TGF β -signaling in squamous cell carcinoma occurring in recessive dystrophic epidermolysis bullosa

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Abstract. *Background:* Recessive dystrophic epidermolysis bullosa (RDEB) is a hereditary skin disorder characterized by mechanical fragility of the skin, resulting in blistering and chronic wounds. The causative mutations lie in the COL7A1 gene. Patients suffering from RDEB have a high risk to develop aggressive, rapidly metastasizing squamous cell carcinomas (SCCs). Cutaneous RDEB SCCs develop preferentially in long-term skin wounds or cutaneous scars. Albeit being well differentiated, they show a more aggressive behavior than UV-induced SCCs. These findings suggest other contributing factors in SCC tumorigenesis in RDEB.

Objective: To analyze factors contributing to RDEB tumorigenesis, we conducted a comprehensive gene expression study comparing a non-malignant RDEB (RDEB-CL) to a RDEB SCC cell line (SCCRDEB4) to achieve an overview on the changes of the gene expression levels in RDEB related skin cancer.

Methods: We applied cDNA arrays comprising 9738 human expressed sequence tags (EST) with various functions. Selected results were verified by Real-time RT PCR.

Results: Large-scale gene expression analysis revealed changes in the expression level of transforming growth factor β 1 (TGF β 1) and several genes under the control of TGF β for RDEB and SCCRDEB4 cell lines. Even the untransformed RDEB keratinocytes show elevated levels of TGF β 1.

Conclusion: Our findings demonstrate a prominent role of TGF β -signaling in RDEB-related skin cancer. Once activated, TGF β signaling either in response to wounding or in order to influence type VII collagen expression levels could facilitate cancer development and progression. Moreover, TGF β signaling might also represent a potentially useful therapeutic target in this disease.

Keywords: Recessive dystrophic epidermolysis bullosa, squamous cell carcinoma, gene expression, cDNA array, real-time PCR, TGF β -signaling

List of abbreviations

CDHE E-Cadherin
EMT Epithelial-mesenchymal transition
EST Expressed sequence tag

FN Fibronectin
GAPDH Glyceraldehyde-3-phosphotase dehydrogenase
GPNMB Glycoprotein nonmetastatic B
HPRT Hypoxanthine ribosyltransferase
KRT18 Keratin 18
LRP-1 Low density lipoprotein receptor-related protein 1
MMP Matrix metalloproteinase

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RDEB-CL	Non-malignant RDEB keratinocyte cell line
RDEB-O	Other generalized RDEB
RDEB	Recessive dystrophic epidermolysis bullosa
SSC	Saline-sodium citrate
SCCRDEB4	SCC RDEB keratinocyte cell line
SPARC	Secreted protein acidic and rich in cysteine
RDEB-sev gen	Severe generalized RDEB
SDS	Sodium dodecyl sulfate
SCC	Squamous cell carcinoma
TSP-1	Thrombospondin-1
TIMP-1	Tissue inhibitor of matrix metalloproteinases
TGF β 1	Transforming growth factor β 1
TRIM27	Tripartite motif-containing protein 27
COL1A2	Type I collagen
COL7A1	Type VII collagen
VCL	Vinculin

1. Introduction

Recessive dystrophic epidermolysis bullosa (RDEB) is a mechano-bullous hereditary skin disorder characterized by extreme skin fragility. Even minor mechanical traumata result in massive blistering of the skin and slowly or non-healing wounds [17]. There are two major subtypes of RDEB: severe generalized RDEB (RDEB-sev gen; previously termed “Hallopeau-Siemens” subtype) and other generalized RDEB (RDEB-O) [36] which differ in clinical severity. Genetic analysis revealed that the causative mutations lie in the COL7A1 gene. This gene encodes for type VII collagen which is known to be the major component of the anchoring fibrils that connect the epidermis to the dermis [54, 110]. In less severe cases mutations result in reduced levels of type VII collagen, while in RDEB-sev gen no or very low levels of type VII collagen (COL7A1) are observed [113]. Apart from blistering, subsequent injury of the whole skin surface (also mucosae can be involved) and mutilating scars [87], RDEB can further be complicated by growth retardation, anemia, pseudosyndactyly and corneal erosions [36]. Patients suffering from RDEB also have a high risk to develop aggressive, rapidly metastasizing squamous cell carcinomas (SCCs) [82,

130] even in early adulthood [65]. This risk increases dramatically with age. Statistical analysis revealed that nearly all patients suffering from RDEB-sev gen develop at least one SCC throughout life and about 80% perish within the following five years of diagnosis [37]. As seen in other chronic, severe scarring conditions after thermal burns, the SCCs generally develop within long-term skin wounds or cutaneous scars [37]. This is in contradiction to UV-induced SCCs, which tend to develop at sun exposed areas [5]. Although RDEB-related SCC appear to be well differentiated, they exhibit highly aggressive behavior which is not indicated for normal SCCs [35]. These findings suggest other contributing factors in SCC tumorigenesis [37]. Several studies focused on the underlying mechanisms of RDEB-related SCC and the comparison of those SCC to UV-induced SCC. Unfortunately, they have not yielded a greater comprehension into carcinogenesis in RDEB and only few peculiarities in RDEB SCC gene expression were revealed. The differences between carcinogenesis of RDEB and normal skin are currently being investigated [35].

This study aims at the elucidation of factors contributing to aggressiveness of SCC in the context of RDEB. A comparative study of comprehensive gene expression profiles of both normal and malignant RDEB keratinocytes should reveal changes in gene expression. Those changes were further evaluated for their contribution to high morbidity rates in RDEB cancer. Here, we focus on expression changes in transforming growth factor β 1 (TGF β 1) and some related genes.

2. Material and methods

2.1. Cell lines and RNA samples

SCCRDEB4 [129] cells were kindly provided by Dr. Andrew South, Dundee, UK and RDEB-CL cells were obtained from Prof. Guerrino Meneguzzi, Nice,

Table 1
Cell lines, RDEB subtype and mutations

Cell line	RDEB subtype	Mutation
RDEB-CL	RDEB-sev gen	COL7A1(7786delG/R578X)
SCCRDEB4	RDEB-sev gen	COL7A1 (8244dupC/8244dupC)
HaCaT	—	Wild type

France. As control for both RDEB cell lines served HaCaT cells. All cell lines were routinely grown in Keratinocyte-SFM, supplemented with bovine pituitary extract and recombinant epidermal growth factor (Invitrogen, USA) (Table 1).

2.2. Probe synthesis

RNA isolation and cDNA labelling was done essentially as described by Aberger et al. [1]. Briefly, after harvesting cells were lysed using Tri Reagent^{TM} (MRC Inc., USA) and RNA was isolated using the provided protocol. Labelling of cDNA to high specific activity by reverse transcription was carried out by using 15 μ g total RNA, 70 μ Ci ^{33} P-dCTP (3000 Ci/mmol, Amersham Biosciences, USA), 10 μ l 3 $\times$ labelling buffer (for 200:120 μ l 5 $\times$ SuperScript^{TM} reverse transcriptase buffer (Invitrogen, USA), 3 μ l dATP/dGTP/dTTP 100 mM each, 60 μ l 0.1 M DTT, 0.8 μ l 1 mM dCTP, 10.2 μ l DEPC treated ddH₂O) and 2 μ l SuperScript II reverse transcriptase (Invitrogen). The labelled probes were subsequently purified using GFX columns (Amersham) according to the manufacturer's protocol.

To measure and verify the incorporation and quality of the labeling reaction, scintillation-counting was performed.

2.3. Array hybridization and data analysis

Nitrocellulose membranes of 22 $\times$ 22 cm (Hybond N+, Amersham Biosciences, UK) were spotted with PCR products of 9738 ESTs of the Incyte Human UniGEM Microarray clone set in duplicates on a MicroGrid II arrayer (BioRobotics, UK). Prehybridization was carried out for 2 h at 65°C. cDNA probes were denatured for 5 min at 95°C. Then the filters were hybridized with labeled cDNAs for 2 days at 65°C in pre-warmed hybridization buffer (5X Denhardt's solution, 5X saline-sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS)). Afterwards the filters were washed four times in buffers with declining SSC concentration (20 minutes and 65°C each): 2 $\times$ SSC/0.1% SDS, 0.2 $\times$ SSC/0.1% SDS (carried out twice) and 0.1 $\times$ SSC/0.1% SDS. Subsequently, filters were rinsed in 2 $\times$ SSC, sealed in Saran-wrap and exposed four days to phosphorimager screens (Fuji, Germany). These screens were scanned using BAS READER 1800 II phosphorimager (Fuji, Germany). For

each RNA sample two arrays were hybridized, so four hybridization spots per EST could be used for statistical analysis.

2.4. Image and data analysis

After spot intensity calculation using AIDA software (Raytest, Germany), data was exported to Microsoft Excel (Microsoft, USA). Each spot signal was normalized for total signal intensity of all spots on the array.

2.5. Real-time RT-PCR

Genes of interest (see Table 2) which showed strong up- or down-regulation exceeding a factor of 1.8 were chosen for verification using real-time RT PCR. Primers were designed using the open-source primer design software PerlPrimer [78] and ordered at Sigma Aldrich (see Table 2).

For PCR reaction True-Start^{TM} Hot Start Polymerase (MBI Fermentas, Germany) was used. The annealing temperature for each primer pair was determined using melting curves and 2% agarose gel banding of PCR products. The expression of three housekeeping genes was monitored (see Results) for normalization. The results of each PCR reaction were calculated using the Bio-Rad 'Genex' MS-Excel macro (Gene Expression Analysis for iCycler iQ® Real-Time PCR Detection System) based on the algorithms outlined by Vandessompele et al. [127].

3. Results

Genes of interest (Table 2) chosen from the array results were those which play a role in the TGF β -signaling. Additionally they exceed a factor of 1.8 for up- or down-regulation compared to the reference. The HaCaT cell line served as reference for both RDEB cell lines. The results of real-time RT-PCR analysis are listed in Table 3. RDEB cells show significant over-expression of TGF β 1, TIMP-1 (tissue inhibitor of matrix metalloproteinases-1), CDHE (E-cadherin), FN1 (fibronectin 1), COL7A1 and COL1A2 (type I collagen), and significant under-expression of LRP1 (low density lipoprotein receptor-related protein 1) and VCL (vinculin).

Table 2
Selected genes and primers

Gene	Abbre-viation	RefSeq ID	Forward primer (5'-3')	Reverse primer (5'-3')
Glyceraldehyde-3-phosphatase dehydrogenase	GAPDH	NM_002046	GTGAAGGTCGGAGTCAA CG	TGAGGTCAATGAAGGGG TC
Hypoxanthine ribosyltransferase	HPRT	NM_000194	GGGCTATAAATCTTTGTC TGAC	CTGGTCATTACAATAGC TCTTCAG
Tripartite motif-containing protein 27	TRIM27	NM_030950	CAGGCACGAGCTGAACT CT	AGCTGCTCAAACCTCCCA AAC
Transforming growth factor beta	TGF β 1	NM_000660	AACCCACAACGAAATCT ATGAC	TAACCTTGAGCCTCAGCA GAC
TIMP metalloproteinase inhibitor 1	TIMP-1	NM_003254	GTCCCTGCGGTCCCAGAT A	GTGGGAACAGGGTGGAC ACT
E-cadherin	CDHE	NM_004360	AAGAGGACCAGGACTTT GAC	CACGAGCAGAGAATCAT AAGG
Thrombospondin-1	TSP-1	NM_003246	CGACCAGAAGGACTCTG ACGGCGAT	GGACGAGTTCTTTACCC TGATGGCG
Low density lipo-protein receptor-related protein 1	LRP1	NM_002332	GCCTGCAGAGATCAAAT AACC	CCTTACTCTGTGGACAA ATCTC
Fibronectin 1	FN1	ENSG0000011541	ATGCCGACCAGAATTGG G	CCCCACGACCATTCCCA ACA
Type VII collagen	COL7A1	NM_000094	CTCAGCAGCTATCACCTG GAC	TGTCCACCACACGTAGT TCAA
Secreted protein acidic and rich in cysteine	SPARC	NM_003118	TCTTCTTTCTCCTTTGCCT G	CTCCACAGATACCTCA GTC
Glycoprotein nonmetastatic B	GPNMB	NM_001005340	CGTGGTAACAGATCAG ATTCTT	AACAAACAGGCCAGTA TTATCC
Vinculin	VCL	NM_003373	GCCTGGGCCAGCAAGG ACAC	CCCTGGGGAGGCACTA GGGT
Type I collagen	COL1A2	NM_000089	CAAGGCTGTCTTCTAC AGG	CCAAAGGTGCAATATC AAGG
Keratin 18	KRT18	NM_199187	GCGAGGACTTTAATCTT GGTG	CTCAGAACTTTGGTGTG ATTGG

SCCRDEB4 cells show significant over-expression of TGF β 1, CDHE, SPARC (secreted protein acidic and rich in cysteine), VCL and GPNMB (glycoprotein nonmetastatic B), and significant under-expression of TSP-1 (thrombospondin-1), LRP1, FN1, COL7A1 and KRT18 (keratin 18). Especially GPNMB is massively up-regulated.

The selection of suitable housekeeping genes for real-time RT PCR analysis was somewhat challenging in the case of RDEB cells. The preliminary results of the nitrocellulose filters indicated that some of classic reference genes show abnormal expression in RDEB (e.g., actins and tubulins), arousing doubts in their usefulness as suitable housekeeping genes for the further verification of over- and under-expressed genes. Others were not included at all in the 9738 expressed

sequence tags (ESTs) spotted on the filters (e.g., cyclophilin).

Structural genes like actins or tubulins were excluded as possible housekeeping genes. As RDEB is a skin disorder, chances are that structural genes are expressed differently (as indicated by the changed intensities on the nitrocellulose filters).

Therefore we chose glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), hypoxanthine ribosyltransferase (HPRT) and tripartite motif-containing protein 27 (TRIM27) as housekeeping genes. These genes take part in different cellular pathways and are therefore unlikely to be co-regulated. All three showed normal expression levels according to the filter results. One is a novel housekeeping gene (TRIM27) and two are "classic" housekeeping genes (HPRT and GAPDH).

Table 3
Real time RT-PCR results (expression ratio vs. HaCaT cells) and main functions

Abbreviation	RDEB-CL (s.d.)	SCCRDEB4 (s.d.)	Main functions
TGF β 1 Transforming growth factor β	2.83 (0.49)	4.91 (1.46)	• Growth-regulatory cytokine; key role in differentiation, apoptosis, matrix protein synthesis and degradation [40], inflammation and angiogenesis [59] • Dual role in cancer, time-dependent model: early phase of tumor development $\rightarrow$ suppressor function later stages/established tumor $\rightarrow$ promotes tumor cell survival, cancer progression, metastasis [59] by facilitating epithelial-mesenchymal transition (EMT) [101, 131], angiogenesis and escape from immune surveillance [101]
TIMP-1 Tissue inhibitor of matrix metallo- proteinases 1	2.61 (1.10)	1.39 (0.31)	• TGFβ1 induces expression of MMP (matrix metalloproteinase) -2 and MMP-9; down-regulates inhibitors TIMPs [40, 59] • TIMPs are the antagonists to MMPs, restrict their activation, bind to MMPs in a 1 : 1 stoichiometry [52] • Can influence activity of growth factors [47], cell morphology [103] and inhibit angiogenesis [62]
CDHE E-Cadherin	2.38 (0.55)	6.47 (1.33)	• Expressed by epithelial cells (hence “E”-cadherin) • Belongs to large family of cell-cell adhesion molecules [134] • Part of adherens junction, plays a role in cell-cell adhesion and recognition (reviewed in [43]), spans intercellular space between two cells • Interacts with other proteins (e.g., VCL) coupling it to the actin cytoskeleton [93]
TSP-1 Thrombospondin-1	1.41 (0.31)	0.14 (0.03)	• Involved in cell adhesion, migration, proliferation, matrix remodeling [2, 23, 67, 76, 88, 100], apoptosis [61, 124], angiogenesis [112] • Able to activate latent TGFβ1 [89] • Strongly influences tumor growth, progression and metastasis • Binds MMP-2 and -9 [10], enhances their cellular uptake and degradation via LRP1 [33, 46, 136]
LRP1 Low density lipo-protein receptor-related protein 1	0.26 (0.03)	0.25 (0.08)	• Important for endocytosis, signal transduction [50], cell migration, proliferation, vascular permeability [72] • $\rightarrow$ binds wide range of ligands [50, 69] (TSP-1, MMP-9, MMP-13, TGFβ1 [73]) • LRP1 binding to TSP-1 mediates degradation of MMP-2 and MMP-9 [84, 85] • Able to modulate TGFβ signaling [19]
FN1 Fibronectin	4.55 (0.72)	0.22 (0.06)	• Ever-present matrix glycoprotein [68] • Binds to various “structural” molecules (collagen, actin or fibrin [135], several integrins [4]) • Functions in keratinocyte adhesion and migration [118, 122] • Cellular transformation causes loss of FN reducing cellular adhesion $\rightarrow$ favorable for tumor metastasis [107] • TGFβ induces synthesis in fibroblasts and fibrosarcoma cells [30, 56, 57]
COL7A1 Type VII collagen	5.46 (0.49)	0.29 (0.03)	• TGFβ1 is enhancer of type VII collagen gene expression in keratinocytes and dermal fibroblasts [26, 91, 109] • Major component of the anchoring fibrils [54] • Mutations in COL7A1 cause RDEB [54], leading to reduced or absent type VII collagen protein [113] • Synthesized as procollagen; forms homotrimers, undergoes further aggregation and maturation processes [102]

(continued next page)

Table 3
(continued)

Abbreviation	RDEB-CL (s.d.)	SCCRDEB4 (s.d.)	Main functions
SPARC Secreted protein acidic and rich in cysteine	0.90 (0.21)	2.59 (0.56)	• Matricellular protein mediating cell-matrix interactions • No primarily structural role [70], expressed when changes in cell-matrix or cell-cell contact are needed [13] • Modulator of growth factor activity, cell-cycle inhibitor [70], regulator of matrix remodeling and turnover [27] • TGFβ1 enhances expression in human fibroblasts [15] • is able to increase TGFβ1 expression <i>in vitro</i> [38]
GNPMB Glycoprotein nonmetastatic B	0.70 (0.11)	143.84 (31.73)	• Transmembrane protein expressed by human keratinocytes and melanocytes [125] • Able to promote TGFβ1 activation • Able to induce MMP-3 and MMP-9 [94, 105, 108]
VCL Vinculin	0.35 (0.11)	6.95 (1.28)	• Component of focal adhesions and adherens junctions; links actin filaments to membrane [31] • Ubiquitously expressed; interacts with various proteins (talin, paxillin, α- and β-catenin) [137] • Transfer of mechanical stress to the cytoskeleton, resulting in its remodeling [34] • Knock-out results in reduced matrix adhesion and elevated migration [133] • tumor suppressor [106]
COL1A2 Type I collagen	79.56 (30.01)	2.01 (0.89)	• Fibrillar collagen made up of two α1(I) and one α2(I) chain encoded by COL1A1 and COL1A2 • Found everywhere in body, including skin, bone and other tissues [53] • Provides mechanical stability and cellular integrity • Fibronectin and different integrins attach to type I collagen • TGFβ induces transcription [25, 55] • Over-expression seems to have tumor suppressive and anti-invasive function in glioma [53]
KRT18 Keratin 18	1.15 (0.27)	0.07 (0.0058)	• Type I keratin • Belongs to simple epithelial keratins • Highly conserved among different species [114, 115] • Involved in preservation of cell polarity and apoptosis • Protective function against mechanical stress (reviewed in [98]) • Often expressed aberrantly in cancer [16, 77, 97, 116]

4. Discussion

cDNA arrays were carried out to analyze the differences in gene expression patterns of normal and transformed RDEB-derived keratinocytes. The results indicate a prominent role of TGF β signaling in the context of RDEB tumorigenesis. Hence, TGF β -related pathways and genes were examined more closely.

Keratinocytes, fibroblasts and cells of the immune system are main producers but also targets of TGF β 1 in the skin [41]. As this regulatory cytokine is able to influence many important processes in the cell, TGF β 1 and TGF β signaling are worthwhile objects in the investigation of cancer development and progression.

TGF β 1 was found to be over-expressed in normal RDEB-CL keratinocytes (2.83 fold). This up-regulation could be related to the different roles of TGF β 1. Lu et al. [75] showed that cells bordering a tumor also often exhibit elevated TGF β 1 levels. They assumed over-expression of TGF β 1 to be an early event in head and neck SCC development. Hence, over-expression of TGF β 1 in RDEB cells could mirror an early phase of tumor development. According to the assumed temporal role of TGF β in cancer [59], it might also have anti-tumoral properties in RDEB-CL keratinocytes and could prevent their transformation into tumor cells.

Another aspect is TGF β 1's role in inflammatory and wound healing processes. It could be argued that elevated TGF β 1 levels in RDEB-CL could also be a result of these processes.

In the cancer cell line, SCCRDEB4, TGF β 1 expression nearly doubles (4.91 fold) compared to RDEB-CL. It is feasible that TGF β 1 over-expression in the tumor cell line could be a sign for aggressive, highly metastasizing cancer.

4.1. TGF β signaling and the promotion of EMT

TGF β signaling is considered to be the main trigger for EMT (reviewed in [132]). A characteristic feature of this process is the down-regulation of cell adhesion molecules, e.g., E-cadherin [40]. In accordance, reduced E-cadherin expression has also been shown after prolonged exposure of early papillomas to TGF β 1 [131]. A study by Deng et al. [32] recently confirmed TGF β 1 as direct inducer of EMT. In contrast, real time RT-PCR results for CDHE mRNA revealed elevated levels of these transcripts (6.47 fold) in SCCRDEB4.

Also VCL, the protein coupling CDHE to the actin cytoskeleton, is up-regulated (6.95 fold).

These findings might be explained by the fact that the effects of TGF β 1 on E-cadherin expression are mediated not only by Smad4 through Snail and Slug induction [121] but also through Smad-independent signaling. Both mechanisms are needed for a full repression of E-cadherin expression [83]. Therefore it might be possible, that in SCCRDEB4 defects in Smad4 signaling and/or Snail inactivation could account for elevated CDHE mRNA levels.

Smad 4 knockdown has been shown to prevent CDHE down-regulation [121] and to suppress type I collagen synthesis [63]. As this reflects the situation present in our study, Smad 4 could be considered a possible candidate that might be defective in RDEB SCC.

The induction of Snail1 or Snail2 by TGF β also results in E-cadherin suppression [9, 20] and elevated expression of FN [20, 96]. Therefore both, Snail1 and Snail2 could be disturbed in SCCRDEB4. On the other hand, keratin 18 has been reported to be a direct target of Snail1: it mediates the down-regulation of keratin 18 [29, 45, 58]. Since we were able to show this down-regulation in SCCRDEB4 (0.07; in contrast to 1.15 in RDEB-CL), defective Snail-related signaling appears to be less likely.

The helix-loop-helix transcription factors E12 and E47 are able to directly repress E-cadherin expression [99]. Beyond that, E47 is able to induce SPARC expression [86]. As SPARC in fact exhibits elevated mRNA levels in SCCRDEB4 (2.59 fold), it could be deduced that E47 defects seem to be unlikely as well.

Cells having acquired the mesenchymal phenotype after EMT show a different gene expression profile. An increase in ECM proteins (like collagens and FN) can be noticed, promoting cell migration by stimulation of integrin signaling and by induction of focal adhesion complexes (reviewed in [132]). By contrast, FN was found to be down-regulated in SCCRDEB4 (0.22 fold) in our study and these cells show only moderately elevated mRNA levels for COL1A2 (2.01 fold). Compared to untransformed RDEB keratinocytes, COL1A2 levels are even massively down-regulated as analysis of COL1A2 mRNA in RDEB-CL yielded a nearly 40-fold over-expression (79.56 fold). Hence, there rather seems to be a down-regulation in type I collagen as compared to untransformed RDEB keratinocytes.

There are many pathways by which TGF β signaling mediates the down-regulation of CDHE characteristic

for EMT. As many of these function independently of each other, it seems to be highly unlikely that all these pathways are turned off or obstructed. Moreover, the results for other genes point out that some of these pathways seem to be working correctly.

Taken together, these findings argue for a preservation of the epithelial phenotype of SCCRDEB4 cells. As loss of CDHE is the hallmark for EMT, the only remaining conclusion is that obviously EMT has not (or not yet) taken place in SCCRDEB4, although elevated TGF β 1 levels would indicate that. Besides, an obstruction of CDHE expression is not sufficient to induce EMT [74] and neither does forced CDHE expression induce a restoration of the epithelial phenotype [92]. It remains to be investigated whether and how the genetic changes due to defects in type VII collagen could be responsible for this observation.

4.2. TGF β signaling in cell-cell contact, adhesion and migration

Genes involved in changes of cell-cell contact, cellular adhesion and migratory potential, which are related to or influenced by TGF β are TSP-1, COL7A1, FN and SPARC.

In many tumor types, TSP-1 is found to be down-regulated [71]. Moreover, it appears that metastatic cells produce 3-fold less TSP-1 than normal cells [90] and that decreased TSP-1 levels correlate with increased recurrence rates and decreased overall survival [44]. Many (but not all [21, 128]) SCCs have been shown to express only little amounts of TSP-1 [18, 49]. Therefore, TSP-1 has been suggested as potent inhibitor of angiogenesis and tumor growth in skin carcinomas [120] and TSP-1 increasing drugs might be a promising strategy for cancer treatment in general [71]. We found this tumor suppressor gene to be down-regulated in SCCRDEB4 (0.14). A lack of TSP-1 might contribute to the aggressiveness of RDEB-related SCCs. Hence, the application of TSP-1 increasing anti-cancer drugs might prove useful.

In accordance with our results showing a strong decrease of FN mRNA levels in SCCRDEB4 (0.22 fold) despite elevated TGF β 1 expression, FN generally seems to be reduced in cancer cells [30]. Therefore, the decline in FN mRNA levels might not be due to some perturbation in TGF β 1 signaling, but might rather result from rapid decay of FN mRNA. However,

more recent studies have implicated high levels of FN with tumor cell proliferation and low FN levels with dormant cancer cells [8]. Yet, all these results have been generated from studies using fibroblasts and not keratinocytes. It has already been shown that those two cell types react differently to the same signal(s) [123]. The role of FN mRNA repression in SCCRDEB4 keratinocytes is therefore difficult to interpret. A study by Berndt et al. in 1998 [11] on tissue samples of oral SCCs and breast cancer cells showed that these cells themselves synthesize only very small amounts of FN *in vivo* and that FN is produced mainly (if not exclusively) by stromal cells. Assuming that stromal cells like fibroblasts are the main source of FN, this might account for the reduced expression of this gene by SCCRDEB4 keratinocytes. On the other hand, RDEB-CL cells are also keratinocytes and they do not show a decrease in FN mRNA. Hence the loss of FN mRNA expression might very well provide for increased migratory potential of SCC cells.

Less information exists on the TGF β -mediated control over SPARC. It seems plausible, that TGF β increases SPARC expression in order to influence processes like migration or proliferation [15]. In cancer, SPARC is often over-expressed [7, 80, 104, 111, 117]. These relations also seem to apply to SCCRDEB4 as we found increased TGF β 1 (4.91) and SPARC mRNA (2.59 fold) levels. Those might contribute to the migratory potential of RDEB SCC cancer cells. Elevated expression levels of SPARC indicate ongoing remodeling processes [15]. This way TGF β signaling seems to increase cancer cell motility and could, *in vivo*, facilitate the development of metastasis.

TGF β 1 is also an enhancer of type VII collagen gene expression in keratinocytes and dermal fibroblasts [26, 91, 109]. In accordance, up-regulation of TGF β 1 in untransformed RDEB keratinocytes seems to increase COL7A1 mRNA levels (5.46 fold) although the present mutations would predict reduced levels of COL7A1 mRNA due to premature mRNA decay. This effect might be cell culture related. On the other hand Martins et al. [79] propose that loss of type VII collagen influences TGF β 1 signaling, leading to increased COL7A1 gene transcription. Although TGF β 1 levels are further elevated in SCCRDEB4, expression of COL7A1 mRNA drops to a very low level (0.29 fold). Reduction or absence of type VII collagen in RDEB keratinocytes has been reported to be related to increased migration and invasiveness [79], as well as enhanced motility and MMP expression [12, 24].

Hence the down-regulation of COL7A1 mRNA could be related to the process of cancer development rather than TGF β signaling. Other pathological processes could intercept the signal stimulating COL7A1 gene expression. TGF β signaling seems to regulate the expression of a higher number of genes in transformed cells [101] and is hence able to influence more cellular processes and pathways in malignant cells. That way, expression of COL7A1 mRNA could be abolished.

4.3. *TGF β signaling and MMP activity*

In tumors, TGF β 1 is able to induce the expression of MMP-2 and MMP-9 whilst simultaneously down-regulating their inhibitors, the TIMPs [40, 59]. Together these proteins contribute to migration and invasion of the tumor. MMPs are already known to be highly over-expressed in tumors [3, 14, 22, 28, 42, 48, 95]. This is also the case for SCCs in RDEB: Kivisaari et al. showed an up-regulation of MMP-7 and MMP-13 [66].

We are now able to demonstrate a dysregulation of their inhibitory molecule as well: while TIMP1 is expressed at elevated levels in RDEB-CL (2.61 fold), its expression is down regulated to normal levels in SCCRDEB4 (1.39 fold). Although there seems to be no reduction in TIMP1 expression as compared to normal keratinocytes, there clearly is a reduction of TIMP-1 levels in comparison to non-transformed RDEB cells. In SCCRDEB4, elevated levels of TGF β 1 seem to result in a down-regulation of TIMP-1. It can be suggested that RDEB-CL keratinocytes innately need higher levels of TIMP-1 to effectively control cell spreading. A reduction in TIMP-1 activity or a reduction of TIMP-1 mRNA to a normal level respectively, might therefore promote abnormal cell behavior and motility and hence favor SCC progression. MMPs might be less hindered from taking action now. As TIMPs bind to MMPs in 1 : 1 stoichiometry [52], elevated MMP levels and simultaneous down-regulation of TIMPs clearly indicates an imbalance between the two molecules and could result in or contribute to uncontrolled MMP activity.

Some of the genes mentioned in the context of cell-cell contact, adhesion and migration are also involved in MMP synthesis, control or degradation.

TSP-1 is able to bind MMPs to facilitate their degradation via the LRP-1. Many carcinoma cell lines

have been shown to produce only little or even no LRP1 [19]. LRP-1 already exhibits reduced levels in untransformed RDEB keratinocytes (0.26), while both TSP-1 (1.41) and TIMP-1 (2.61) expression is not altered or even elevated. This could also be interpreted as a factor that facilitates SCC development. SCCRDEB4 cells show reduced levels of TSP-1 (0.14) and LRP-1 (0.26) mRNA which might further contribute to uncontrolled activity of MMPs.

A down-regulation of type VII collagen has been associated with increased MMP expression as well [12, 24]. The finding that type VII collagen shows reduced expression in SCCRDEB4 (0.29) might propose increased MMP expression in SCCRDEB4. This assumption is supported by the results of Kivisaari et al. [66].

SPARC increases the production [126] and stimulates the activation of various MMPs in different cell types [39, 64, 81, 119]. However, untransformed RDEB-CL keratinocytes show normal SPARC mRNA levels, despite TGF β 1 up-regulation indicating that TGF β signaling still has an anti-tumor activity.

Obviously, there is not only a down-regulation of MMP-inhibitory molecules but also MMP degradation appears to be hindered. Additionally, TGF β signaling appears to boost the expression and activation of MMPs. TGF β 1 seems to be a key player in MMP control in RDEB SCC. The ability of untransformed RDEB keratinocytes to degrade MMPs might already be weakened and it is possible that the up-regulation of TIMP-1 could result from or be meant to compensate this deficiency. Once TGF β 1 levels exceed a certain level and/or are put in a malignant context, this last line of defense against uncontrolled MMP activity could fail and promote tumor growth and metastasis. Hence TGF β is likely to cause or contribute to the extraordinary aggressive behavior of SCCs in RDEB. Consequently, targeting this pathway might represent a useful supplementary strategy in cancer therapy of RDEB patients.

4.4. *TGF β signaling and GPNMB*

Of certain interest is also the massive up-regulation of glycoprotein nonmetastatic B expression in SCCRDEB4 (144 fold). For some cancer types, GPNMB has already been proposed as possible tumor and/or prognostic marker [51, 108]. So, next to being a possible tumor marker for RDEB related

SCC as well, GPNMB might also actively (although indirectly) take part in cancer promotion by further activating TGF β 1 through MMP activity. Besides, this GPNMB-mediated TGF β 1 activation could account for the increased TGF β 1 mRNA levels in the SCCRDEB4 as GPNMB expression is only elevated in the tumor cell line.

5. Conclusion

In conclusion, we have found clear indications that TGF β signaling seems to be of general importance in RDEB as even untransformed RDEB keratinocytes show elevated levels of TGF β 1 mRNA. In a non-malignant background this effect could be caused by wound healing processes or might act to elevate the expression of type VII collagen in order to counteract weakened cellular adhesion.

While TGF β -induced EMT apparently does not have a high relevance in RDEB related SCCs, its signaling appears to be of crucial importance in the control of MMP activity. Elevated TGF β 1 mRNA levels in the tumor cell line SCCRDEB4 seem to provide for reduced MMP inhibition and degradation, while simultaneously promoting their expression and activation. Apart from MMP activity enhancement, TGF β signaling is known to cause changes in cellular adhesion molecules that favor tumor spread and metastasis.

Importantly, the negative effect of elevated TGF β expression can only be seen in SCCRDEB4. Hence, an over-expression of TGF β 1 itself does not seem to cause malignant consequences. Probably either a certain threshold of TGF β 1 has to be exceeded or additional mutations have to be acquired. Also the time period of exposure to TGF β 1 has been shown to be of vital importance [131]. Considering this, elevated TGF β 1 expression in RDEB-CL might represent a reason, why SCCs in RDEB preferentially develop on sites that are subject to constant wounding. TGF β signaling is active in the context of wound healing, so body sites prone to blistering necessitate pronounced activity of this pathway. The prolonged exposure to TGF β 1 at elevated levels might promote SCC development and progression via the different actions described, especially by facilitating the loss of control over MMP activity. Targeting this signaling pathway might therefore contribute to RDEB cancer therapy. TGF β 1 becomes even more interesting as ele-

vated expression and protein levels of this gene seem to be causative for the failure of tamoxifen treatment and tumor immunotherapy [6, 60].

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