperproliferation over exposed dermal collagens. Therefore, MKα3^{+} and MKα3^{-} cells were grown to confluency on Col-I, deprived of supplements and growth factors overnight. The cell monolayer was then scratched with the tip of a pipette, after which the cells 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 in vivo observations, the MKα3^{-} cells migrated significantly faster into the denuded area than the MKα3^{+} cells (Fig. 7A). This was probably not due to altered growth factor signaling, because stimulation with EGF after growth factor depletion induced a similar activation of the ERK pathway (Fig. 7B). To rule 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 videomicroscopy. Consistent with the observed differences in length of the Ln-332 trails (Fig. 6C), the migration tracks of MKα3^{-} cells were clearly longer than those of MKα3^{+}

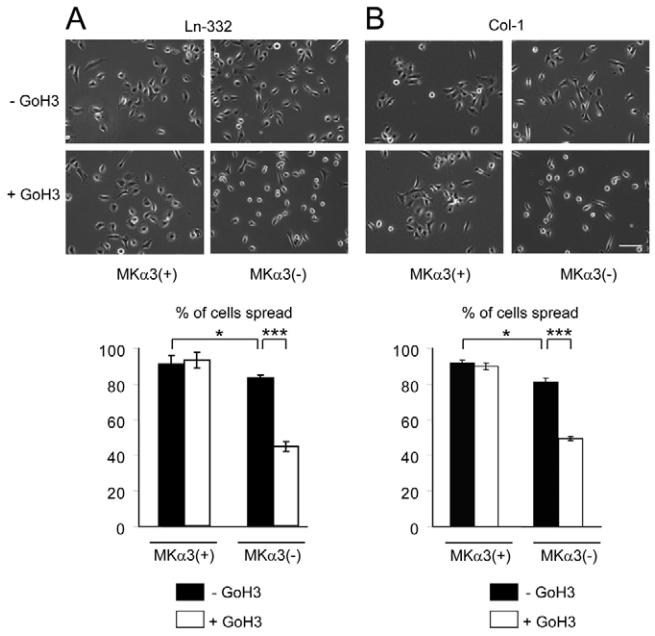

Fig. 5. The integrin α6β1 mediates cell spreading on endogenous Ln-332 when α3β1 is absent. MKα3$^+$ and MKα3$^-$ cells were seeded on Ln-332 (A) or Col-1 (B), allowed to spread, and then incubated with α6-blocking antibody GoH3 (10 μg/ml). After 3 hours, the number of spread cells was scored and expressed as a percentage of the total number of cells. In each independent experiment, approximately 500 cells were counted for each condition. The graphs depict the averages of three experiments (*P<0.05, ***P<0.0005). Scale bar: 50 μm.

cells within the same time frames (Fig. 7C). Quantification of the average velocity confirmed that MKα3$^-$ cells migrated faster (~80 μm/hour vs ~60 μm/hour). 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 twofold 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 fashion (Fig. 7C). In line with this, a significantly larger proportion of the MKα3$^-$ cells remained stably polarized over time, as determined by the adoption of a fan-shaped morphology in time-lapse movies (Fig. 7C). The effect of EGF was also investigated in single-cell migration assays. The EGF-induced increase of the migration velocity followed comparable kinetics in the absence and the presence of α3. However, under growth-factor-depleted conditions and after EGF stimulation, the average velocity was always higher in the MKα3$^-$ cells, which was observed both on Col-1 and on Ln-332 (Fig. 7D).

To verify that the observed effects were indeed due to the absence of α3, MKα3$^-$ cells were reconstituted with the cDNA encoding human α3A by retroviral transduction followed by FACS sorting, generating a cell line which we designated MKα3^R (Fig. 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 integrin was no longer required to maintain cell spreading as in α3$^-$ cells (Fig. 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 that of endogenous α3 (Fig. 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

In this study, we have investigated the function of the α3β1 integrin in the biology of adult skin by a gene targeting approach. We generated mice with a specific deletion of α3 in the epidermis, and examined skin organization and wound healing. We found that the loss of epidermal α3β1 causes skin abnormalities, including microblistering at the DEJ, duplication of the basement membrane, and progressive hair loss. Whereas 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, for example, that applied during cleansing, causes repeated dissociation of the epidermis from the dermis because of a reduced strength of adhesion. The duplication of the basement membrane is either a compensation mechanism, or a result of rupture within the plane of the basement membrane in regions where

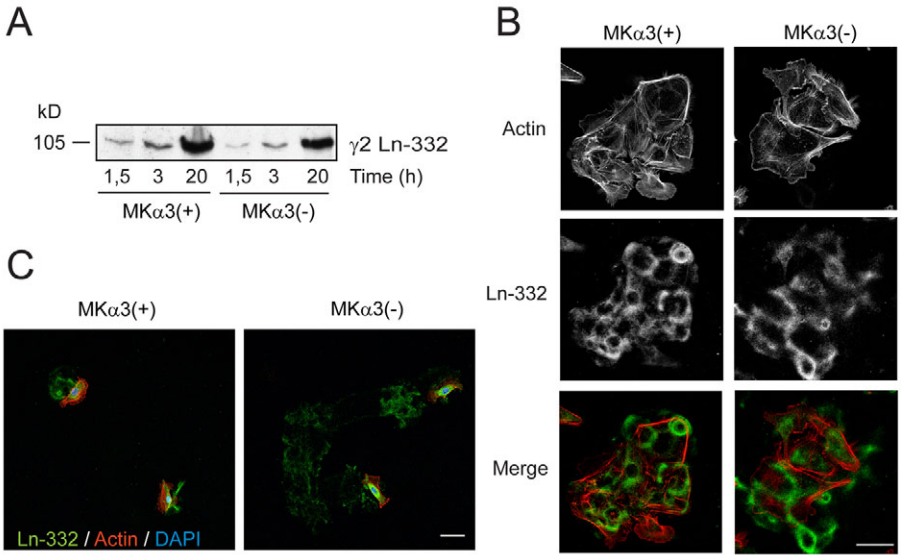

Fig. 6. Ln-332 synthesis and deposition are not affected by the deletion of the integrin α3 subunit. (A) MKα3$^+$ and MKα3$^-$ cells were seeded on Col-I and then detached with EDTA at the indicated time points after which the ECM was dissolved in sample buffer, subjected to SDS-PAGE, and the γ2 chain of Ln-332 was detected by western blotting. (B) Immunofluorescence images demonstrating patches of deposited Ln-332 in spread MKα3$^+$ cells (left panel) and MKα3$^-$ cells (right panel). Scale bar: 10 μm. (C) Low-magnification immunofluorescence images demonstrating Ln-332 trails left behind by MKα3$^+$ cells (left), and MKα3$^-$ cells (right) migrating over Col-I. Scale bar: 10 μm.

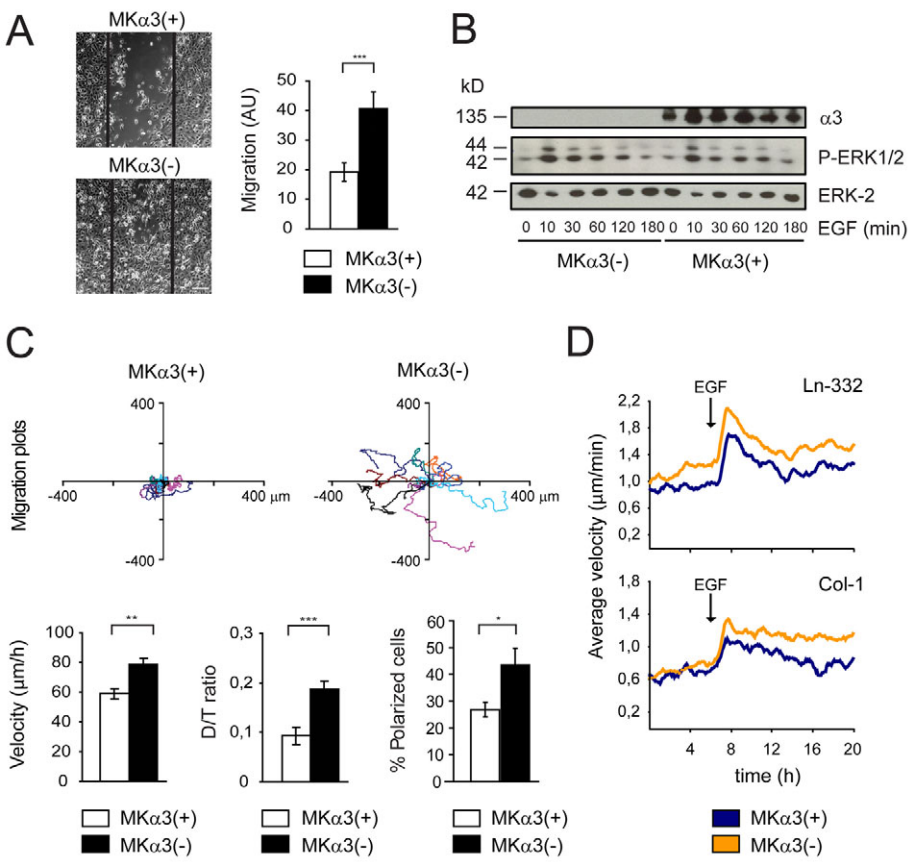

Fig. 7. Loss of α3 enhances directionality and velocity of keratinocyte migration. (A) Confluent MKα3$^+$ and MKα3$^-$ cells were deprived of growth factors, incubated for 2 hours with mitomycin C (10 µg/ml), and scratched with the tip of a pipette prior to EGF stimulation. The black bars indicate the wound edges at t=0. Scale bar: 100 µm. Wound areas were measured using ImageJ, 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 ± s.e.m. of three independent experiments (***P<0.0005). (B) EGF-induced phosphorylation of ERK1/2 was monitored by western blotting in lysates of MKα3$^+$ and MKα3$^-$ 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 for 16 hours. An image was captured every 5 minutes. Cell tracks were then determined using ImageJ. The migration plots indicate tracks from ten individual cells from four independent experiments. To quantify the average velocity and directionality (D/T ratio), data from four independent experiments were pooled. The graphs represent the means ± s.e.m. from ~50 cells (*P<0.05, **P<0.005, ***P<0.0005). To determine whether polarization was stable, cells were sparsely seeded on Col-I and monitored by time-lapse recordings. An image was captured every 5 minutes. A cell was considered stably polarized when maintaining a leading lamellipodium for 1 hour. The number of polarized cells was counted and expressed as a percentage of the total number of cells. The graph shows the means ± s.e.m. from 250 cells pooled from four 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 minutes. 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.

blistering occurs. In fact, it has been proposed that α3β1 is actively involved in basement membrane assembly (DiPersio et al., 1997). The relative mildness of the blisters can probably be explained by a rescue of the adhesion by α6-subunit-containing integrins. Indeed, hemidesmosomes, which are predominantly involved in the stable adhesion of epidermal cells to the basement membrane and in which α6β4 integrin is an essential component, are normal in *Itga3*$^{flox/flox}$; K14-Cre mice. The progressive hair loss is reminiscent of that in mice deficient for Ln-511, a high-affinity ligand for α3β1 integrin and the major laminin isoform expressed in hair follicles (Li et al., 2003). Several abnormalities in the morphology of the hair follicle have been described previously in transplantation studies grafting skin from newborn α3-deficient mice onto wild-type recipients (Conti et al., 2003). The observed phenotype was partly due to an aberrant organization of the actin cytoskeleton. We are currently investigating the cause of hair loss in our model.

β1 integrins are required for keratinocyte migration in vitro and during wound healing (Grose et al., 2002). Since the deletion of α2 integrin had no effect on wound re-epithelialization (Chen et al., 2002; Zweers et al., 2006), we expected α3β1 integrin to be essential for keratinocyte migration. Surprisingly, we found that wounds heal slightly faster in *Itga3*$^{flox/flox}$; K14-Cre mice than in *Itga3*$^{flox/flox}$ mice, suggesting an inhibitory effect of α3 on wound healing. We did not detect any difference in the distribution of integrins during re-epithelialization, suggesting that the absence of α3 integrin had no effect on other integrins. A similar conclusion was reached previously when the skin of neonatal α3-null animals was investigated under steady-state conditions (Hodivala-Dilke et al., 1998). This suggests that it is the absence of α3 integrin itself that causes the increased migration and that α3β1 integrin slows down keratinocyte migration during wound healing. We assume that the β1 integrin driving epidermal migration is α5β1, α9β1 or αvβ1, or combinations of two or three of these integrins. The integrins α5β1 and αvβ1 are expressed de novo upon wounding and bind fibronectin that is deposited in the provisional matrix (Cavani et al., 1993; Juhasz et al., 1993; Larjava et al., 1993).

To confirm our observations in vitro, we isolated keratinocytes from newborn *Itga3*$^{flox/flox}$ mice and deleted *Itga3*, thus generating cells identical to those of the original clones except that they do not contain α3 integrin. Although a role of α3β1 integrin in the establishment of cell-cell contacts has been suggested (Carter et al., 1990b; Wang et al., 1999), we found no obvious defects in the localization of tight junction or adherens junction proteins in the knockout cells. Adhesion to Ln-332 was somewhat compromised, whereas adhesion to Col-1 was not impaired. Cell spreading, however, 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 integrin α6β1, which also binds Ln-332 (Delwel et al., 1994), probably rescues cell spreading 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

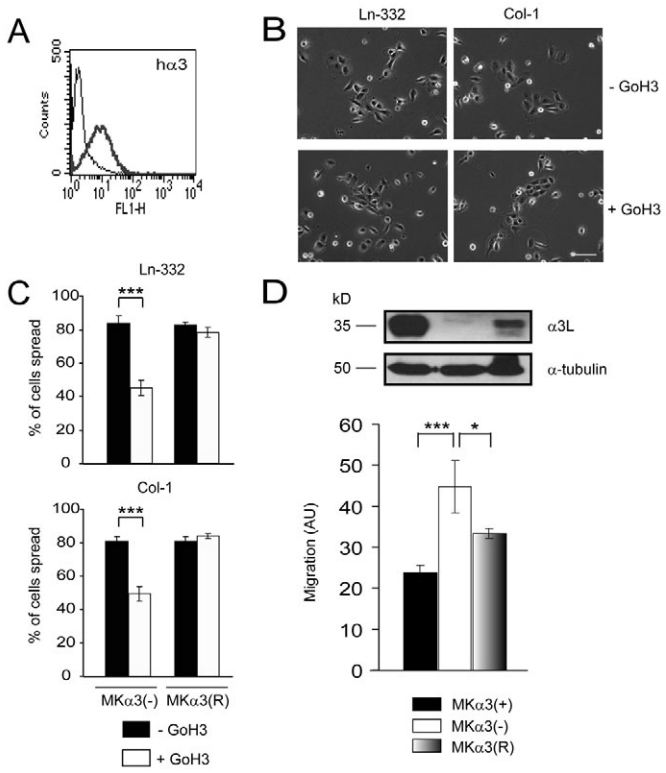

Fig. 8. The phenotype of MKα3⁻ cells is reversed by reconstitution with human α3A. (A) MKα3⁻ cells were transduced with the cDNA encoding human α3A, and cell surface expression was verified by FACS analysis using monoclonal antibody J143 against human α3. Negative control (secondary antibody only) is indicated by thin 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 hours with GoH3 (10 μg/ml). Scale 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 three 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 hours 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 ± s.e.m. of three independent experiments (*P<0.05, ***P<0.0005).

Tiam1-Rac signaling (Hamelers et al., 2005). From the results presented here, it is obvious that α3β1 integrin is not essential for Ln-332 deposition. There are probably other integrins that can create a platform for Rac activity (DeMali et al., 2003) and thus, in this case, for Ln-332 deposition. Indeed, Rac activity levels were the same in the presence or absence of α3 integrin (data not shown). Whereas in vitro adhesion and cell spreading was rescued by increased surface expression of α6β1 integrin, we only detected very low amounts of α6β1 integrin in vivo in the epidermis of both *Itga3*$^{flox/flox}$; K14-Cre and *Itga3*$^{flox/flox}$ animals, and there it was not upregulated after α3 was deleted (our unpublished data).

We investigated migration in more detail using in vitro migration assays. Whereas MKα3^{+} keratinocytes moved in a random fashion, mainly in circular and 'back-and-forth' patterns, α3⁻ cells migrated faster and with a higher directional persistence. This is a direct consequence of the absence of α3β1, which would suggest that α3β1 integrin under normal conditions retards keratinocyte migration, or an effect of the upregulation of α6β1 integrin, suggesting that it specifically enhances the velocity and directionality of migration. Alternatively, since in some studies α6β4 integrin has been implicated in migration, this integrin might become activated and stimulate migration when α3 integrin is absent (reviewed by Wilhelmsen et al., 2006; Margadant et al., 2008). However, this is probably not the case, because (1) we found no difference in the cell surface levels of α6β4 in the absence or the presence of α3, (2) neither did we detect a redistribution of α6β4 from hemidesmosomes into migration-associated structures, which would be expected if β4 was activated to stimulate migration, and (3) the enhanced migration of α3-null cells is not only observed on Ln-332 but also on Col-1, which is not a ligand for α6β4. It is unlikely that the enhanced migration is caused by the upregulation of α6β1 integrin, because an increased migration velocity was also reported of keratinocytes derived from α3-null mice, in which α6β1 was not upregulated (Hodivala-Dilke et al., 1998; DeHart et al., 2003). Similarly, inhibiting α3 integrin function with blocking antibodies in human keratinocytes increased migration, without affecting the levels of other integrins (Kim et al., 1992; O'Toole et al., 1997). Finally, enhanced migration of the α3-negative keratinocytes is observed on Col-1 to which α6β1 integrin cannot bind.

We thus conclude that α3β1 integrin slows down migration because it binds to the Ln-332 that the cells deposit when they migrate over the provisional matrix. Consistent with this conclusion, the Ln-332 deposits are always observed at the cell rear and in migration tracks but not at the front of the migrating cells (Frank and Carter, 2004). Why in the absence of α3 integrin, the additionally formed α6β1 integrin is not equally efficient in inhibiting migration as α3β1, can be explained by the much higher affinity of α3β1 than α6β1 for Ln-332 (Delwel et al., 1994; Nishiuchi et al., 2006), as well as by the relatively low amounts of α6β1 on MKα3⁻ cells compared with those of α3β1 on MKα3^{+} cells. This is underscored by the reduced adhesion and cell spreading of the MKα3⁻ cells. Thus, we consider the enhanced motility in the absence of α3β1 integrin to be mainly a direct effect of the loss of inhibition of migration caused by the binding of the cells to Ln-332 via α3β1 integrin. In agreement with this supposition is the hypermotility of keratinocytes isolated from an epidermolysis bullosa patient who does not express the γ2 chain of Ln-332 (Miquel et al., 1996), which is reversed by the restoration of Ln-332 expression (Gagnoux-Palacios et al., 1996). However, the exact effect of the α3β1–Ln-332 interaction on keratinocyte migration is controversial, because promotion of migration of normal human keratinocytes by this interaction was also reported (Zhang and Kramer, 1996; Nguyen et al., 2000). Similarly, human keratinocytes defective in Ln-332 expression because of a deletion in the *LAMB3* gene were found to migrate with decreased velocity and directional persistence (Hartwig et al., 2007). The apparent paradox is most likely explained by differences in the expression of laminin-binding integrins and the matrices used; in the absence of α6β1 integrin, α3β1 is required for polarization, cell spreading and migration (Choma et al., 2004), 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 whereas the laminin-binding integrins maintain adhesion to endogenous Ln-332 deposits at the rear of the cell. Interaction with these deposits regulates cell polarization (Frank and Carter, 2004), and is crucial to maintain adhesion during migration. This is illustrated by the observation that GoH3 induced detachment of both sessile

and migrating MKα3⁻ cells (unpublished data). 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. In conclusion, we show that integrin α3β1 inhibits the velocity and directionality of keratinocyte migration in vitro, and wound healing in vivo.

Materials and Methods

Generation of *Itga3* conditional-knockout mice

A genomic fragment of 15 kb encompassing exon 1 to exon 3 of *Itga3* was isolated from a Lambda-FixII SV129 library and subcloned into plasmid vector pBS-SK$^+$. A single *loxP* site and a *loxP*-PGK*neo*^{r}-PGK*tk*-*loxP* (floxed *neo/tk* cassette) were inserted into *Hpa*I and *Bam*H1 sites, respectively. The targeting construct (excised from the plasmid with *Not*I and *Swa*I) 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. One recombinant clone harboring the conditional *Itga3* allele was injected into blastocysts from C57BL/6 mice, which were transferred to mothers of the same strain. The chimeric male offspring was mated with FVB/N females. Agouti offspring were screened for the presence of the conditional allele by genotyping tail DNA with primers P1 (GAACAACATCTGCCTGGAGT) and P2 (GTATGACTTCTGCCATGTAGC). Heterozygous mice were intercrossed and homozygous mice were used to generate animals that were transgenic for the K14-Cre recombinase (Huelsken et al., 2001) and carried the conditional *Itga3* alleles. The K14-Cre transgene was detected by PCR with primers K14-Cre3 (CGATGCAACGAGTGATGAGGTTC) and K14-Cre5 (GCACGTTCACCGGCATCAAC). Removal of exon 1 by Cre-mediated recombination was confirmed by PCR using primers P1 and P3 (CAACAGCACTGCTGTAGCA). To obtain *Itga3*$^{flox/flox}$; K5-Cre mice with a keratinocyte restricted deletion of the *Itga3* gene, we crossed the floxed *Itga3* mice with transgenic mice expressing Cre under the control of the bovine keratin-5 promoter (Ramirez et al., 2004). 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*$^{flox/flox}$ mice and Cre-mediated deletion of the *Itga3*$^{flox/flox}$ allele was performed essentially as described previously (Raymond et al., 2005). For all experiments, three clones were used with similar results. Throughout this study, results obtained with one 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 (Sterk et al., 2000). Cells were cultured at 37°C and 5% CO_2 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 hours in DMEM (Gibco BRL) containing 10% FCS and 100 U/ml penicillin/streptomycin. Rac-11P cells (Sonnenberg et al., 1993) 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, Carpinteria, CA), integrin α3A (29A3) (de Melker et al., 1997), human integrin α3 (J143) (Kantor et al., 1987) and pan-actin (Chemicon International). Rat mAbs were: GoH3 against α6 (Sonnenberg et al., 1988), 4G6 against Ln-511 from Lydia Sorokin (University of Münster, Münster, Germany), 346-11A against β4 from Steve J. Kennel (Oak Ridge Laboratories, Oak Ridge, TN) and BMA5 against α5 and MB1.2 against β1, both from Bosco 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 polyclonal antibody against α3 from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies were directed against human integrin β1 (U19E, from Ulrike Mayer, University of East Anglia, Norwich, UK), ZO-1 and occludin (Zymed laboratories), BP180 (mo-NC16a) from Leena Bruckner-Tuderman (University of Freiburg, Freiburg, Germany), Col-IV from Eva Engvall (The Burnham Institute, La Jolla, CA), Ln-332 and Nd from Takako Sasaki (Shriners Hospital for Children Research Center, Portland, OR), CD3 from Thermo Fisher Scientific (Fremont, California), and filaggrin, involucrin and keratin 5, keratin 6 and keratin 14 from Covance Research Products. 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 (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 minutes at 37°C. Ln-332-containing matrix was prepared by growing Rac-11P cells to confluency, before overnight detachment with 10 mM EDTA at 4°C. The plates were then washed twice with PBS, blocked with 2% BSA for 1 hour 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 (Hamelers et al., 2005). Sections were photographed on an Axiovert S100 wide-field 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 intraperitoneally (50 μg/g body weight) at 2 or 4 days after wounding, 3 hours before sacrifice. The number of BrdU$^+$ cells was determined from sections using ImageJ and expressed as a percentage of the total number of cells. At least 30 sections were examined.

Immunoprecipitation and western blotting

Immunoprecipitation of integrins was performed as described previously (Sterk et al., 2000). Total cell lysates were prepared in SDS sample buffer, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and analyzed by western blotting. Bound antibodies were detected using the ECL detection system from GE Healthcare.

Ultrastructural analysis, immunofluorescence microscopy and flow cytometry

Electron microscopy on mouse skin was performed essentially as described previously (Raymond et al., 2005). Skin cryosections and coverslips with cells were incubated with antibodies as previously described (Raymond et al., 2005). Images were acquired at room temperature with a confocal Leica TCS NT or AOBS microscope using ×20 (NA 0.7) dry, ×40 (NA 1.25) oil and ×63 (NA 1.32) oil objectives (Leica) and AxioVision 4 software (Carl Zeiss MicroImaging). Pictures were processed and cell debris was masked using Photoshop 7.0 and ImageJ. Flow cytometry and cell sorting were performed as previously described (Sterk et al., 2000).

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 3×10^4 cells per well. After 30 minutes at 37°C, nonadherent cells were washed away with PBS. The adherent cells were fixed in 4% paraformaldehyde, washed twice with H_2O, stained for 10 minutes with crystal violet, washed twice with H_2O, 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 cells were allowed to spread for 5 hours, after which the cells were maintained with or without GoH3 (10 μg/ml) for an additional 3 hours. Cells were then photographed on a Widefield CCD system using ×10 and ×20 dry lens objectives (Carl Zeiss MicroImaging) 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 three 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, Breda, The Netherlands; 10 μg/ml) was added 2 hours prior to scratching with a yellow pipette tip. After washing twice 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 minutes at 37°C and 5% CO_2 on a Widefield CCD system using a ×10 dry lens objective (Carl Zeiss MicroImaging). 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 ± s.e.m. of three independent experiments. The number of polarized cells was counted from ~250 cells pooled from four 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 hour. 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 ± s.e.m. pooled from four independent experiments, or the averages of one representative experiment as indicated.

Statistical analysis

Data were analyzed using a homoscedastic two-tailed *t*-test. $P<0.05$ was considered statistically significant.

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Note added in proof

During the revision of this manuscript, an interesting paper was published on the role of α3 integrin in wound healing. Confirming the results presented in our paper, migration was enhanced in the absence of α3 integrin in unstimulated conditions. When stimulated with TGFβ, migration was retarded in the absence of α3, as was wound re-epithelialization in vivo. The authors conclude that integrin α3β1 is important for wound re-epithelialization, not as a mediator of migration but by modulating TGFβ signaling. However, the in vivo experiments were performed by transplanting skin grafts from α3-null mice onto nude mice, which creates several drawbacks. For example, there is still a wild-type environment around the α3-negative graft. Furthermore, the dermis is also α3-negative in this system. In addition, immunocompromised mice were used, which must have implications for normal wound healing (Reynolds et al., 2008).

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