Injury-Driven Stiffening of the Dermis Expedites Skin Carcinoma Progression

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Abstract

Recessive dystrophic epidermolysis bullosa (RDEB) is a genetic skin fragility disorder characterized by injury-driven blister formation, progressive soft-tissue fibrosis, and a highly elevated risk of early-onset aggressive cutaneous squamous cell carcinoma (cSCC). However, the mechanisms underlying the unusually rapid progression of RDEB to cSCC are unknown. In this study, we investigated the contribution of injury-induced skin alterations to cSCC development by using a genetic model of RDEB and organotypic skin cultures. Analysis of RDEB patient samples suggested that premalignant changes to the dermal microenvironment drive tumor progression, which led us to subject a collagen VII hypomorphic mouse model of RDEB to chemical carcinogenesis. Carcinogen-treated RDEB mice developed invasive tumors phenocopying human RDEB-cSCC, whereas wild-type mice formed papillomas, indicating that the aggressiveness of RDEB-cSCC is mutation-independent. The inherent structural instability of the RDEB dermis, combined with repeated injury, increased the bioavailability of TGFβ, which promoted extracellular matrix production, cross-linking, thickening of dermal fibrils, and tissue stiffening. The biophysically altered dermis increased myofibroblast activity and integrin β1/pFAK/pAKT mechanosignaling in tumor cells, further demonstrating that cSCC progression is governed by pre-existing injury-driven changes in the RDEB tissue microenvironment. Treatment of three-dimensional organotypic RDEB skin cultures with inhibitors of TGFβ signaling, lysyl oxidase, or integrin β1-mediated mechanosignaling reduced or bypassed tissue stiffness and limited tumor cell invasion. Collectively, these findings provide a new mechanism by which RDEB tissue becomes malignant and offer new druggable therapeutic targets to prevent cSCC onset. Cancer Res; 76(4); 940–51. ©2015 AACR.

Introduction

Cutaneous squamous cell carcinoma (cSCC) is among the most commonly diagnosed cancers with invasive and metastatic potential. Although mostly moderately progressive and easily resectable, approximately 4% of cSCCs metastasize (1). Besides UV irradiation as the major cause of cSCC in the general population (2), also chronic inflammation, scarring, papilloma virus infection, or genetic factors have been postulated to contribute (1, 3).

In certain genetic skin disorders, the incidence of cSCC is highly elevated (1). One example is recessive dystrophic epidermolysis bullosa (RDEB), a skin fragility disorder (4). The cumulative risk of invasive cSCC in the most severe form of RDEB is >90% by the age of 55, and 80% of the patients die of metastatic tumors within 5 years after their first cSCC (4).

RDEB is caused by mutations in the COL7A1 gene encoding collagen VII (C7), an epidermal–dermal adhesion protein and a component of the anchoring fibrils (5). Loss of C7 functions leads to friction-induced separation of the skin layers, clinically visible as blisters, and healing with scarring. Typically, aggressive cSCCs arise at strongly scarred sites (5).

Very little is known about the molecular mechanisms underlying the belligerent behavior of RDEB-cSCC (6). Although C7 supports adhesion and migration of keratinocytes in vitro (7), it is unlikely that its loss drives carcinogenesis, because keratinocytes do not usually come into direct contact with C7 in situ. Further, no consistent genetic changes distinguish RDEB-cSCC from less-aggressive common cSCCs, except for COL7A1 mutations (8).

Alternatively, the dermal tissue may play an enabling role for carcinoma progression in RDEB. Many cancers generate an adjacent stroma with altered composition and mechanical properties of the extracellular matrix (ECM) that facilitates invasion (9, 10). However, important tissue-specific differences exist (11), and the influence of the ECM on cancer development cannot be generalized for all tumor types.

Here, we addressed the mechanisms of the dermal contribution to cSCC progression using in vivo and in vitro genetic models, RDEB mice, and three-dimensional (3D) organotypic skin cultures populated with RDEB and cSCC cells. Complete absence of C7 in mice causes early postnatal lethality (12). Therefore, we used a C7 hypomorphic mouse model, here referred to as the RDEB mouse, which shows all signs of human RDEB (13, 14). The results of our study indicate that in RDEB, cSCCs do not interact with a self-generated tumor stroma, but rather with a pre-existing, genetically altered and injury-modified stiff microenvironment, which enables rapid carcinoma progression. In therapeutic terms,
inhibition of advancing tissue stiffness reduced invasion and is likely to have an impact on carcinoma prevention in RDEB.

Materials and Methods

Study approval

Studies using patient material were approved by the ethics committee of the University of Freiburg (approval no. 127/04) and conducted according to the declaration of Helsinki. The patients provided informed consent for use of their biomaterials for research. All animal experiments were approved by the regional review board (Regierungspräsidium Freiburg, Freiburg, Germany; approval no. G-08/50).

Transgenic mice

The RDEB mouse, which has been described in detail elsewhere (13, 14), was kept on mixed C57BL/6, 129sv background. The mice were housed in the pathogen-free facility at the University of Freiburg and given food and water ad libitum; in addition, the RDEB mice were provided with a soft food diet for extra support.

Tumor induction in mice

Cutaneous tumors were induced by two-stage 7,12-Dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) chemical carcinogenesis (15) following the treatment regimen displayed in Fig. 2A. One week after the final TPA application, the mice were sacrificed, tumors counted, and photographed with Canon power shot S3IS camera (Canon). Tumor-primed skin was isolated from mice treated with the initial dose of DMBA and twice-weekly applications with TPA for a period of 7 weeks. Untreated skin specimens were obtained from back skin of 10-week-old mice. Skin and tumor specimens were embedded either in optimal cutting temperature compound (Sakura) or paraffin for immunohistochemical or immunofluorescence analyses.

Human tissue specimens and cells

All patients had a genetic diagnosis of RDEB, and complete C7 deficiency was molecularly confirmed. Skin biopsy specimens were taken from perilesional skin adjacent to blisters on limbs; age- and site-matched biopsies from healthy human donors served as controls. RDEB-cSCC tumor specimens were derived from surgical excision material. RDEB-cSCC keratinocytes (RDEB-SCCK) were isolated from primary invasive RDEB-cSCC and spontaneously immortalized in culture. Normal human and RDEB patient keratinocyte cell lines were generated by transduction with retroviruses containing HPV-E6/E7 oncogenes. SCC25 cell lines were originally immortalized in culture. Normal human and RDEB patient keratinocytes. Similarly, keratinocyte and fibroblasts were cultured under standard conditions in Keratinocyte Growth Medium (Gibco) and DMEM (Gibco) with 10% FCS, respectively.

3D cocultures

3D cocultures were set up as follows: rat tail collagen I (BD Biosciences) and matrigel (BD Biosciences) were mixed to a final concentration of 4.0 mg/mL and 1.35 mg/mL, respectively. After neutralizing with 1 mol/L NaOH, 1 × 10^6 fibroblasts/mL were added, and 1.0 mL of this mixture was pipetted into each 12-well insert (BD sciences). Next day, 5 × 10^3 RDEB-SCCK were added onto each gel, and the cultures were raised to the air-liquid interface. Ascorbic acid (50 μg/mL) and the following concentrations of inhibitors were added freshly to the culture medium, every other day: SB505124 (10 μmol/L); PT373228 (5 μmol/L); β-aminopropionitrile (BAPN; 600 μmol/L); and LY294002 (20 μmol/L). Each experiment was repeated at least three times in duplicates. The cocultures were harvested at 21 days, fixed in 4% paraformaldehyde and embedded in paraffin, and analyzed for invasion by hematoxylin and eosin (H&E) staining. The invasion index, presented as the average values of (1 − [non-invading area/total area]), was calculated by quantifying the total area over which RDEB-SCCK cells had dispersed (including invading and non-invading cells) and the area of non-invading cells.

Additional materials and methods are described in Supplementary Methods.

Results

Human RDEB skin is mechanosensitive and exhibits hallmarks of fibrosis

Due to the loss of physiologic adhesion provided by C7, RDEB skin undergoes cycles of mechanically induced blistering, healing with scarring and, ultimately, develops cSCC with poor outcome. Morphologic hallmarks of human RDEB skin are dermal–epidermal microblistering, dense matrix, and inflammatory infiltrates in the dermis (Fig. 1A). H&E staining of early lesions suspected of RDEB-cSCC revealed invading epithelial cell columns and tumor cell islands in the upper dermis, accompanied by inflammatory infiltrates (arrows, Fig. 1A). Immunofluorescence staining confirmed complete lack of C7 in RDEB skin and cSCC used for this study (Fig. 1B). Earlier data from our laboratory have demonstrated that loss of C7 increases expression of TGFβ (13, 14) that has been widely implicated in inflammation, fibrosis, and cancer progression. In order to identify the TGFβ-expressing cell types, we performed double immunofluorescence staining for TGFβ and vimentin, a marker specific to fibroblasts. High levels of TGFβ expression were observed in RDEB skin and RDEB-cSCC fibroblasts (Fig. 1C), which was in accordance with previous findings (6, 16, 17). The staining pattern also suggested that TGFβ was highly expressed by inflammatory cells. The TGFβ signaling activity in both RDEB skin and RDEB-cSCC was confirmed by the elevated expression of pSMAD2/3 as compared with normal human skin (Fig. 1D). The staining also suggested slightly elevated levels in RDEB skin epidermis. Therefore, next we determined the levels of TGFβ expression in keratinocytes isolated from RDEB and non-RDEB skin and SCC. In accordance with previous findings (14), qPCR analysis revealed that RDEB keratinocytes expressed slightly but not significantly more TGFβ1 than normal human keratinocytes. Similarly, TGFβ1 transcripts were slightly but not significantly upregulated in RDEB-cSCC as compared with the non–RDEB-cSCC, indicating limited contribution of keratinocyte-derived TGFβ1 expression to differences in tumor progression (Supplementary Fig. S1). In order to determine the extent of fibrotic remodeling in the dermal microenvironment, collagen I and picrosirius red stainings were carried out. Both RDEB skin and RDEB-cSCC not only contained excessive collagen I and dense collagen fibrils (Fig. 1E and F), but also activated (myo) fibroblasts (Fig. 1G). These results demonstrate that RDEB skin not yet bearing tumors exhibits fibrotic changes of the dermal microenvironment similar to a well-developed solid tumor stroma that
supports tumor progression. Importantly, the results implicate injury-driven tumor-unrelated dermal changes as a determinant of the aggressive nature of RDEB-cSCC.

Chemically induced tumors in RDEB mouse skin closely resemble human RDEB-cSCC

To corroborate our hypothesis that the unusual aggressiveness of cSCC in RDEB depends on injury-induced changes in RDEB skin, we subjected RDEB mice to two-stage chemical carcinogenesis. This procedure generates homogeneous mutations primarily in the Hras1 gene followed by repeated cycles of injury (15). Five-week-old wild-type and RDEB mouse littermates were topically treated with DMBA followed by twice-weekly promotion with TPA for 14 weeks (Fig. 2A). In the wild-type mice, the onset of tumors commenced at week 6 of TPA promotion and reached 89% by week 9, whereas the onset of tumors in the RDEB mice was

Figure 1.
Human RDEB skin and cSCC display fibrotic stiff dermis. Healthy human skin (left), mechanically injured RDEB patient skin (middle), and moderately differentiated RDEB-cSCC (right) were subjected to H&E staining (A), immunofluorescence stainings (B–E and G), and picrosirius red staining (F). A, in injured RDEB skin, the epidermis was slightly separated from the dermis (microblistering), and the dermis contained dense ECM fibril bundles oriented parallel to the skin surface and inflammatory infiltrates (arrows). RDEB-cSCC shows invading tumor cell formations along with inflammatory infiltrates (arrows). B, linear immunofluorescence signal of C7 was seen at the dermal epidermal junction in healthy human skin but not in RDEB skin and RDEB-cSCC. C, double immunofluorescence staining with antibodies to TGFβ1 and vimentin displayed high expression of TGFβ1 by fibroblasts in RDEB patient skin and RDEB-cSCC. D, pSMAD2/3 staining confirmed enhanced TGFβ signaling in injured RDEB patient skin and RDEB-cSCC, as compared with normal human skin. E, immunofluorescence staining showed enhanced collagen I deposition in the dermis in injured RDEB patient skin and RDEB-cSCC. F, increased collagen fibril thickness/density was demonstrated by picrosirius red staining. G, αSMA staining (arrows) displays activation of (myo)fibroblasts (red). Nuclei were stained with DAPI (blue). Scale bar, 50 μm.
Chemically induced tumors in RDEB mouse skin are invasive and phenotypically similar to human RDEB-cSCC. A, timeline of 15-week chemical carcinogenesis in mice. B, graph showing the percentage of tumor incidence in wild-type and RDEB mice in relation to duration of TPA application. About 34% of wild-type mice (n = 18) developed tumors at 6 weeks and reached a maximum of 89% at 9 weeks of TPA application. RDEB mice (n = 14) displayed a 2-week delay in tumor initiation compared with wild-type siblings. The percentage of tumor incidence in RDEB mice was 7% at 8 weeks and reached a maximum of 93% at 11 weeks of TPA application. C, line graph showing the average number of tumors per mouse during the TPA treatments. A total of 41 tumors developed in wild-type mice (n = 18) and 15 tumors in RDEB mice (n = 14) after DMBA/TPA treatment. Values represent mean ± SEM. *, P < 0.05; **, P < 0.001. P values = wild-type vs. RDEB mouse tumors for each time point during TPA promotion; significance as indicated in the figure. D and E, macroscopic appearance of the tumors in wild-type (D) and in RDEB (E, red arrow) mice. F, cSCC in a patient with severe RDEB. Note the similarity in appearance between human and RDEB mouse tumors. G and H, H&E staining of wild-type (G) and RDEB (H) tumor sections. Red arrows, small horn pearls; white arrows, infiltrating tumor cells. I, graph shows the number of invasive tumors. In RDEB mice, 12 of 15 tumors were invasive. In wild-type mice, 20 of 41 tumors were analyzed, and all showed characteristic morphology of noninvasive benign papillomas. ***, P < 0.0001. J and K, immunofluorescence staining with E-cadherin antibodies (green) of tumors in wild-type (J) and RDEB (K) mice. In comparison with wild-type tumors, E-cadherin staining is clearly reduced in RDEB tumors. L and M, immunofluorescence staining with pan-cytokeratin antibodies (green) of the lung of a wild-type (L) and an RDEB (M) mouse reveals metastatic colonization in the RDEB mouse. Nuclei visualized with DAPI (blue). Scale bar, 50 μm.
delayed until week 8 and reached 93% by week 11 of TPA promotion (Fig. 2B). At the end of the treatment, the majority of wild-type mice showed multiple benign papillomas (15) with an average of $2.27 \pm 1.74$ tumors per mouse (Fig. 2C and D). In contrast, RDEB mice displayed erythematous flat papules with an overlying scale/crust (average $1.07 \pm 0.47$ tumors per mouse; Fig. 2C and E) that were strongly reminiscent of RDEB-cSCC in patients (Fig. 2F). Careful histologic analysis showed that 80% of the lesions in RDEB mice were invasive (Fig. 2H and I). To assess the tumor cells undergoing epithelial-to-mesenchymal transition (EMT), one of the hallmarks of cancer progression, tumor sections were stained for E-cadherin. The expression of E-cadherin, an epithelial cell adhesion marker, was progressively lost in RDEB tumors, as compared with wild-type tumors, indicating the loss of epithelial identity (Fig. 2K). Further, RDEB tumors displayed increased tumor cell proliferation and reduced rate of apoptotic cell death compared with wild-type tumors (data not shown). In addition to invasive cSCC, one RDEB mouse had lung metastasis. Although not significant, this finding supports the observation of increased aggressiveness of the induced RDEB mouse tumors (Fig. 2M; Supplementary Fig. S2). Taken together, chemical carcinogenesis on RDEB mouse skin leads to tumors that phenocopy naturally occurring RDEB-cSCC in patients and can serve as models to assess the mechanisms underlying tumor progression.

**Murine RDEB skin displays elevated TGFβ signaling**

The finding that chemically induced tumors in RDEB mice differed from wild-type papillomas clearly supported key involvement of the dermis in RDEB-cSCC. Studies on human tissue (13, 14) and our previous work (13, 14) implicated altered TGFβ activity as a major determinant of the disparate tumor-influencing properties of wild-type and RDEB mouse dermis. Indeed, immunostaining revealed that both the level of TGFβ and its downstream activity, as measured by pSMAD2/3, were highly increased in 7-week DMAB/TPA-treated murine RDEB skin, hereafter referred to as tumor-primed skin (Fig. 3). Similar to human RDEB skin (Fig. 1C), double immunofluorescence staining showed colocalization of vimentin and TGFβ in the fibroblasts in both tumor-primed RDEB skin and tumors (Fig. 3A). A slight decrease in TGFβ levels and pSMAD2/3 activity was observed in immediate RDEB-cSCC tumor microenvironment as compared with tumor-primed RDEB skin not yet carrying tumors (Fig. 3A and B). These findings were validated by qPCR and Western blot analyses (Fig. 3A and C). The presence of heightened TGFβ signaling and the lower number of tumors in RDEB mice reflect the paradoxical effect TGFβ has on tumor initiation and growth (18).

**Proliferative ECM in chemically injured RDEB skin**

Next, we assessed the effects of TGFβ activity on the biomechanical properties of the ECM in tumor-primed murine RDEB skin and showed that they were similar to those observed in mechanically injured human RDEB skin. Significantly increased collagen I deposition was observed in tumor-primed murine skin and cSCC (Fig. 4A). To validate the increase as TGFβ-related, murine RDEB fibroblasts (RDEBF) were cultured in the presence or absence of the selective TGFβ type 1 receptor inhibitor SB505124 (Supplementary Fig. S3A). The substantial reduction of collagen I synthesis observed in the presence of SB505124 demonstrated that TGFβ regulated collagen I expression in RDEBF.

In line with this, picrosirius red staining revealed significantly increased collagen fibril thickness in murine RDEB dermis as compared with controls (Fig. 4B). High fibril thickness/rigidity can be associated with collagen cross-linking (10, 19) and, indeed, strongly elevated levels of the collagen cross-linking enzyme, lysyl oxidase (LOX), were found in the ECM of tumor-primed murine and injured human RDEB skin and tumors (Fig. 4C; Supplementary Fig. S4). LOX is expressed by both keratinocytes and fibroblasts, and is essential for the normal development of the skin (20, 21). In order to identify LOX-expressing cell types, staining with antibodies to cytokeratins (keratinocyte marker) and vimentin (fibroblast marker) was carried out. LOX was present in both keratinocytes and fibroblasts. However, its expression was strongly and specifically increased in fibroblasts in tumor-primed murine and injured human RDEB skin and tumors (Fig. 4C; Supplementary Fig. S4) and also in the metastatic region of RDEB lung (Supplementary Fig. S2). Further, to assess the influence of TGFβ on LOX expression, murine wild-type and RDEB keratinocytes and fibroblasts were cultured in presence of recombinant TGFβ or TGFβ type 1 receptor inhibitor SB505124. Western blot analysis showed that whereas LOX expression in keratinocytes was largely unresponsive to TGFβ stimuli, TGFβ greatly governed LOX expression in fibroblasts (Supplementary Fig. S3B). Further, the analysis demonstrated that presence or absence of C7 did not affect the ability to respond to TGFβ (Supplementary Fig. S3B). Stimulation with TGFβ increased both the proenzyme and mature LOX, whereas the inhibitor attenuated both (Supplementary Fig. S3B).

In accordance with the fact that both the TGFβ expression and stiff matrix in RDEB skin facilitate a phenotypic switch of fibroblasts to αSMA-positive myofibroblasts (22), αSMA-positive...
cells were more prominent in tumor-primed murine RDEB skin than in controls (Supplementary Fig. S5A). Thus, the unrestrained TGFβ activity in C7-deficient RDEB skin concomitantly drives expression of collagen I and LOX, resulting in a stiffer proinvasive dermal matrix.

**RDEB-ECM facilitates integrin β1-mediated mechanosignaling in cSCC cells**

The effects of biomechanically altered ECM on RDEB-cSCC cell signaling were first assessed with immunofluorescence staining using antibodies to β1 integrin and focal adhesion kinase (FAK). Enhanced levels of β1 integrin and activated pFAKtyr397 were observed in RDEB tumors (Fig. 4D; Supplementary Fig. S5B and S5C), specifically colocalized at the surface of invading RDEB-cSCC cells. This, in combination with increased active pAKTser473 in the tumors (Supplementary Fig. S5B and S5C), indicated focal adhesion/stiff ECM-mediated mechanosignaling. The fact that treatment of human RDEB-SCCK with PF573228, an inhibitor of pFAKtyr397 autophosphorylation, reduced pAKTser473 validated the focal adhesion-mediated pAKT signaling axis (Fig. 4E).

Taken together, these data conclusively demonstrate an invasion-permissive chain of events in RDEB skin: loss of C7 and repeated mechanical or chemical injury lead to unrestrained TGFβ activity; this results in gradual stiffening of the dermal matrix; the stiff microenvironment triggers integrin β1-mediated mechanosignaling in cSCC cells, which promotes malignant progression.

**Potential for therapeutic intervention in RDEB-cSCC**

3D organotypic cocultures (23) were used to evaluate cell-matrix interactions relevant for RDEB-cSCC progression and to assess possibilities to modify the process. Cocultures constructed with human RDEB-SCCK as epithelial cells and normal human fibroblasts (NHF) formed a skin-like bilayered structure, whereas RDEBF promoted invasion of RDEB-SCCK (Fig. 5A). Significant increase of collagen fibril density, as indicated by picrosirius red staining, was evident in RDEBF-containing cocultures (Fig. 5B). Atomic force microscopy (AFM) demonstrated that 3D collagen matrices populated with RDEBF were significantly stiffer and had about 3-fold higher Young’s modulus than 3D collagen matrices constructed with NHF (Fig. 5C). Treatment of these matrices with SB505124 reduced stiffness substantially (Fig. 5C).

The picrosirius red staining intensity was correlated with stiffness values obtained by AFM. A clear positive correlation was observed between the median values of Young’s modulus of 3D matrices and median values of area intensity of picrosirius red stainings obtained from their respective 3D organotypic cocultures (Fig. 5D). Hence, in this system, picrosirius red staining intensity directly corresponded with stiffness and could be used as a read-out for it.

With the perspective of identifying therapeutic targets to prevent or at least delay RDEB-cSCC, different strategies were used to inhibit matrix stiffness, mechanosignaling, and tumor cell invasion in 3D organotypic cultures. The first strategy targeted early events in RDEB fibrosis by inhibiting TGFβ signaling with SB505124, which efficiently reduced matrix stiffening and invasion (Fig. 5A, B, E, and F). The second strategy targeted mid events using BAPN, an irreversible LOX inhibitor. This treatment reduced collagen fibril thickness and invasion (Fig. 5A, B, E, and F). To inhibit invasion at later stages, i.e., in extensively fibrotic stiff dermis, we targeted stress sensing in tumor cells and used the ATP-competitive FAK inhibitor PF573228 and the phosphoinositide 3-kinase inhibitor LY294002. These compounds blocked invasion without altering dermal stiffness (Fig. 5A, B, E, and F). Taken together, depending on the extent of injury-driven dermal fibrosis, RDEB-cSCC progression can be combated by inhibiting specific targets along the tissue stiffness-promoting and responding pathway.

**Discussion**

Almost every adult patient with severe generalized RDEB will develop aggressive cSCC, a leading cause of premature death in this disorder (4). Except for surgical removal, there is no therapy for RDEB-cSCC; development of treatments has been hampered by lack of understanding of the underlying cellular and molecular events. Here, we delineated the contributing mechanisms (Fig. 6) and generated new knowledge that facilitates design of prophylactic and therapeutic measures for delaying tumor progression and extending cancer-free periods in RDEB.

The triggers of malignant transformation in the epidermis were not in our focus, because non-RDEB and RDEB-cSCC display the same nature of genetic changes, apart from the individual COL7A1 mutations (8). For this reason, tumors were induced by DMBA treatment, which generates a rather homogeneous oncogenic change, i.e., the Hras1 mutation c.182A>T, instead of other methods like exposure to UV light, which results in heterogeneous genetic changes (15).

The observation that RDEB mice developed fewer but more aggressive tumors implicated TGFβ activity as a major regulatory factor (18). TGFβ displays a paradoxical role on tumor growth. During initial tumorigenesis, it inhibits proliferation...
Figure 5.
Tampering with RDEBF-associated matrix stiffening enhances mechanosignaling and reduces tumor cell invasion. H&E staining of 3D cocultures constructed with RDEB-SCCK as epithelial cells and either NHF or RDEBF as dermal cells. A, 3D coculture with RDEB-SCCK and NHF formed an ordered skin-like tissue structure (leftmost). When RDEBF were used as dermal cells, RDEB-SCCK invaded the dermal compartment (second plot from the left). Treatment of this 3D coculture with SB505124 (10 μmol/L), BAPN (600 μmol/L), PF573228 (5 μmol/L), or LY294002 (20 μmol/L) inhibited invasion to a varying extent (middle and right plots). B, corresponding picrosirius red staining of the 3D cocultures shown in A. Thin fibers appear green, and dense rigid collagen bundles indicating stiff matrix appear orange red. Note the reduced collagen fibril density in 3D cocultures treated with SB505124 and BAPN. C, Young’s modulus measured by AFM on 3D matrices populated with NHF or RDEBF and treated with DMSO (vehicle) or SB505124. The matrices populated with RDEBF showed a 2.9-fold increase (median in Pa) in stiffness, as compared with matrices constructed with NHF. The matrices cultured with SB505124 exhibited significantly reduced stiffness. ***, P < 0.0001 for NHF vs. RDEBF, vehicle treated (n = 3) and NHF vs. RDEBF, SB505124-treated (n = 2) 3D matrices. D, graph indicates the correlation (linear regression) between the median values of Young’s modulus of 3D matrices populated either with NHF receiving vehicle, or RDEBF receiving vehicle or SB505124 were correlated with median values of picrosirius red-positive areas obtained from the respective 3D organotypic cocultures (R² = 0.9926). E, the bar graph shows the invasion index of the 3D cocultures in A; n ≥ 11 images quantified per condition; values represent mean ± SD. ***, P < 0.0001 for vehicle-treated RDEBF vs. RDEBF treated with either SB505124 or with BAPN or with PF573228 or with LY94002. F, scatter graph shows quantification of picrosirius red-positive areas shown in B; n ≥ 7 images quantified per condition; values represent mean ± SD. ***, P < 0.0001 for vehicle-treated RDEBF vs. RDEBF treated with either SB505124 or with BAPN. Scale bar, 50 μm.
Injury-Driven Dermal Stiffness Drives cSCC

Schematic representation of signaling events driving tumor progression in RDEB skin. C7 deficiency is associated with proliferation of keratinocytes and increased ECM deposition, which accelerates initial invasion. Stiff dermal matrix increases integrin-mediated mechanosignaling in RDEB-cSCC cells and facilitates cell survival and migration via FAK- and AKT-mediated signaling axis.

The DMBA/TPA-induced tumors on RDEB mice, in contrast with those in wild-type mice, displayed hallmarks of cancer, i.e., increased proliferation, lower cell death, signs of EMT, invasion, and ability to metastasize. In vitro and in vivo analyses showed that these events were mediated through the integrin-FAK-AKT mechanosensing pathway. Our observations are in line with the growing awareness that biophysical characteristics of tumor-generated stroma influence different cancers. The tissue/tumor constellations are variable and context-dependent, but a stiff environment has been reported to lower expression of tumor suppressors, increase telomerase activity, promote cell-cycle progression, lead to apoptotic resistance, promote malignant transformation by enhancing TGFβ-induced EMT, and increase invasion and metastasis (11). However, it is very clear that the influence of the ECM on cancer development must be delineated specifically for each tissue/carcinoma constellation (11). Here, we show that RDEB-cSCC fate is profoundly affected by the ECM, but not via tumor-conditioned stroma. Instead, injury-driven cancer-independent biophysical alterations of the dermis are pivotal in this context.

The emerging concept of repeated injury, unrestrained fibroproliferative healing, and subsequent tissue stiffening as enablers of aggressive carcinoma behavior have high relevance for other pathologies, too, including burn scar neoplasms and SCC complicating common leg ulcers (26). However, tissue stiffening alone is not sufficient to drive carcinoma. For example, progressive systemic sclerosis, a multiorgan autoimmune disease, exhibits endogenously stiffened skin but not cSCC proneness. In contrast, systemic sclerosis patients who have pulmonary fibrosis, which is injury-related, have an increased risk of lung cancer (27, 28). Hence, our observations and published data from other investigators collectively argue that injury-driven changes in the...
biophysical performance of the mesenchyme are a general determinant of carcinoma progression (Fig. 6).

Causative treatment for RDEB will offer ultimate protection against aggressive cSCC, but in spite of progress, clinical implementation of such therapies is still in distant future (5). Our study offers promise for a disease-modulating, cSCC-limiting therapy. Three major mechanisms were identified as potential druggable targets, namely excessive TGFβ activity, tissue stiffening, and dysregulated mechanosensing. It is important to state that the drugs BAPN and LY294002 used to target LOX and PI3K, respectively, cannot be clinically used due to dermal toxicity and adverse effects (29, 30). However, several more clinically relevant FDA/European Medicines Agency–approved drugs already exist for the indentified targets, or candidate drugs are in advanced stages of clinical trials. Examples are losartan or neutralizing TGFβ antibodies to target initial fibrotic processes, LOX antibodies to reduce crosslinking, and small molecules inhibiting FAK activity to correct mechanosensing in tumor cells and keratinocytes (11, 31). Such drugs could be repurposed for rapid clinical implementation as prophylactic cSCC therapy in RDEB and other related, more common conditions.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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