Fibroblast-derived dermal matrix drives development of aggressive cutaneous squamous cell carcinoma in patients with recessive dystrophic epidermolysis bullosa

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Abstract

Patients with the genetic skin blistering disease recessive dystrophic epidermolysis bullosa (RDEB) develop aggressive cutaneous squamous cell carcinoma (cSCC). Metastasis leading to mortality is greater in RDEB than in other patient groups with cSCC. Here we investigate the dermal component in RDEB using mRNA expression profiling to compare cultured fibroblasts isolated from individuals without cSCC and directly from tumor matrix in RDEB and non-RDEB samples. While gene expression of RDEB normal skin fibroblasts resembled that of cancer-associated fibroblasts, RDEB cancer-associated fibroblasts exhibited a distinct and divergent gene expression profile, with a large proportion of the differentially expressed genes involved in matrix and cell adhesion. RDEB cancer-associated fibroblasts conferred increased adhesion and invasion to tumor and non-tumor keratinocytes. Reduction of COL7A1, the defective gene in RDEB, in normal dermal fibroblasts led to increased type XII collagen, thrombospondin-1 and Wnt-5A, while re-expression of wild type COL7A1 in RDEB fibroblasts decreased type XII collagen, thrombospondin-1, and Wnt-5A expression, reduced tumor cell invasion in organotypic culture, and restricted tumor growth in vivo. Overall our findings demonstrate that matrix composition in patients with RDEB is a permissive environment for tumor development, and type VII collagen directly regulates the composition of matrix proteins secreted by dermal and cancer-associated fibroblasts.
Introduction

Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited skin blistering disease caused exclusively by mutations in the gene encoding type VII collagen, \textit{COL7A1} (1). Type VII collagen is the main component of anchoring fibrils, structures which support anchorage of the epidermis to the underlying dermis (2). The central dogma is that defective anchoring fibrils leads to skin fragility characterised by long term wounds and healing with scarring resulting in considerable disruption to dermal architecture (3). This devastating condition is further complicated by the development of numerous, aggressive cutaneous squamous cell carcinoma (cSCC) suggestive of a field effect. Tumors frequently metastasise resulting in over 80% mortality by age 50 (4). This rate of metastasis far exceeds other patient groups in which cSCC is also a major complication, such as organ transplant patients and patients with the hereditary disease xeroderma pigmentosum, the reasons for which remain unclear (5). Protocols for the early detection and aggressive management, including amputation, radiation therapy and chemotherapy, have not been proven to increase survival in RDEB cSCC (6). Previous studies have failed to identify consistent differences between RDEB cSCC and cSCC from the general population or organ transplant recipients in spite of the different clinicopathological behavior. In fact, in virtually all studies to date, RDEB cSCC exhibit similar characteristics to non-RDEB cSCC and a causative relationship with tumor progression has yet to be unequivocally identified (7-15). With few exceptions these studies have focussed on tumor keratinocytes and have ignored the surrounding stroma. Cancer-associated fibroblasts are the main cell type present in tumor stroma and have been shown to contribute toward cancer invasion (16), initiation and progression via stromal-epithelial interactions (17-19) and can provide oncogenic signals such as fibroblast growth factors which act in a paracrine manner to transformed tumor keratinocytes.
(20). Prompted by our recent observations that very few genetic and behavioral differences exist between primary cultures of cSCC keratinocytes derived from RDEB and non-RDEB individuals (15) we now apply a similar approach to study primary cultures of fibroblasts derived from RDEB patient skin and cSCC.
**Materials and Methods**

**Tissue Samples**

This study was conducted according to the Declaration of Helsinki Principles and was approved by the appropriate Ethics Committees. RDEB patient diagnosis was confirmed by characteristic immunofluorescence findings and clinical criteria (3). Biopsies from normal skin or cSCC tissue from non-RDEB and RDEB patients were obtained after informed consent. Supplementary Data Table 1 gives details of all samples used for fibroblast isolation in this study. Normal non-RDEB, non-cSCC samples from redundant control skin were obtained from non-malignant aesthetic plastic surgery procedures.

**Cell Culture**

Fibroblasts were isolated as follows. Briefly, after mechanical disassociation and trypsin digestion, biopsy fragments were subjected to collagenase D (Roche Diagnostics, Burgess Hill, UK) overnight. Fibroblasts were grown in high glucose DMEM media supplemented with 10% FBS. All fibroblast cultures used in this study were of early passage, <8.

**Cell Proliferation Assay**

Fibroblasts were seeded at 20,000 cells/cm² density in 96 well plates in triplicate and a methylthiazolylidiphenyl-tetrazolium bromide (MTT) cell proliferation assay (#30-1010K, ATCC, Teddington, UK) was performed according to manufacturer’s instructions every 24hrs, over 5 days. Biological replicates from each fibroblast type were then analyzed in Prism 5 (Graphpad software, La Jolla, CA).
Agilent One Colour Microarray and Expression Analysis

Full details of microarray and analysis are given in Supplementary Data. Briefly, Fibroblasts seeded at 20,000 cells/cm² density were lysed after 4 days and 100ng/μl of RNA was amplified, labelled and hybridised onto Agilent Single Colour Whole Human Genome Oligo Microarray 4x44k cDNA array (G4112F, Design ID 14850; Agilent Technologies) according to the manufacturer's guidelines. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (21) and are accessible through GEO Series accession number GSE37738 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37738).

Antibodies

The following primary antibodies were used for immunoblotting and immunostaining: anti-αSMA (1A4, Abcam, Cambridge, UK), anti-FAP (ab53066, Abcam), anti-FSP1 (ab27597, Abcam), GAPDH (71.1, Sigma-Aldrich, Poole, UK), anti-keratin cocktail (LL001 and LP34, produced in house), anti-MUC1 (VU4H5, Santa Cruz, Santa Cruz, CA), anti-TSP1 (A6.1 and A4.1, Santa Cruz), anti-type V collagen (1E2E4, Millipore, Billerica, MA and ACL50511AP, Accurate Chemicals, Westbury, NY), polyclonal anti-type VII collagen raised against the NC1 domain, anti-type XII collagen (A11, Santa Cruz), anti-vimentin (V9, Santa Cruz) and anti-WNT-5a (AF645, R&D, Minneapolis, MN). The following secondary antibodies were used: goat anti-mouse Alexa Fluor 488 (Invitrogen, Paisley, UK), goat anti-rabbit Alex Fluor 568 (Invitrogen), goat anti-mouse HRP (Dako) and swine anti rabbit HRP (Dako).

Immunoblotting
Samples were resolved by 1D SDS-PAGE gels using standard techniques. Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was subsequently blocked with 5% non-fat milk or 5% bovine serum albumin (BSA) for 1 hour and probed with specific primary antibodies, followed by the appropriate peroxidase-conjugated secondary antibody. Proteins were detected using the ECL Western Blotting Detection Reagents (GE Healthcare, Chalfont St. Giles, UK) or SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Cramlington, UK). The membrane was then stripped and re-probed for loading control.

Tissue preparation, histology, and immunostaining

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Four μm thick sections were cut and air-dried overnight before being de-paraffinized and then rehydrated through a decreasing concentration of alcohol to water. Standard heat-based antigen retrieval method was used. Sections were stained using Vectastain® ABC kits (Vector Labs, Peterborough, UK) or Bond Autostainer (Leica Microsystems GmbH, Wetzlar, Germany) according to the manufacturer’s protocol. Liquid Diaminobenzidine (DAB) (DAKO) was subsequently applied for 3-10 mins and sections were counterstained with haematoxylin. Slides were photographed using AxioImager Z1 (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Morphogenic Analysis and Immunohistochemistry quantification

A coherent single square lattice was applied to each photograph of DAB stained tissue to produce a total of 100 test points determined by intersect of the 10x10 lattice. At each intersect a 20x20 pixel area was sampled. Those areas which did not fall onto stroma (i.e. were either not
occupied by tissue or were obviously epithelial or tumor cells) were discarded. Using color deconvolution in FIJI (http://fiji.sc/), DAB was converted to a 256 grey scale and the average intensity across all areas for all sections was calculated. The fraction of the area occupied by those pixels greater than the average signal intensity was calculated and used to generate box and whisker plots shown in Figure 4.

**Cell-derived Matrix and Invasion Assay**

Cell-derived matrix (CDM) was made *in vitro* based on the protocol by Larouche and colleagues (22) with some modifications. Briefly, 10,000 fibroblasts per cm² were seeded on plastic and the media was supplemented with 0.1mM L-ascorbic acid 2-phosphate (A8960, Sigma-Aldrich) with re-feeding every 2-3 days. After 7-10 weeks, a single layer of cell-derived matrix (CDM) was formed. RDEBSCC tumor cells were then seeded onto this CDM, which was raised to air-liquid interface the next day. Tumor cells were left to invade for 7-14 days before fixation in 4% paraformaldehyde.

**Invasion quantification**

Sections of the cell derived matrix were immunostained with the antibody LL001 against keratin 14 and photographed using AxioImager Z1 (Carl Zeiss MicroImaging GmbH). The obtained images were analysed using FIJI. The images were first processed using colour deconvolution into 8-bit images and then a threshold set to select for the tumor cells stained by LL001. The invasion index was calculated based on the total number of invading particles (excluding the surface epithelium) times the total area of the particles for each image.
RNA interference

We used the top three siRNA oligonucleotides against COL7A1 as ranked by Sigma-Aldrich (SASI_Hs01_00155170, SASI_Hs01_00155171, SASI_Hs01_00155173) and a non-targeting control (NTC) (MISSION siRNA Universal Negative Control #1, Sigma-Aldrich). NHF were seeded in 6-well plates at 100,000 cells/well and transfected 24 h later with pooled siRNA or NTC (40 nM final concentration) using Lipofectamine 2000 (Invitrogen) diluted in Opti-MEM (Invitrogen) according to the manufacturer’s instructions.

Recombinant type VII collagen expression

Full details of recombinant type VII collagen expression are given in Supplementary Data. Briefly, wild-type COL7A1 was delivered with infectious replicative defective retrovirus and target cells were selected with puromycin (2 μg/mL).

Tumorigenicity assays

All animals were used in accordance with UK Home Office regulations. Full details of the protocol are given in Supplementary Data. Briefly, a suspension of 4x10^6 SCCIC1 cells and 1x10^6 of the indicated population of primary fibroblasts were mixed with high concentration Matrigel® (Becton Dickinson, Oxford, UK) and injected sub-cutaneously into the flanks of SCID balb/c mice. Tumors were regularly measured by caliper.
Results

RDEB fibroblast cultures display comparable proliferation rates to equivalent non-RDEB fibroblast cultures

We isolated dermal fibroblasts from RDEB patients without a clinical diagnosis of cSCC (herein referred to as RDEBF), from RDEB cSCC (RDEBSCCF) as well as from non-pathological skin from aesthetic plastic surgery procedures (normal human fibroblasts, or NHF) and spontaneous UV-induced cSCCs excised from non-RDEB individuals (UVSCCF), as described in Materials and Methods. Supplementary Data Table 1 details the samples used in this study. No distinct morphological differences between cells isolated from the 4 patient groups were observed (Figure 1A). Using an approach similar to our previous analysis of cultured keratinocytes (15) we seeded fibroblasts at a relative high density and assayed proliferation over 5 days using the MTT assay (Figure 1B). This demonstrated no significant difference between the proliferation rates under these conditions and identified that cultures were relatively quiescent at day 4. We screened 2 RDEB cSCC fibroblast isolations for genetic rearrangement using 10K SNP mapping arrays and observed no abnormalities (data not shown). No contaminating keratinocytes were observed in any fibroblast isolations and no expression of keratinocyte markers such as mucin-1 (23) or keratin was evident (Figure 1C). All populations tested expressed similar levels of vimentin and varied in the expression of other fibroblast markers such as α-smooth muscle actin (α-SMA), fibroblast specific protein 1 (FSP1), and fibroblast activated protein (FAP) (24, 25) (Figure 1D).
mRNA expression profiling demonstrates that non-cancer RDEB skin fibroblasts behave as cancer-associated fibroblasts

We chose to assay mRNA expression at day 4 when cells were relatively quiescent as we have previously shown that a similar approach analysing keratinocytes was capable of identifying targets necessary for cSCC cell survival (15). Unsupervised clustering of all 20,095 probes passing quality control on the Agilent array platform clearly separated NHF from RDEBSCCF but was unable to separate RDEBF from UVSCCF (Figure 2A). One-way ANOVA analysis identified 1,098 probes as being differentially expressed between disease state and normal (Figure 2B). The majority of these probes were differentially expressed in RDEBSCCF while the majority of probes differentially expressed in RDEBF were also differentially expressed in UVSCCF and shared with RDEBSCCF (Figure 2C). Analysis of all 1,098 probes as well as 159 probes common to all 3 disease groups showed a stepwise progression starting with UVSCCF and RDEBF, followed by RDEBSCCF, demonstrating that the level of deregulation in this gene set was greatest in RDEBSCCF (Figure 2D).

An activated gene signature clusters normal fibroblasts but cannot separate non-cancer RDEB skin fibroblasts from cancer-associated fibroblasts

Previous array experimentation using normal dermal cultured fibroblasts has identified a core serum response (CSR) gene set which is differentially expressed in response to serum exposure (26). Upon exposure to serum fibroblasts become activated and display similar features to those observed in a wounded or cancer environment. Importantly this CSR gene signature is evident in various tumor tissues and is associated with a worse prognosis in breast, lung and gastric cancer, suggesting that tumors and their associated microenvironment have a wound-like phenotype.
We observed little overlap between the CSR gene signature and our 1,098 probes; 61 of 904 probes on our array representing the 591 CSR genes were differentially expressed. However, we investigated whether the CSR signature alone was capable of separating disease state. Clustering of our fibroblast samples based on the CSR gene signature resulted in a separation of NHF from the three disease states but was unable to cluster RDEBSCCF (Supplementary Figure 1). The CSR gene signature clustering highlights the role of serum response in all three disease states, corroborating similarities in the transcriptional profiles of RDEBF, UVSCCF and RDEBSCCF that we observe in our ANOVA analysis.

Matrix associated gene expression is increased in RDEB, UV cSCC and RDEB cSCC fibroblast cultures

KEGG pathway analysis of the 1,098 differentially expressed genes identified a significant enrichment of genes associated with adhesion and extracellular matrix, with 6/13 pathways being involved in these processes (Figure 3A and Supplementary Data Table 2). The differences identified in COL5A1, COL12A1, ITGA3, ITGA6 and TSP1 were confirmed at the level of protein expression by Western blotting (Figure 3B). Other differentially expressed genes of note included Wnt5A (confirmed by Western, Figure 3B) TLR4, TGFBR3, DAP, and ACVRL1.

Type V collagen, type XII collagen and thrombospondin-1 are up-regulated in cSCC stroma
We analysed type V collagen, thrombospondin-1 and type XII collagen expression in normal skin (n=3), UV cSCC (n=6) and RDEB cSCC (n=8) tissue using immunohistochemistry. Type V collagen expression was detected in the dermis of normal skin and was significantly increased in the tumor stroma in 7/8 RDEB cSCC and 2/6 UV cSCC (Figure 4 upper panels). Thrombospondin-1 expression was virtually undetectable in the dermis of normal skin and was significantly increased in the stroma of 12/13 cSCC samples (Figure 4 middle panels). Both type V collagen and thrombospondin-1 were also expressed by normal epidermal keratinocytes as well as tumor keratinocytes (data not shown). Type XII collagen was weakly detected in the dermis of normal skin and significantly increased in the stroma of 5/6 UV cSCC and 5/7 RDEB cSCC samples (Figure 4 lower panels). These data demonstrate that the changes in protein expression identified in cultured fibroblasts are also apparent in cSCC in vivo.

**RDEB cSCC fibroblast derived extracellular matrix promotes adhesion of cSCC and skin keratinocytes isolated from RDEB and non-RDEB individuals**

We hypothesised that the difference in protein expression in RDEB and cSCC fibroblasts would influence the behavior of cSCC keratinocytes. In order to assess the fibroblasts’ own secreted matrix in the absence of an artificial input we coated tissue culture plastic dishes with primary fibroblast secreted matrix as described in Materials and Methods. We assessed fibroblast secreted matrix from 7 separate primary populations, three NHF, one RDEBF, one UVSCCF and two RDEBSCCF. Adhesion was expressed relative to collagen I for 6 separate keratinocyte lines: one HPV immortalised axilla, one spontaneously immortalised foreskin, one HPV immortalised RDEB axilla, one cSCC and two RDEB cSCC. Every keratinocyte population we tested was more adhesive in the presence of RDEBSCCF matrix compared with all other fibroblast secreted
matrix (p<0.001, combined analysis of 3 experiments performed in quintuplicate; Supplementary Data Figure 2).

**RDEB cSCC fibroblast-derived matrix promotes RDEB cSCC keratinocyte invasion**

We next examined the invasion of RDEB cSCC keratinocytes into 3D organotypic models constructed from fibroblast-derived cellular matrix. Rather than embed patient fibroblasts into a synthetic matrix or cadaveric dermis with or without the basement membrane generated 3D organotypic cultures using cell-derived matrices as described (22). 3D matrices derived from RDEBSCCF increased invasion compared with corresponding NHF or UVSCCF (Supplementary Data Figure 3) supporting the hypothesis that RDEB cSCC-derived matrix influences tumor cell behavior.

**Manipulation of \textit{COL7A1} in primary normal dermal fibroblasts and RDEB fibroblasts modulates matrix composition**

Fibroblasts from different anatomical sites retain their site-specific gene expression profiles in culture (27). In order to assess whether the difference in gene expression observed in RDEBSCCF, RDEBF and UVSCCF were a result of positional information acquired as a result of a wounded environment (in the case of RDEB and RDEB cSCC) and/or a tumor environment (in the case of cSCC and RDEB cSCC) or whether the presence of full-length type collagen VII influences the expression of extracellular matrix components identified in our array, we examined the effect of \textit{COL7A1} knockdown in normal dermal fibroblasts populations (n=3). Of 6 proteins tested we observed an increase in the level of type XII collagen, thrombospondin-I and Wnt-5A expression after \textit{COL7A1} knockdown (Figure 5A and B). We confirmed the observation
that COL7A1 modulates the expression of matrix associated proteins, by re-expressing wild-type cDNA in four RDEB fibroblast populations (3 RDEBSCCF and 1 RDEBF): we then observed a significant reduction in type XII collagen, thrombospondin and Wnt5A (Figure 5C).

Expression of full length recombinant type VII collagen in RDEB fibroblasts retards cancer cell invasion and in vivo tumor progression

In order to assess the functional consequences of type VII collagen re-expression in RDEB patient fibroblasts we assessed EB29F expressing either recombinant type VII collagen or empty vector control in organotypic invasion assays and cSCC xenograft tumor progression. Recombinant type VII collagen significantly retarded the invasion of RDEB cSCC keratinocytes into 3D organotypic models constructed from fibroblast derived cellular matrix in separate experiments, (performed in duplicate and triplicate respectively) (Figure 6). Co-inoculation of SCCIC1, a UV induced cSCC keratinocyte line derived from an immunocompetent individual, with RDEB fibroblasts expressing empty vector alone significantly accelerated tumor growth after 50 days when compared to the same RDEB fibroblasts expressing recombinant type VII collagen or NHF (Figure 7A and B). Histological examination of these tumors showed that NHF and RDEB fibroblasts expressing type VII collagen induced significant differentiation compared with RDEB fibroblasts expressing empty vector alone (Figure 7C).
Discussion

Here we demonstrate through gene expression profiling of skin and tumor isolated fibroblasts that the RDEB cSCC dermal microenvironment is strikingly distinct compared to normal skin and UV induced cSCC. Cultured RDEB cSCC fibroblasts account for 86.8% (953) of the 1,098 differentially expressed genes identified in this study of which 61.4% (674) are differentially expressed only in this sample group when compared with normal dermal fibroblast cultures (Figure 2C). Furthermore, considerable overlap between the expression changes which separate RDEB skin and UV cSCC from control skin demonstrate that RDEB dermal fibroblasts are indistinguishable from cancer-associated fibroblasts (Figure 2). We show that the differences identified here are not a result of physical genetic change or occult tumor cells within our primary isolations (Figure 1 and data not shown) and by identifying changes in quiescent cultures supported by the cells’ own secreted matrix we have removed potential consequences of migratory and proliferative stimuli which have previously been shown to separate tumor fibroblasts from normal (28, 29). Greater than 89% of the differentially expressed genes separating UV cSCC and RDEB skin from normal skin are also found in RDEB cSCC and these changes increase in a stepwise manner from normal to UV cSCC and RDEB through to RDEB cSCC suggesting a progression in the degree of gene expression changes (Figure 2B+C). A large proportion of the differences between disease and normal skin are in genes encoding components of, or receptors which interact with, the extracellular matrix (Figure 3 and 4). The changes in extracellular matrix correlate with increased adhesion and invasion of cSCC keratinocytes which can be attributed to the lack of functional type VII collagen expression (Supplementary Data Figures 2 and 3 and Figure 6). We show that knockdown of \textit{COL7A1} in normal fibroblasts and
re-expression of \textit{COL7A1} in RDEB fibroblasts results in similar matrix associated changes to those identified by microarray, providing a mechanism by which RDEB fibroblasts parallel cancer-associated fibroblasts (Figure 5). The functional outcomes of these matrix changes are clearly demonstrated by the decreased cSCC cell invasion in 3D organotypic cultures and decreased tumor progression in xenograft studies upon restoration of type VII collagen in RDEB fibroblasts (Figures 6 and 7). Our proposed model is that RDEB skin already mimics aspects of cSCC due to the shift in gene expression in skin fibroblasts resulting from absence of type VII collagen, and is further modified by the process of repeated tissue injury and inflammation, leading to cancer initiating changes in the keratinocytes that rapidly progress in the absence of a normal inhibitory dermal architecture. Decreased tumor cell differentiation in the presence of RDEB fibroblasts compared with normal or RDEB fibroblasts expressing full-length type VII collagen further support this (Figure 7C).

Tumor stromal cells are said to be “activated”, being more proliferative and secreting more extracellular matrix proteins such as type I collagen and fibronectin (25). Here we have focussed on differences in extracellular matrix and adhesion protein expression, and show that type V and XII collagens as well as integrin subunits alpha 3 and alpha 6 and thrombospondin-1 are up-regulated in RDEBF and UVSCCF fibroblasts and further up-regulated in RDEBSCCF fibroblasts. Type V and XII collagens are reported to have roles in cellular adhesion and migration (30, 31) and the increased fibroblast mediated contraction of type V collagen-containing gels is integrin dependent (32) in agreement with integrin alpha 3 dependent increased SCC invasion into fibroblast remodelled gels (16). Type XII collagen is a FACIT collagen (Fibril Associated Collagens with Interrupted Triple helices) which does not aggregate.
to form fibrils but instead binds in a periodic manner to the surface of fibrils formed by fibrillar collagens (such as type I collagen) (33). How an up-regulation of FACIT type XII collagen alters the cSCC tumor microenvironment is not yet known. Studies in v-myc or v-src transformed avian fibroblasts and prostate cancer cells have indicated down-regulation of type XII collagen (34, 35), while more recently type XII collagen has been reported to be highly expressed by cancer-associated fibroblasts in colon cancer (36) suggesting that cancer-associated type XII collagen expression is context dependent. Thrombospondin up-regulation in the stroma of esophageal adenocarcinoma identifies patients with poor prognosis (37) and a recent gene expression study correlating stromal activation with metastasis in head and neck SCC identified \textit{COL5A1} and \textit{TSP1} as the top two differentially expressed genes (38) supporting their clinical relevance in other aggressive cancers.

We show through both siRNA knock-down and re-expression of \textit{COL7A1} that, as with normal breast epithelial cells (39), the composition of the extracellular matrix directly influences the composition of cell-secreted extracellular matrix. Moreover, we show that the expression of type VII collagen directly influences the extracellular matrix secreted by fibroblasts, data which is not mirrored in keratinocytes – our previous analysis of RDEB and non-RDEB tumor and normal keratinocytes did not identify changes in extracellular matrix gene expression (15) and knockdown of \textit{COL7A1} in UV cSCC keratinocytes induced cytokine rather than extracellular matrix changes (40).

A normal tissue microenvironment is protective and suppresses the development of cSCC (41). Moreover, inhibiting the interactions between tumor cells and their surrounding matrix is
effective at arresting or delaying tumor progression (42). In RDEB there appears to be a situation in which the composition of the matrix per se is tumor promoting or tumor permissive in the epidermis. Certainly our in vivo data would support this notion. Additional contributions from other well defined processes in tumor progression and wound healing such as inflammation (43) and/or tumor stroma cross talk (44) need to be further investigated. The clinical behavior of cSCC in RDEB also differs from UV cSCC. In RDEB, the majority of tumors are well- or moderately-differentiated, yet surgical excision with clear tumor excision margins does not lead to a cure (unlike the vast majority of UV cSCCs treated similarly). Thus a tumor which would normally be considered less aggressive in a non-RDEB setting frequently recurs in RDEB patients, often with further multiple primary cSCCs that are less well-differentiated and which can metastasize and become fatal (45). RDEB cSCC usually occur in areas of chronic scarring and non-healing ulceration, correlating positively with the extent of scarring, clinical observations that support a tumor promoting or permissive stromal microenvironment in the pathophysiology of RDEB cSCC(4). Moreover, no other primary epithelial tumors have been reported to frequently manifest in RDEB patients (4) even though COL7A1 is expressed in other tissues (46) and extracutaneous/mucosal symptoms occur, especially in more severe RDEB subtypes (47). These observations would support the hypothesis that COL7A1 has a more direct role in epidermal homeostasis than in other epithelial tissues, arguing against a systemic tumor promoting role and agreeing with the recent proposition that factors which modulate malignant transformation through the tumor microenvironment are likely tissue dependent (48). Certainly the context dependent nature of the expression of COL7A1 and type VII collagen across a breadth of different cancers would support this idea (5).
COL7A1 is mutated in RDEB fibroblasts yet similar changes in matrix composition were observed in UV cSCC fibroblasts which, compared with normal fibroblasts, express increased levels of wild type COL7A1 and type VII collagen (Supplementary Data Figure 4). This and our knockdown experiments would suggest that convergent evolution of the overall matrix composition of UV cSCC fibroblasts and RDEB fibroblasts, rather than simply reduced COL7A1 in UV cSCC fibroblasts, is responsible for these changes in vivo. Therefore it is likely that these tumor-promoting matrix changes can arise through other processes, as evidenced in other cancers (discussed above). We cannot rule out that the common environment of inflammation is responsible for a proportion of the expression differences we identify and further work will be necessary to explore this likely possibility. The large proportion of gene expression changes unique to RDEB cancer-associated fibroblasts have not been the focus of this study and will be followed up separately. It is important to note that the majority of matrix genes we identify here are either direct or indirect targets of TGF-beta signalling, COL7A1 included (49). As TGF-beta signalling directs cellular processes in a context dependent manner (50) this pathway could have important bearing on the switch from normal to a cancer-associated fibroblast behavior.

In summary our data suggest that dermal composition is cancer predisposing in patients with RDEB which, rather than suppressing tumor development, is permissive to the progression toward metastatic disease. Down-regulation of COL7A1 switches gene expression in dermal fibroblasts toward a cancer-associated fibroblast phenotype. This cancer-associated fibroblast phenotype in turn promotes substrate adhesion and invasion of tumor keratinocytes and tumor progression in vivo. By understanding the underlying mechanisms behind the aggressive nature of cSCC in RDEB patients, more effective therapies which target the dermal architecture and
microenvironment may help in combating this devastating complication to an already severe
disease and may also translate to aggressive cSCC and other epithelial cancers in the general
population.

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Figure Legends

Figure 1 - RDEB fibroblast cultures display proliferation rates and marker expression comparable to corresponding non-RDEB fibroblast cultures

A: Representative images of confluent cultured primary fibroblasts (Day 4) (100x Magnification). B: MTT assay average colorimetric reading relative to day 1 for each fibroblast sample group seeded at high density (20,000 cells/cm²) and cultured over 5 days. Results are triplicate mean ±SD, n=3-5 per group. C: No evidence of MUC-1 or cytokeratin expression in fibroblast cultures. (NHK: Primary normal human keratinocyte; RSK: RDEBSCC keratinocyte line, SCCRDEB2). D: Expression of markers α-SMA, FSP1, FAP-α, and vimentin are consistent with cultured dermal fibroblasts and do not separate sample groups.

Figure 2 - mRNA expression profiling demonstrates RDEB normal skin fibroblasts behave as cancer-associated fibroblasts and RDEB cancer-associated fibroblasts represent a distinct sample group

A: Unsupervised clustering based on 20,095 probes which passed filtering criteria clearly separates fibroblast cultures into 3 groups: NHF, UVSCCF/RDEBF and RDEBSCCF. B: Supervised hierarchical clustering of 1,098 differentially expressed transcripts across all three disease states demonstrates RDEBSCCF represent a distinct sample group. C: Venn diagram depicting the overlaps in 1,098 differentially expressed gene transcripts from pair-wise comparisons with NHF. Most of the gene transcripts identified are differentially expressed in RDEBSCCF (953/1,098 probes). RDEBF and UVSCCF show very similar gene expression
changes which overlap considerably with RDEBSCCF. D: Non-parametric ranking of all 1,098 probes (top) and 159 probes dysregulated in all three fibroblast disease groups (bottom) compared to NHF reveals a stepwise progression in gene dysregulation from NHF to RDEBSCCF. Each probe was ranked according to level of gene dysregulation (either increased or decreased expression) compared to NHF.

**Figure 3 – Matrix associated gene and protein expression is increased in RDEB, UV cSCC and RDEB cSCC fibroblast cultures**

A: KEGG pathway analysis identifies an enrichment of gene expression associated with extracellular matrix and cell adhesion in RDEB and cSCC sample groups. B: Array signal intensity (graph, multiple dots correspond to multiple probes where present) is reflected by protein expression of type V collagen, integrin alpha 3, integrin alpha 6, Wnt-5A, thrombospondin-1 and type XII collagen as determined by Western blotting.

**Figure 4 – Immunohistochemical labeling identifies increased matrix associated protein expression in cSCC**

Labeling with anti-type V collagen, anti-thrombospondin-1 and anti-type XII collagen antibodies identifies increased expression in the stroma of UV induced (n=6) and RDEB cSCC (n=7-8) compared with normal skin (n=3). The percentage of pixels staining with intensity above the average signal is expressed as average area fraction (graph, left panels). Box and Whisker plots show upper and lower quartile (box), median (central line) and 10th and 90th percentile values.
Right panels show a representative 200x200 pixel region of stroma from individual normal or tumor samples. Negative control (omitting primary antibody) shows no labeling. Bar = 50μm.

**Figure 5 – COL7A1 knockdown in normal dermal fibroblasts increases type XII collagen, thrombospondin-1 and Wnt-5A expression while COL7A1 over-expression in RDEB fibroblasts reduces type XII collagen, thrombospondin-1 and Wnt-5A expression**

A: siRNA mediated knockdown of COL7A1 in three separate normal dermal fibroblast populations yields reduction in type VII collagen expression in whole cell lysates after 4 days as identified by Western blotting. C7 Pooled = pool of 3 COL7A1 targeting siRNA. Scr = non-targeting control siRNA. B: Western blotting identifies increases in type XII collagen, thrombospondin-1 and Wnt-5A after COL7A1 knockdown. Right panel graphs show Western densitometry from each separate fibroblast population normalised to GAPDH and scrambled control. C: Over-expression of COL7A1 in 4 separate RDEB patient derived fibroblast populations results in decreased thrombospondin-1, type XII collagen and Wnt-5A expression. C7 = pbabe-COL7A1 vector expressing full length wild type COL7A1, pBabe = pBabe-puro empty vector alone. Right panel graphs show Western densitometry normalised to GAPDH and scrambled control.

**Figure 6 – Full length type VII collagen expression in RDEBF cell derived matrix retards cSCC keratinocyte invasion**
Invasion of SCCRDEB4 keratinocytes into RDEB cell derived matrix is retarded in RDEBF cells expressing recombinant full-length type VII collagen (EB29F C7) compared with identical cells expressing empty vector alone (EB29F pBabe). An invasion index was calculated from 3-13 images for each cell derived matrix organotypic culture over two experiments and normalised to pBabe. A: Normalised invasion index from a representative experiment ± SEM. B: representative section stained with a keratin 14 antibody highlighting SCCRDEB4 keratinocytes. Magnification 200X. * = p value <0.05.

**Figure 7 – Expression of full length recombinant type VII collagen in RDEB fibroblasts retards tumor progression in vivo leading to increased tumor differentiation compared with RDEB fibroblasts expressing empty vector control**

Female SCID Balb/c mice were subcutaneously injected in the right flank with 4×10^6 SCCIC1 cells mixed with 1x10^6 indicated fibroblast populations and high-concentration Matrigel® (Becton-Dickinson, Oxford, UK). NHF = normal dermal fibroblasts, EB29FC7 = type VII collagen expressing RDEB fibroblasts, EB29FpBabe = vector only control RDEB fibroblasts. A: Tumors were regularly measured by caliper and individual volume was calculated using the formula V = π4/3[(L + W)/4]^3, were L is the length and W is the width. Graph shows average +/- SEM. * = p value <0.05. B: images of all tumors harvested at the end of the experiment. C: H&E stained frozen sections of a representative xenograft tumor from each of the 3 experimental groups shows increased differentiation in NHF and EB29FC7 compared with EB29FpBabe. Magnification = 100X.
Figure 1

A

B

MTT Proliferation Assay

Proliferation (%)

Days

0 1 2 3 4 5

NHF  UVSCCF  RDEBF  RDEBSCCF

C

D

Keratinocyte

Fibroblasts

Pan-Keratin

(LP34)

GAPDH

Pan-Keratin

(MNF116)

GAPDH

MUC1

GAPDH

Keratinocyte

Fibroblasts

α-SMA

FSP1

FAP

GAPDH

Vimentin

GAPDH

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
D Non-parametric ranking of Gene Dysregulation in each Fibroblast Group

i) Using all 1,098 differentially expressed probes

![Bar chart showing average ranks for different conditions.

ii) Using 159 differentially expressed probes shared by all three diseases states compared to NHF

![Bar chart showing average ranks for different conditions using the subset of 159 probes.]

Figure 2

A

NHF66  NHF88  NHF2  SCCIC15  RDEB48F  SCCT7F  SCCT8F  SCCIC17  RDEB23F  RDEB26F  SCCIC16  RDEB27F  RDEB3SCC1F  RDEB2SCC1F  RDEB43SCC1F  RDEB7SCC2F

B

Legend - Dendrogram

Algorithm: Hierarchical Clustering

Parameters:

Cluster on = Both rows and columns

Distance metric = Euclidean

Linkage rule = Average

C

Legend:

RDEBSCCF vs NHF 953 probes

UVSCCF vs NHF 201 probes

RDEBF vs NHF 287 probes

159

674

RDEBF vs NHF

UVSCCF vs NHF

RDEBSCCF vs NHF

D

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**KEGG Terms**

1. Ribosome
2. Heparan sulphate biosynthesis
3. Chondroitin sulphate biosynthesis
4. Glutamate metabolism
5. D-Glutamine and D-glutamate metabolism
6. 3-Chloroacrylic acid degradation
7. Hematopoietic cell lineage
8. Nicotinate and nicotinamide metabolism
9. Vibrio cholerae infection
10. Focal adhesion
11. ECM-receptor interaction
12. Axon guidance
13. Cell adhesion molecules

**Figure 3**

**A**

![Graph showing expression of ECM and cell adhesion related terms](image)

**B**

- **ITGA3 mRNA expression level**
  - [Graph showing expression levels](image)
- **TSP1 mRNA expression level**
  - [Graph showing expression levels](image)
- **COL12A1 mRNA expression level**
  - [Graph showing expression levels](image)

- **ITGA6 mRNA expression level**
  - [Graph showing expression levels](image)
- **COL5A1 mRNA expression level**
  - [Graph showing expression levels](image)
- **WNT5A mRNA expression level**
  - [Graph showing expression levels](image)
Figure 6

A

Invasion Index

* 

Invasion Index (Normalised to pBabe)

RDEB29F pBabe  RDEB29F C7

Cell Derived Matrix

B

<table>
<thead>
<tr>
<th>1 Week</th>
<th>EB29F pBabe</th>
<th>EB29F C7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7

Tumour Growth Over Time

A

B

C

SCCIC1+NHF

SCCIC1+EB29FC7

SCCIC1+EB29FpBabe

SCCIC1+NHF

SCCIC1+EB29FC7

SCCIC1+EB29FpBabe

Days

Tumour Volume mm³

0

100

200

300

400

500

Figure 7 shows the tumour growth over time for different conditions: SCCIC1+NHF, SCCIC1+EB29FC7, and SCCIC1+EB29FpBabe. The graph indicates a significant increase in tumour volume for SCCIC1+EB29FpBabe compared to the other two conditions, highlighting the potential impact of EB29FpBabe.
Fibroblast-derived dermal matrix drives development of aggressive cutaneous squamous cell carcinoma in patients with recessive dystrophic epidermolysis bullosa

Yi Z Ng, Celine Pourreyron, Julio C Salas-Alanis, et al.

Cancer Res Published OnlineFirst May 7, 2012.

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