Epidermal-specific deletion of CD44 reveals a function in keratinocytes in response to mechanical stress

CD44, a large family of transmembrane glycoproteins, plays decisive roles in physiological and pathological conditions. CD44 isoforms are involved in several signaling pathways essential for life such as growth factor-induced signaling by EGF, HGF or VEGF. CD44 is also the main hyaluronan (HA) receptor and as such is involved in HA-dependent processes. To allow a genetic dissection of CD44 functions in homeostasis and disease, we generated a Cd44 floxed allele allowing tissue- and time-specific inactivation of all CD44 isoforms in vivo. As a proof of principle, we inactivated Cd44 in the skin epidermis using the K14Cre allele. Although the skin of such Cd44<sup>Δ<sub>ker</sub></sup> mutants appeared morphologically normal, epidermal stiffness was reduced, wound healing delayed and TPA induced epidermal thickening decreased. These phenotypes might be caused by cell autonomous defects in differentiation and HA production as well as impaired adhesion and migration on HA by Cd44<sup>Δ<sub>ker</sub></sup> keratinocytes. These findings support the usefulness of the conditional Cd44 allele in unraveling essential physiological and pathological functions of CD44 isoforms.

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The function of CD44 in skin epidermis

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mild changes observed in the \( \text{Cd44}^{+/−} \) mice, transgenic animals expressing an antisense CD44 cDNA under the control of a keratin-5 promoter displayed a drastic skin phenotype.\(^{16} \) Second, \( \text{Cd44}^{+/−} \), \( \text{Met}^{+/−} \) mice are haplo-insufficient and most mutants die briefly after birth from a breathing defect that results from impaired synaptogenesis and axon myelination.\(^{7} \) This result suggests that CD44 and MET co-operate in vivo and that \( \text{Cd44}^{+/−} \) mice establish a rescue function to maintain activity of the MET pathway, which is not sufficient when MET levels are reduced. Such a rescue function is exemplified in hepatocytes where the intercellular cell adhesion molecule ICAM-1 takes over the co-receptor function of CD44 for MET when \( \text{Cd44} \) is inactivated.\(^{17} \)

For all these reasons, the best way to study CD44 functions in vivo is by cell-specific conditional inactivation. Therefore, we engineered a \( \text{Cd44} \) floxed allele which when combined with Cre eliminates an essential constant exon (exon 3 designated c3, Figure 1a) and thereby all CD44 isoforms. Using this novel allele, we first investigated the role of CD44 in the skin. The skin is a protective barrier against influences of the external environment and the tightly controlled proliferation and differentiation of epidermal keratinocytes along the three epidermal layers is essential for homeostasis of this highly regenerative tissue. On wounding, a complex process involving inflammation, proliferation and remodeling\(^{18} \) takes place by which basal keratinocytes together with hair follicle stem cells are able to replace the epidermal cell population.\(^{19,20} \) Deregulation of these processes can lead to severe skin diseases such as hyperkeratosis, hyperplasia or cancer.

In this paper we show that while the skin of keratinocyte-deficient \( \text{Cd44} \) mice (\( \text{Cd44}^{−/−} \)) appears normal, epidermal stiffness is decreased and in vivo wound healing delayed. Moreover, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell proliferation and differentiation is also disturbed. These defects are cell autonomous and \( \text{Cd44}^{Δ\text{ker}} \) keratinocytes display a decreased production of HA, impaired adhesion, morphological dynamics and locomotion.

Results

Consequence of targeted \( \text{Cd44} \) deletion in K14 expressing cells on skin homeostasis. A \( \text{Cd44} \) floxed allele was generated by gene targeting in ES cells. Exon 3, present in all CD44 isoforms, was flanked by LoxP sites. Cre recombinase leads to excision of exon 3, to the generation of an in-frame stop codon and subsequent disruption of protein synthesis in the cells where Cre is expressed leading to loss of all CD44 isoforms (Figure 1a). After germ-line transmission of the \( \text{Cd44} \) floxed allele, \( \text{Cd44} \) floxed mice were crossed with \( \text{K14Cre} \) mice\(^{21} \) to generate \( \text{Cd44}^{Δ\text{ker}} \) animals. The \( \text{K14Cre} \) transgene is expressed as early as 9.5 days post-coitum in complex epithelia such as the epidermis.\(^{21} \) The removal of Cd44 in the whole epidermis and in isolated primary keratinocytes of \( \text{Cd44}^{Δ\text{ker}} \) mice was confirmed by histological stainings (1b) PCR, Southern blot and Western blot analyses while qRT-PCR revealed a 70% decrease in CD44 mRNA (Figures 1b, c and Supplementary Figure S1). The corresponding mRNA is not expressed as protein as shown in the western blot (Figure 1c). The western blot analysis also reveals that the majority of CD44 proteins expressed in the epidermis of control mice contain the v6 exon since the staining pattern with CD44v6 specific antibodies is nearly identical to that obtained with a pan CD44 antibody that recognizes all CD44 isoforms (Figure 1c). We confirmed that \( \text{Cd44} \) was also inactivated in other K14-expressing organs such as the oral epithelium, tongue, mammary epithelium and thymus (data not shown).

To analyze potential morphological changes in the skin, \( \text{Cd44}^{Δ\text{ker}} \) mice and control littermates were killed at different ages and the skin harvested for histological analysis (Figure 1d). A hematoxylin-eosin (H&E) staining, a Gomori staining showing the distribution of the reticulin fibers, a Verhoeff’s Van Gieson staining detecting collagen fibers and a Masson Goldner staining visualizing connective tissue were performed. As shown in Figure 1d (new born) but also in 2, 6 and 30 weeks old mice, (data not shown) no structural changes could be detected in the skin of \( \text{Cd44}^{Δ\text{ker}} \) mice. Specifically, no edema was observed and the number or morphology of the dermal fibroblasts did not significantly differ. Moreover, fibers orientation seemed not to be affected and dermal stability was not disturbed. Finally, no epidermal hyperplasia or atypical keratinocytes were identified. Therefore, no gross abnormalities were observed in the dermis or the epidermis. Observation of \( \text{Cd44}^{Δ\text{ker}} \) mice for a long time period (a year) did not reveal any spontaneous skin phenotype (data not shown).

Lack of \( \text{Cd44} \) in the skin interferes with wound healing.

We next addressed the response of the skin to stress and performed wound healing experiments in \( \text{Cd44}^{Δ\text{ker}} \) mice. To this end 4 mm full-thickness excisional wounds were performed on 10–12-week-old \( \text{Cd44}^{Δ\text{ker}} \) mice and control littermates (Figure 2a) and the size of the wound determined. A difference in the relative wound area was observed between the two cohorts from day 1 on. The most drastic and statistically significant difference was observed at day 8, where \( \text{Cd44}^{Δ\text{ker}} \) mice showed a striking delay in wound closure while the wounds of \( \text{Cd44}^{+/−} \) mice were already completely closed (Figure 2a).

To analyze the contribution of keratinocytes in the re-epithelialization (Figure 2b), wound sections were stained for keratin 6 (K6), which is specifically induced in hyperproliferative and migrating keratinocytes during wound healing.\(^{22–24} \) At each time point the distance between the...
migration fronts of the keratinocytes was larger in \( \text{Cd44}^{\Delta \text{ker}} \) mice, further demonstrating that inactivation of \( \text{Cd44} \) in the epidermis leads to delayed wound healing (Figure 2b). In striking contrast, we did not observe any difference in wound closure between the \( \text{Cd44}^{+/−} \) germ-line knockout and control mice (Supplementary Figure S2A). This observation confirms that functions of \( \text{Cd44} \) are substituted in the germ-line knockout keratinocytes as was also the case in hepatocytes.\(^{17} \) This substitution apparently does not occur if the inactivation of the \( \text{Cd44} \) gene arises at later times in embryogenesis, as is the case in the conditional knockout (the K14 promoter for Cre expression is active only from E9.5) mice.

**Impaired keratinocyte proliferation and differentiation in \( \text{Cd44}^{\Delta \text{ker}} \) mice.** We next tested the response of epidermal \( \text{Cd44}^{\Delta \text{ker}} \) keratinocytes to hair plucking and to TPA treatment\(^{25} \) (Figure 3).

As reported,\(^{26} \) hair plucking induced keratinocytes proliferation. Strikingly, quantification of Ki67 staining revealed that the proliferative response of \( \text{Cd44}^{\Delta \text{ker}} \) keratinocytes was much less pronounced than in controls (Figure 3a). Interestingly, however, hair regrowth did not appear delayed in \( \text{Cd44}^{\Delta \text{ker}} \) mice (not shown).

TPA treatment induced epidermal thickening of the skin as compared with acetone treatment as measured by H&E staining (Figure 3b). This increase was much less pronounced in \( \text{Cd44}^{\Delta \text{ker}} \) mice (Figure 3b). Taken together, these results indicate an impaired proliferative response of \( \text{Cd44}^{\Delta \text{ker}} \) keratinocytes. In support of this, isolated \( \text{Cd44}^{\Delta \text{ker}} \) keratinocytes showed reduced clonogenic efficiency as compared with controls and germ-line knockout keratinocytes (Figure 3c).

The epidermis consists of four different layers expressing various sets of keratins: The basal layer is characterized by expression of K5 and K14, the spinous layer by expression of K5 and K14, the granular layer produces filaggrin,\(^{28,29} \) and the cornified layer, loricrin.\(^{30,31} \) We made use of these markers to analyze the impact of \( \text{Cd44} \) on epidermal differentiation. Skin sections of TPA treated animals were stained immune fluorescently with the markers K14, K10, filaggrin and loricrin (Figure 3b). Fluorescence intensity increased for all markers in TPA treated animals consistent with the induction of epidermal marker expression by TPA. When calculating the ratio of the stained layers relative to the total epidermal thickness, we observed that the relative thickness of each epidermal layer was not changed between acetone-treated controls and TPA-treated \( \text{Cd44} \)-proficient animals. However, in \( \text{Cd44}^{\Delta \text{ker}} \) mice, we observed a reduction in the relative thickness of the K10 and filaggrin-positive layers while the relative thickness of the K14 and loricrin layers appeared unaffected (Figure 3b). In addition, overall fluorescence intensity appeared weaker. These results indicate that genetic inactivation of \( \text{Cd44} \) in the K14-expressing cells has also an impact on differentiation of keratinocytes. The lack of effect on relative K14 or loricrin levels might be due to the timing of the experimental endpoint.

Consistent with the *in vivo* data, we observed a delayed induction of keratin genes *in vitro*, when treating keratinocytes isolated from \( \text{Cd44}^{\Delta \text{ker}} \) mice for 2 h with TPA. This was most apparent for Keratin 5 and Keratin 14, and to a lesser extent for Keratin 1 (Supplementary Figure S2B upper part). Interestingly, TPA induction of several genes encoding for AP-1 family members that are robustly induced by TPA, was also delayed (Supplementary Figure S2B lower part).

**Removal of \( \text{Cd44} \) decreased the adhesion of keratinocytes to HA and their migration on HA surfaces.** A prominent component of the extracellular matrix in the skin is HA.\(^{32} \) As \( \text{Cd44} \) is the main receptor for HA\(^{10} \) we next performed a HA staining on isolated keratinocytes and on skin sections using the biotinylated HA-binding protein (Bio-HABP). Interestingly, the HABP staining did not reveal changes in the overall HA distribution in the skin sections neither during homeostasis nor during wound healing (Supplementary Figure S3A). This might be due to the contribution of cells from the dermis that still express \( \text{Cd44} \). In contrast, in the isolated keratinocytes, a positive staining was detected only in \( \text{Cd44}^{+} \) keratinocyte cultures as shown in Figure 4a. The decreased HA staining in \( \text{Cd44}^{\Delta \text{ker}} \) keratinocytes suggests that the cells are either unable to bind to HA and/or to produce HA. We quantified the amount of HA produced by \( \text{Cd44}^{+} \) and \( \text{Cd44}^{\Delta \text{ker}} \) keratinocytes using solid-phase ELISA in cell culture supernatants (Figure 4b). A significant decrease of HA production was observed in the supernatant of \( \text{Cd44}^{\Delta \text{ker}} \) cultures consistent with previous reports indicating that \( \text{Cd44} \) directly influences HA production.\(^{33,34} \)

We next investigated the adhesion of cells on oligo-HA functionalized membranes (scheme in Supplementary Figure S3B) utilizing a combination of phase contrast and non-invasive live-cell imaging by reflection interference contrast microscopy (RICM). Figure 4c represents phase contrast and corresponding RICM images for \( \text{Cd44}^{\Delta \text{ker}} \) and \( \text{Cd44}^{+} \) (control) keratinocytes. The area of tight cell adhesion can be identified from the dark area in RICM images without any additional fluorescent labeling (Figure 4c, highlighted by a red line).\(^{35} \) The fraction of adherent cells (\( \chi \)) was significantly lower for \( \text{Cd44}^{\Delta \text{ker}} \) cells as compared with \( \text{Cd44}^{+} \) keratinocytes (Figure 4d, left). In addition to this defect in the collective cell adhesion, the adhesion area \( A_{\text{adh}} \) of individual keratinocytes was also significantly decreased by a factor of 3 on \( \text{Cd44} \) inactivation (Figure 4d, right). These results indicate that the binding ability of \( \text{Cd44}^{\Delta \text{ker}} \) cells to HA is impaired.

We next investigated the morphological dynamics and migration of \( \text{Cd44}^{\Delta \text{ker}} \) and \( \text{Cd44}^{+} \) keratinocytes. The \( \text{Cd44}^{\Delta \text{ker}} \) keratinocytes that lack HA binding capability, show much longer and more persistent trajectories on oligo-HA functionalized membranes as compared with \( \text{Cd44} \)-proficient control keratinocytes (Figure 4e, i–iii) similarly to control keratinocytes plated on pure membrane (no HA; Figure 4e, iii).

Dynamic morphological fluctuations of cells are active processes due to bending and stretching of their membranes and remodeling of their cytoskeleton by ATP consumption.\(^{36,37} \) The corresponding deformation energy can be estimated from the power spectrum analysis as reported previously.\(^{38} \) Figure 4f illustrates power spectra of \( \text{Cd44}^{\Delta \text{ker}} \) and \( \text{Cd44}^{+} \) keratinocytes on oligo-HA functionalized membranes and the corresponding data for \( \text{Cd44}^{+} \) cells on pure membranes (no HA; Supplementary Figure S3C). A
maximum of the curve at mode $m=2$ suggested that keratinocytes predominantly undergo elliptic deformation as schematically depicted in the inset (Figure 4f, left). The summation over all modes of deformation $\sum f_{\text{deform}}(m)$ reflecting the total energy consumption was strikingly decreased in Cd44$^{\text{Δker}}$ keratinocytes plated on HA functionalized membranes as compared with control keratinocytes while cells plated on pure membranes (no HA) did not show any deformations (Figure 4f, right).

**Decreased epidermal stiffness in Cd44$^{\text{Δker}}$ mice.** One essential role of the skin is to provide elasticity and resistance to stretch and the HA-related defects of keratinocytes lacking Cd44 could affect these intrinsic properties of the skin epidermis. We therefore examined the skin mechanical properties using atomic force microscopy (AFM) for nanoindentation, a method that quantifies tissue mechanical and elastic properties. AFM elasticity measurements were performed on mouse skin tissue slices and the indenter bead was positioned over the dermal or epidermal regions (Figure 4g, shown for new born mice, sections of 2-, 6- and 30-week-old mice gave similar results) in the same experimental set-up.

AFM analysis revealed a striking decrease in epidermal stiffness in Cd44$^{\text{Δker}}$ mice, whereas the dermis was not affected. Reasons for the decrease in epidermal stiffness in Cd44$^{\text{Δker}}$ mice could be reduced HA production and/or lack of CD44-mediated adhesion to HA (Figure 4f). Since the mechanical environment of the wound site is important for the healing process, delayed skin wound healing in Cd44$^{\text{Δker}}$ mice could thus be the collective result of impaired keratinocyte proliferation, CD44/HA-mediated migration and adhesion together with altered biomechanical properties of the epidermis.

**Discussion**

CD44 is a multi-tasking family of proteins involved in signaling pathways essential for embryonic development. Therefore the mild phenotype of the Cd44 germ-line knockout mice suggested that CD44 is functionally substituted during embryogenesis. Such a lack of phenotype in germ-line knockout mice was described for several other genes and substitutions based on up-regulation of members of the same family or functionally related proteins have been identified. For instance fibroblast growth factor-2 (Fgf-2) knockout mice
develop normally and display normal liver regeneration.\textsuperscript{41} VEGF was shown to substitute for FGF-2 and the VEGF inhibitor SU5416 inhibited liver regeneration in Fgf-2 knockout mice but not in control animals.\textsuperscript{42} Similarly, the inactivation of the \textit{tau} gene encoding for a major neuronal microtubule-associated protein essential for neuronal cell morphogenesis ...
and axonal elongation and maintenance only led to mild defects in microtubule stability due to compensation by another protein, MAP-1A.43

In the case of CD44, several evidences support substitution events in the Cd44Δ−/− mouse. For example, the presentation of FGF produced in the apical ectodermal ridge to its receptor FGFR on the underlying mesenchymal cells by CD44v3 during limb development1 is overtaken by another heparan sulfate proteoglycan, upregulated in the Cd44Δ−/− mice.44 Furthermore, RHAMM, another HA receptor, compensates for CD44 in the Cd44Δ−/− mice.45 Finally, we have shown that ICAM-1 substitutes for CD44v6 in the Cd44Δ−/− mice as a coreceptor for MET in vivo and in vitro.17

The most appropriate strategy to identify functions of CD44 in vivo in various tissues and different stages of development is the use of a conditional inactivation of the Cd44 gene with the expectation that a substitute molecule will not be expressed. This strategy additionally allows deciphering cell autonomous functions of CD44 in the context of an otherwise wild-type tissue. Using the Cre-Lox technology to inactivate Cd44 we show in this paper that specific abrogation of CD44 in the skin epidermis has little apparent impact on the gross morphology of the skin. However, strikingly decreased epidermal stiffness as measured by AFM was detected on removal of Cd44. In addition, keratinocytes isolated from Cd44Δker mice show reduced HA production, impaired adhesion to HA, reduced collective and individual migration, and morphological dynamics on HA functionalized surfaces. These results support a physiological function of CD44 in keratinocytes. Consistently, TPA leads to impaired epidermal thickening and differentiation in Cd44Δker mice. Delayed TPA response of Cd44Δker keratinocytes was also observed in vitro. Interestingly, we also saw a delayed TPA induction of members of the AP-1 transcription factor, an important regulator of epidermal differentiation.46–48 This suggests a possible modulation of AP-1 expression by Cd44. As Cd44 is itself a well-known target gene of AP-1 (ref. 49) a positive feedback regulation of AP-1 by Cd44 might be in place in keratinocytes subjected to differentiation signals.

We also observed a proliferation delay in Cd44Δker epidermis subjected to hair plucking. In addition, in vivo wound healing was delayed in Cd44Δker animals. Interestingly, this delay was not observed in Cd44 germ-line knockout mice, further supporting a substitution of Cd44 in early embryogenesis.

The most likely explanation for the delayed wound healing in Cd44Δker mice is an impairment of HA synthesis by keratinocytes and the inability of keratinocytes to attach and migrate on exogenous HA. The importance of HA synthesis for keratinocytes migration has been previously demonstrated by comparison of two different keratinocyte cell lines, carrying the HA synthase 2 (Has2) gene in sense or antisense orientation to increase or decrease HA synthesis, respectively.50 Keratinocytes expressing the Has2 sense gene migrated faster in an in vitro wounding assay, whereas Has2 antisense cells migrated more slowly. Interestingly, addition of exogenous HA to keratinocyte cultures did not restore the reduced migratory ability of Has2 antisense cells suggesting that the dynamic synthesis of HA controls keratinocyte migration, a key function during wound closure.50

The defects described above might result in a change in epidermal stiffness, which in turn affects wound healing. The AFM technology applied to a tissue layer allowed discriminating between the contribution of the dermis and epidermis, and suggests that the decreased epidermal stiffness might prevent rapid wound healing. Indeed, it has been previously demonstrated that stiff surfaces promote wound healing by stimulating epidermal cell proliferation and migration.51,52 Cd44Δker keratinocytes lack CD44 as the main HA receptor and also produce reduced levels of HA. The absence of CD44-dependent HA binding might thus decrease the stiffness of the wound surface and thereby delay wound healing. Furthermore, the change in the dynamic fluctuation of Cd44Δker keratinocytes (Figure 4f) might also contribute to the decreased stiffness of the epidermis.

In contrast to our observation, however, one report described delayed wound healing in Cd44Δ−/− mice due to impaired vascularization.53 In another case, decreased HA-mediated keratinocyte differentiation and lipid synthesis that interfered with normal epidermal permeability and barrier function was reported in Cd44Δ−/− mice.54,55 These contradictory results might be due to the different genetic backgrounds of the mice used in the different experimental settings as reported for genes such as Egfr.56

The gross morphology of the skin is not altered in Cd44Δker mice. This is in contradiction with drastic alterations in the structure of the dermis and epidermis of mice in which Cd44 was abolished in keratinocytes using an antisense CD44 construct expressed under the control of a K5 promoter.15 In addition, these Cd44 knockdown keratinocytes displayed a defect in HA uptake resulting in an accumulation of HA in the dermis. Some of the severe structural changes in the skin of these CD44 antisense mice could be attributed to off-target effects of antisense oligonucleotide expression in vivo.57 Interestingly, however, a change in skin elasticity, wound repair and keratinocyte proliferation were also observed in these antisense mice consistent with our observations in the Cd44Δker genetic model.

In conclusion, genetic inactivation of Cd44 in the keratinocytes allowed to more precisely defining the role of the CD44/HA pair in epidermal repair during wound healing and in the
regulation of keratinocyte differentiation, migration and proliferation. We genetically demonstrated important functions of CD44 in skin epidermis in homeostasis and acute stress conditions. Such genetic tools will allow dissecting CD44 functions in skin aging and in pathological situations such as skin hyperplasia and cancer.
Materials and Methods
Generation of Cd44 floxed mice, other mouse strains. pCd44-KO vector consisting of genomic Cd44 exons 2 and 3 (10.7 kb) derived from the mouse strain 129/Ola and a single Lox-P site inserted downstream from exon 3 were obtained from Therese Tsuchy (Beaverton, OR, USA). The short arm of Cd44 (3623 bp) was cut out from pCd44-KO vector with PspOMI and Nhel, and was ligated in pT载体Ox-5’ (constructed by Dieter Rietschel and Karin Gottschlich) from the neo-cassette with Nof and Xbal to obtain the pT载体Ox-s.a. vector. The long arm of Cd44 (6862 bp) was cut out from the pCd44-KO vector with Hpal and BamHII, and inserted into pT载体Ox-s.a. vector with Hpal and BglII 3’ from the neo-cassette. The final construct abbreviated as pCd44-KO was linearized by BssHII and electroporated into 129S2 ES cells. Correctly targeted ES cells were identified, the neo-cassette removed using pCMV-Cre and positive clones injected into C57BL/6 blastocysts and transferred to 3Hl foster mothers. After germline transmission, Cd44 floxed mice (Cd44flox) were crossed to K14Cre mice (Daniel Metzger, Strasbourg, France) to result in Cd44flox/lox mice. All experiments were performed with Cd44flox/lox mice backcrossed to C57BL/6 mice for eight generations. Controls and mutants were identified by PCR genotyping on tail DNA using the following primers: Cd44 forward 5’-GGG AGC TGA TCA TGG CTC TC-3’ and reverse 5’-TTA TTC TGC ATG TCA TAC GCG TC-3’. Cd44 reverse 5’-ACAC GTT TTG TTT TCG GA-3’ and control mice (pictures). Young keratinocytes were isolated from mouse tail skin epidermis. Mice were sacrificed by cervical dislocation and the tail skin was immediately dissected. Skin samples were immediately immersed in the fixative solution (2% paraformaldehyde, 2% sucrose, 0.1 M sodium phosphate buffer, pH 7.4) and kept overnight at 4°C. Then, samples were rinsed twice with PBS and embedded in OCT compound (Sakura Finetek, USA). Three to 5µm sections were cut on a cryostat and dried on a silane-coated glass slide overnight at 4°C. Sections were fixed with 4% paraformaldehyde for 10 minutes at room temperature, washed three times with PBS, and blocked with 2% BSA in PBS for 30 minutes at room temperature.

Primary keratinocyte culture. All experiments were exclusively performed with keratinocytes isolated from mouse tail skin epidermis. Mice were sacrificed by decapitation and the tail was removed, vertically cut with a razor and the skin was pulled off. The epidermis was separated from the dermis by placing the skin (dermal side down) into 1% trypsin for 1 h at 37 °C. The epidermis was cut into small pieces, transferred into Defined Keratinocyte-SFM (Serum Free Medium) (Life Technologies) containing 10% FCS and 0.25 mg/ml DNAse and incubated 1 h at 37 °C. Disaggregated cells and tissue clumps were poured through a sterile cell strainer (70 µm), centrifuged and re-suspended in Defined Keratinocyte-SFM medium, seeded on coated plates (Coating Matrix, Life technologies) and incubated at 37 °C, 5% CO2. On the next day the cells were washed with PBS and suspended in new Keratinocyte-SFM medium. Cells were used after 2-5 days of culture.

Preparation of genomic DNA from different tissues and from keratinocytes. Genomic DNA from skin, liver, kidney and lung were isolated as described.58 Keratinocytes were lysed in a buffer containing 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl, 0.2% SDS and proteinase K (100 µg/ml) for 2 h at 60 °C. DNA was precipitated by addition of 100% ethanol followed by centrifugation for 2 min at 10,000 r.p.m. Pellets were washed with 70% ethanol, centrifuged for 2 min at 10,000 r.p.m., dried and suspended in TE buffer.

Southern blot analysis. Genomic DNA (15 µg) from mouse keratinocytes was digested with Hind III (Fermentas, St. Leon-Rot, Germany) and resolved on a 0.8% agarose gel. 0.4 M NaOH was used to transfer DNA fragments from the gel to a Zeta Probe GT Membrane (Bio-rad, Munich, Germany). The membrane was baked for 2 h at 80 °C. Hybridization was performed at 65 °C overnight using the Church buffer. A probe was produced via PCR from the bacterial artificial chromosome RP23-40814 (BACPAC Resources-CHORI, Oakland, CA, USA) using the primers Cd44 forward 5’-AGAAC CAC TTC ACT GGC TGA GC-3’ and Cd44 reverse 5’-TCT GCA CAG TCA AGA CTC TG-3’. RNA isolation and RT-PCR. Total RNA was isolated using Trizol (Sigma-Aldrich, Taufkirchen, Germany), complementary DNA was synthesized using Ready-To-Go-You-Prime-First-Strand Beads (GE Healthcare (VWR), Bruchsal, Germany) and RT-qPCR performed using GoTaq RT-qPCR Master Mix (Promega, Mannheim, Germany) and Eppendorf fluorescence thermocyclers (Eppendorf, Wesseling-Berzdorf, Germany), all following manufacturers’ instructions. The 2−ΔΔt method was used to quantify amplified fragments. Expression levels were normalized using at least one housekeeping gene (Rps29 and/or Rp4).

RNA sequence analysis. The sequence of the qPCR primers can be found in ref. 47. The sequences of the primers used for Cd44 are: 5’-GGACTGTCAGTCTGAGATCC-3’ present in exon 16 and 5’-GCACTGTGACTCAGTGGATC-3’ in exons 17-18.

Wound healing experiments. Ten to twelve-week-old male Cd44flox mice and control animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight)/xylazine (10 mg/kg body weight). The dorsal hair was shaved and the exposed skin was cleaned with 70% ethanol. Full-thickness excisional skin wounds (usually two wounds) were performed on both sides of the dorsal middle line using a 4 mm biopsy-punch. The clot was removed daily and...
pictures of the wound area were taken. The wound area was calculated using the Image J Program (National Institutes of Health, Bethesda, MD, USA). The wound closure was expressed as percentage of recovery with respect to the initial wound area.

To specifically measure the contribution of keratinocytes, the wound healing experiment was performed without removing the clot. Animals were killed at indicated time points and the wounded tissue including the surrounding wound margin skin was harvested and embedded in Tissue Freezing Medium (Leica Microsystems, Wetzlar, Germany). Wound sections were stained immune fluorescently for the activation marker keratin 6 and the distance between the migration fronts of the keratinocytes was determined.

For each experiment six wounds per genotype were analyzed. Error bars represent the S.E.M. statistical analysis was performed by Student’s t-test. P < 0.05 or P < 0.005 is indicated by one and two asterisks respectively.

TPA treatment. Seven-week-old female mice were shaved on the dorsal area of the skin and 48 h later TPA (5 nm/100 μl aceton) or acetone alone was locally applied topically on the back skin. This treatment was performed two times every 48 h. 48 h after the last treatment, the skin was harvested and fixed in 4% paraformaldehyde overnight, processed in a tissue processor for dehydration and embedded in paraffin for histological analyses. Sections were stained using H&E or embedded in paraffin for histological analyses. Sections were stained immune fluorescently for the proliferation marker Ki67. For quantification of Ki67-positive cells were counted. As a control, skin samples were taken at a distance from the plucked surface.

Colony formation assay. Murine keratinocytes of each genotype were seeded on coated (Coating Matrix mix, Thermo Fisher Scientific, Cat.nr. R-011-K) 6-well plates at 1000 cells/well. The cells were grown for 10 days. Plates were gently washed once with PBS and fixed with 3.7 % formalin for 10 min at RT. After fixation, plates were rinsed once again with PBS and colonies stained with 0.2 % crystal violet solution in 10 % ethanol for 15 min at RT. To remove the excess staining, the plates were rinsed several times with PBS. Colonies of more than 50 cells were counted under the microscope.

Immunofluorescence and immunohistochemistry. For immunofluorescence analysis, frozen or paraffin sections of the skin were cut into 7 μm-thick sections and mounted on glass slides. The unspecific binding of the antibody was blocked by 5% FCS in PBS for 1 h at room temperature followed by overnight incubation with primary antibodies diluted in blocking solution: IM7 (2 μg/ml); 9A4 (2 μg/ml); ollaggrin (1 μg/ml); αkeratin 6 (1 μg/ml); αkeratin 10 (1 μg/ml); αkeratin 14 (1 μg/ml); αloricrin (1 μg/ml). After 5 washes with PBS, the respective Alexa Fluor labeled secondary antibodies (1:500) were applied with DAPI (Sigma-Aldrich) in blocking solution for 1 h at room temperature, washed three times with PBS and mounted.

Keratinocytes were fixed in 4% paraformaldehyde for 20 min at room temperature. After washing for three times with PBS the cells were incubated with 5% FCS in PBS for 1 h at room temperature treated with IM7 (2.5 μg/μl) over night, rinsed in PBS for 3 times, incubated with Alexa Flour labeled secondary antibody (1:500) for 1 h at room temperature, washed and then mounted using the Fluorescent Mounting Medium (Dako).

For H&E staining, paraffin sections were deparaffinized in xylene, rehydrated (100% EIOH 1 min, 95% EIOH 1 min, 80% EIOH 1 min, 70% EIOH 1 min, H2O), stained with hematoxylin for 1–5 min, rinsed in H2O and stained with eosin for 1 min. Thereafter, slides were dehydrated (95% EIOH 1 min, 100% EIOH 1 min, Xylene 5 min 2X) and mounted.

To detect HA by means of a biotinylated HA-binding proteins (Bio-HABPs, 2.5 μg/ml, Merk, Darmstadt, Germany) cells were seeded in 12-well plates on sterile cover slips and stained for 24 h. Cells were fixed for 10 min with 4% paraformaldehyde (PFA) in PBS, then blocked with 5% FCS in PBS and afterwards incubated overnight with the biotinylated HA-binding protein. After incubation with Streptavidin/Alexa488 (0.4 μg/ml Invitrogen) for 45 min, nuclei were stained with DAPI (Dako, Hamburg, Germany). For controls, cells were incubated with 50 U/ml Bovine Tests hyaluronidase (Sigma-Aldrich) before fixation.

Functionalization of supported membranes with biotinylated HA oligomers

Preparation of biotinylated hyaluronic acid oligomers: HA oligomers (13–16 monomers, 0.334 mg/ml) in 0.1 M MES buffer at pH 4.5 (1.00 mg, 91 nmol) were incubated with a 10-fold molar excess of hydrazide-PEG5-Biotin (0.50 mg, 910 nmol) and 1-ethyl-3-(3-dimeThylaminopropyl)carbodiimide (0.17 mg, n = 912 nmol) at pH 4.5 (addition of 1 mM HCl). The reaction mixture was stirred for 12 h in an end-to-end motion at 25 °C, transferred to a pre-wetted Slide-A-Lyzer dialysis cassette with a molecular weight cut-off of 2 kDa (Thermo Fisher Scientific, Waltham, MA, USA) and dialyzed exhaustively against HBS buffer (10 mM Heps, 150 mM NaCl, pH 7.5).

Preparation of HA functionalized supported membranes. Cell incubation chambers were prepared according to our previous protocol.38 Then, supported membranes consisting of 2 mol% biotin-DOPC in SOPC were deposited inside the cell incubation chambers by vesicle fusion.30 Subsequent functionalization of the supported membrane with neutravidin (10 nm) and oligo-HA (10 nm) were performed as described.38 As the anchor lipids biotin-DOPC are monomerly incorporated into the matrix lipids, the average lateral distance between lipid anchors <d> and thus oligomers can be estimated from the molar fraction x of lipid anchors by inserting the value of the lipid area of A_{lipid} = 65 Å^2.

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Excess HA oligomers were removed by rinsing with Keratinocyte-SFM medium. The measurement chambers were equilibrated at 37 °C before use for cell adhesion and migration experiments.

Reflection interference contrast microscopy. Keratinocytes (5 × 10^4 cells/cm^2) were incubated for 5 h at 37 °C and 5% CO2 in cell incubation chambers in Keratinocyte-SFM medium. RICM was undertaken as described.38 Image corrections for shot noise and parabolic illumination profile were performed as described.38 The measured intensity was converted into heights, applying the RICM theory for finite INA and multiple reflecting layers, with refractive indices n = 1.525 (glass substrate), n = 1.486 (lipid membrane), n = 1.335 (cell membrane) and n = 1.37 (cytosol).60 The adhesion area A_{Adh} was calculated from the average height and s. d. in each pixel. The fraction of adherent cells χ was determined by dividing the number of adherent cells, which were identified by RICM by pixel thresholding, by the total number of cells visible on phase contrast images.

Statistical image analysis of stochastic morphological dynamics. Keratinocyte migration was followed in cell incubation chambers (5 x 10^4 cells/cm^2) in Keratinocyte-SFM medium by recording time-lapse movies over 12 h with a frame rate of 0.017 Hz with a Keyence BZ-9000 microscope (Keyence, Osaka, Japan), which was equipped with a Plan Fluor 40x/0.6 air objective. The movies were drift-corrected utilizing cross-correlation analysis. Morphological dynamics of migrating keratinocytes were quantified as described previously.38 The peripheral edge of each cell r in each phase contrast image of a time-lapse series of 60 min was detected by pixel contrast and plotted as a function of θ for 0–360° in laboratory coordinates. An amplitude map |rθ|/t) was obtained by displaying the time evolution of the radial distances r. Thereafter, the corresponding autocorrelation map 1rθ(r, t) was calculated according to

\[
1rθ(r, t) = \frac{(r(\theta + Aθ, t + A t) - r(\theta, t))}{<(r(\theta, t))^2>}
\]

A Fourier transform of the radial distance in θ and t resulted in the determination of different modes m of deformation.

\[
\hat{1}rθ(r, t) = \hat{F}(r(\theta, t)) + \hat{F}(r(\theta, t))
\]

Finally, an integration over all modes m of deformation was performed to quantify the total power of cell deformation ∑r_m. Statistics. The underlying number of experiments per data point corresponds to three experiments. In summary, the fraction of adhered cells and the adhesion area A_{Adh} were calculated for a total number of 30 cells per data point, which were presented in the corresponding figures as means ± s.d. Data characterizing the morphological dynamics of keratinocytes, comprise a total number of 20 cells per
AFM elasticity measurements. AFM elasticity measurements were performed using a NanoWizard II AFM (JPK Instruments) mounted on top of an inverted optical microscope (Carl Zeiss AxioObserver A1). Indenter probes were prepared by gluing a single 10 μm silica bead (Kisker Biotech) to the apex of a tipless V-shaped cantilever (NP-O, Veeco, Germany). Colloidal indenter probes were calibrated before and after each measurement. Frozen mouse skin tissue slices were attached to a microscopy slide and rehydrated in PBS for 20 min at room temperature. Subsequently, the indenter bead was positioned over dermal and epidermal regions as identified by light microscopy. Indentation measurements were performed by extending the closed-loop z-scanner with a constant rate of 1 μm/s until reaching a pre-set force of 2 nN, typically corresponded to cell indentation depths of <1.5 μm. Cell elasticity values were calculated from the obtained force-distance curves by applying a Hertzian mechanics model to the initial 500 nm of indentation. Elasticity measurements were performed on at least 15 different locations within the dermal or epidermal regions, using skin slices from at least three different animals per condition. The statistical significance between experimental groups was determined using a one-way ANOVA test.

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
MS performed most of the experiments and analyzed the data. ASB performed all RCM measurements and analyzed the data and wrote the corresponding text. CMF performed the AFM measurements, analyzed the data and wrote the corresponding text. GP KB and EH performed part of the experiments. AD and TK contributed to the development of the CD44 floxed mice. EFW, TNH and MT designed part of the experiments and analyzed the data. HP analyzed the data and participated in the writing of the manuscript. LB performed part of the experiments and contributed to the writing of the manuscript. VDR contributed to the production of the CD44 floxed, analyzed the data and wrote the manuscript.

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