Increased invasive behaviour in cutaneous squamous cell carcinoma with loss of basement-membrane type VII collagen

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Summary
Type VII collagen (ColVII) is the main component of anchoring fibrils, attachment structures within the lamina densa of the basement membrane that are responsible for attachment of the epidermis to the dermis in skin. Mutations in the human ColVII gene, COL7A1, cause the severe inherited blistering disorder recessive dystrophic epidermolysis bullosa (RDEB) affecting skin and mucosae, associated with a greatly increased risk of skin cancer. In this study, we examined the effect of loss of ColVII on squamous cell carcinoma (SCC) tumourigenesis using RNAi in a 3D organotypic skin model. Our findings suggest that loss of ColVII promotes SCC migration and invasion as well as regulating cell differentiation with evidence for concomitant promotion of epithelial-mesenchymal transition (EMT). Immunostaining of RDEB skin and a tissue array of sporadic cutaneous SCCs confirmed that loss of ColVII correlates with decreased involucrin expression in vivo. Gene-expression-array data and immunostaining demonstrated that loss of ColVII increases expression of the chemokine ligand-receptor CXCL10-CXCR3 and downstream-associated PLC signalling, which might contribute to the increased metastatic potential of SCCs with reduced or absent ColVII expression. Together, these findings may explain the aggressive behaviour of SCCs in RDEB patients and may also be relevant to non-RDEB skin cancer, as well as other tumours from organs where ColVII is expressed.

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Key words: Collagen, Skin, Squamous cell carcinoma, Invasion

Introduction
Type VII collagen is the main component of anchoring fibrils, attachment structures that anchor the basement membrane to the dermis in skin. Mutations in the human type VII collagen (ColVII) gene, COL7A1, cause autosomal dominant or recessive dystrophic epidermolysis bullosa (RDEB), a disease characterised by chronic skin fragility and blistering (Christian et al., 1994). In the severe generalised subtype of RDEB (Hallopeau-Siemens RDEB), recurrent blistering leads to extensive scarring with a cumulative risk of squamous cell carcinoma (SCC) of 70% by age 45 (Finn et al., 2009). These patients frequently have premature termination codon mutations in COL7A1 resulting in truncated protein expression (Variki et al., 2007). ColVII collagen expression is greatly reduced, and anchoring fibrils are reduced in number or absent, in RDEB skin (McGrath et al., 1993).

The role of absent or reduced expression of ColVII in SCC development is controversial. There is an inverse correlation between the severity of RDEB and ColVII expression (Christian et al., 1994; McGrath et al., 1993). Surprisingly, data from an activated Ras/Ikb/Beta-driven skin tumourigenesis model suggested that the amino-terminal noncollagenous (NC1) domain of ColVII was necessary for tumour formation by RDEB keratinocytes (Ortiz-Urda et al., 2005). In the same model, a specific domain of the laminin 332 β3 chain binds to ColVII, promoting phosphoinositide-3-kinase signalling (Waterman et al., 2007). More recently, using a panel of RDEB SCC tumour samples, it was demonstrated that some RDEB SCC tumours do not have ColVII expression (Pourreyron et al., 2007).

It has been proposed that prolonged wounding and associated scarring in RDEB may lead to loss of differentiation and an ‘activated’, pre-malignant, phenotype (Goldberg et al., 1988; Smoller et al., 1990a). Other factors implicated in the development of SCC in RDEB include an increase in plasma basic fibroblast growth factor, mutations in the p53 gene, hypermethylation of p16[KNA] and decreased expression of insulin growth factor-binding protein 3, a mediator of apoptosis (Arbiser et al., 2004; Arbiser et al., 1998; Mallipatdi et al., 2004). Increased expression of metalloproteinase (MMP) 7 has also been observed in RDEB SCC tumours compared with sporadic SCC and is most abundant in poorly differentiated tumours, particularly within the invasive edge of tumour cells (Kivisaari et al., 2008). Mutations in the MMP1 promoter were recently identified to modify the severity of RDEB and may provide an explanation for variation in phenotype between patients with the same mutation in COL7A1 (Title et al., 2008).

In the current study, we used siRNAs targeting ColVII in a 3D organotypic skin model to examine the specific effect of loss of
ColVII on SCC biology. Our results indicate that loss of ColVII promotes invasion, disorganised differentiation and epithelial-mesenchymal transition (EMT). Analysis of gene expression array data demonstrates that decreased ColVII expression is associated with increased chemokine expression, specifically CXCL10-CXCR3, mediated through altered TGFβ signalling. These findings provide an explanation for the aggressive nature of SCC in severe RDEB and are also relevant to sporadic SCC and other tumours in organs where ColVII is expressed in the basement membrane: for example, mucosal, breast and gastrointestinal tract cancers.

**Results**

ColVII siRNAs specifically knockdown ColVII expression in cutaneous SCC

In order to examine the effect of loss of ColVII on cutaneous SCC with the same genetic background, a pool of four 19-bp synthetic siRNAs (si1, si2, si3 and si4) targeting different regions in the ColVII mRNA (supplementary material Fig. S1A,B) was transfected into two non-RDEB cutaneous SCC keratinocyte cell lines, MET1 and SCC-IC1 (siCOL7). A pool of four non-targeting siRNAs was used as a negative control (siα3). Western blotting (WB) analysis on conditioned media for secreted ColVII showed complete absence of the 290-kDa full-length protein and its NC1 domain in both cell lines, at day 4 post-transfection with siCOL7 (Fig. 1A). There was significant downregulation of ColVII in media and in cells, up to days 10 and 8 post-transfection with siCOL7, respectively (Fig. 1B). Titration of the siRNA concentration, from 20 to 2.5 nM, and the use of the individual siRNAs from the pool, demonstrated the specificity and efficiency of the siRNAs against ColVII (Fig. 1C,D).

Loss of ColVII in SCC increases in vitro cell migration

An in vitro scratch assay was performed to analyse migration in siRNA-transfected MET1 and SCC-IC1 cell lines. ColVII knockdown in SCC keratinocytes increased cell migration 2-fold (Fig. 2A). Live cell time-lapse microscopy of an in vitro scratch assay with MET1 cells confirmed this finding (supplementary material Movies 1 and 2). SCC keratinocytes transfected with different concentrations of the siRNA pool targeting ColVII, from 20 nM to 2.5 nM, showed a consistent increased cell migration for the different tested concentrations demonstrating that this effect is specific for ColVII-RNAi (data not shown). In a rescue experiment, siCOL7 cells were incubated with conditioned media from siC cells, which resulted in a 2-fold decrease in cell migration. In contrast, addition of siCOL7-conditioned media to siC cells did not have a significant effect on cell migration (Fig. 2B). When recombine full-length ColVII or the ColVII NC1 domain were added to siCOL7 cells we also observed a reduction in cell migration (Fig. 2C). Moreover, knockdown of ColVII in RDEB (severe generalised) SCC keratinocytes (no detectable ColVII) had no effect on cell migration (Fig. 2D). These results confirm that the increased migration phenotype observed in siCOL7 cells is specific to ColVII knockdown and can be rescued by extracellular ColVII. However, the reduction in siCOL7 cell migration in response to incubation in siC-conditioned media was greater than the response to incubation in media containing either recombinant ColVII or ColVII NC1 domain. This may be due to differences in conformation or post-translational modifications between recombinant and secreted ColVII. Another possible explanation is that other secreted factors differentially expressed by siC versus siCOL7 cells might contribute to the differences observed.

**Fig. 1.** Specific knockdown of ColVII in cutaneous SCC by siRNA. (A) WB analysis of ColVII from conditioned media of MET1 (left panel) and SCC-IC1 (right panel) cells, 4 days post-transfection with a pool of 19-bp targeting ColVII mRNA (siCOL7) or a pool of non-targeting siRNAs (siC). Laminin 332 (Lam332) WB was performed to demonstrate equal protein loading. (B) Timecourse expression of ColVII over a period of 10 days after siRNA transfection in MET1 cells. The upper panel shows a WB of conditioned media and cell extracts from siC (lane 1) and siCOL7 (lane 2) cells at days 2 to 10 post-transfection. Lam332 and GAPDH WBs were included as internal controls. The bottom panel shows densitometry analysis after normalisation against the respective loading controls, demonstrating that a significant ColVII knockdown is maintained up to day 8 in cells, or day 10 in conditioned media, post-transfection. (C) The upper panel shows a WB of conditioned media from siC, siCOL7, si1, si2, si3 and si4 MET1 cells at day 4 post-transfection. The lower panel shows densitometry analysis after normalisation against the pool induce significant ColVII knockdown. (D) WB analysis of conditioned media from siC and siCOL7 MET1 cells transfected with 20 to 2.5 nM final concentration of siRNA (lanes si20, si2.5, si7.5, and si2.5, respectively). The position of the bands corresponding to full-length 290 kDa ColVII (ColVII-FL), 145 kDa NC1 domain of ColVII (ColVII-NC1) and the 160 kDa reduced (cleaved) α3 chain (α3 chain-r) of Lam332 is indicated.
Knockdown of ColVII in SCC increases in vitro cell invasion accompanied by increased MMP2 activity

To examine whether expression of ColVII had an effect on SCC invasion, siRNA-transfected MET1 cells were cultured on collagen:matrigel gels with incorporated fibroblasts (supplementary material Fig. S2A). Collagen:matrigel gels have been previously shown to be a good model for analysis of cell invasion (Nystrom et al., 2005). ColVII knockdown in SCC cells increased the number of invading cells as well as the depth of invasion into the gel. Quantification of depth of invasion showed that SCC cells without ColVII invaded almost twofold deeper into the gel (Fig. 3A). Cancer invasion and EMT may be associated with overexpression of ECM-degrading proteinases, such as the gelatinase MMP2 (Yokoyama et al., 2003). ColVII knockdown in SCC keratinocytes resulted in increased expression and activity of pro-MMP2 (72 kDa), as detected by WB and gelatin zymography on conditioned media (Fig. 3B). Furthermore, in MET1, expression of active MMP2 (64 kDa) was detected only in siCOL7 cells. Loss of ColVII did not affect MMP9 activity in either cell line. Densitometry analysis showed that there was a 2.4- and 1.6-fold increase in pro-MMP2 activity in siCOL7 MET1 and SCC-IC1 cells, respectively (supplementary material Fig. S3A). In addition, siCOL7 cells showed increased expression of MMP2 in the cells at the migrating front in an in vitro scratch assay, when compared with siC cells (Fig. 3C). Neutralisation of MMP2 with a functional antibody reduced siCOL7 cell migration in an in vitro scratch assay to control levels, confirming the functional role of MMP2 in the increased migration of ColVII-null SCC cells (supplementary material Fig. S3B).

Loss of ColVII in SCC increases EMT

To analyse the effect of loss of ColVII on EMT, 3D organotypic cultures were generated on de-epidermalised dermis (DED) (supplementary material Fig. S2A) with siRNA-transfected MET1 cells. When seeded on DEDs and cultured at the air-liquid interface, keratinocytes form a stratified epidermis and express morphological markers of epithelial differentiation (Ojeh et al., 2001). Immunofluorescence (IF) staining confirmed that ColVII was removed by dispase (supplementary material Fig. S2B) and was

Fig. 2. Loss of ColVII in cutaneous SCC increases in vitro cell migration, which can be rescued by secreted ColVII. (A) The upper panel shows representative pictures of an in vitro scratch assay using siC and siCOL7 MET1 cells, taken before (t0) and 36 hours (t36) after migration. The lower panel shows the percentage change in migration in an in vitro scratch assay calculated using ImageJ. Data represent the mean ± s.d. (n=8). The experiment was repeated three times with similar results. (B) Computer-generated migration values of an in vitro scratch assay of siC and siCOL7 MET1 cells incubated with conditioned media from both siC and siCOL7 cells. Conditioned media were harvested 24 hours after incubation and incubated with the respective cells for 36 hours. A twofold decrease in cell migration was observed in siCOL7 cells when incubated with siC-conditioned media. Data represent the mean ± s.d. (n=8). (C) Computer-generated migration values of an in vitro scratch assay for 36 hours of siC and siCOL7 MET1 cells incubated with recombinant full-length ColVII or the recombinant NC1 domain. Recombinant proteins were added at a concentration of 5 μg/ml in media. BSA was used as a control. Both recombinant proteins partially reduced cell migration of siCOL7 cells. Data represent the mean ± s.d. (n=6). (D) An in vitro scratch assay for 36 hours showing that in RDEB SCC keratinocytes (Ks), which have no detectable ColVII expression, siRNAs for ColVII did not have an effect on cell migration. Data represent the mean ± s.d. (n=6). All experiments were performed at day 4 post-transfection. *, ** and *** indicate P<0.05, P<0.01 and P<0.001, respectively. Scale bars: 100 μm.
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not present in the basement-membrane zone (BMZ) from siCOL7 sections after 14 days, but linear expression was detectable in siC sections (Fig. 3D). Decreased membranous E-cadherin (also known as cadherin 1) was observed, with increased expression of MMP2 and vimentin-positive elongated cells in the papillary dermis adjacent to the BMZ, in siCOL7 organotypic cultures (Fig. 3D). We speculated that the latter were fibroblasts that were preferentially attracted to the BMZ in siCOL7 sections, or SCC keratinocytes undergoing EMT. This observation was further analysed by performing IF staining on sections from collagen:matrigel gels in which increased epithelial cell invasion was previously demonstrated (see Fig. 3A). Vimentin-positive invading epithelial cells were observed specifically in siCOL7 sections. Moreover, there was also an increase in vimentin-positive, non-epithelial cells around the invading cells in these sections (Fig. 4A, top and middle panels).

There is evidence that the vimentin intermediate filaments associate with αvβ3 integrin-rich endothelial focal contacts, playing a role in angiogenesis (Gonzales et al., 2001). Increased αvβ3 integrin was observed in siCOL7 invading epithelial cells (Fig. 4A, bottom panel). To explore whether these findings are relevant in vivo, IF staining was performed on RDEB (severe generalised subtype) patient peritumoural skin with varying levels of ColVII expression (decreased, patient A/absent, patient B). Expression of αvβ3 integrin inversely correlated with ColVII expression in vivo (Fig. 4B).

Loss of ColVII in SCC decreases terminal epithelial differentiation

Analysis of organotypic cultures on DED also revealed changes in markers of epithelial differentiation. In siCOL7 sections, expression

Fig. 3. Loss of ColVII in cutaneous SCC increases invasion, accompanied by increased MMP2 expression/activity and altered expression of EMT markers. (A) Organotypic cultures of siCOL7 and siC MET1 cells on collagen:matrigel gels with incorporated fibroblasts (top panel) immunostained with a pan-cytokeratin antibody and visualised with DAB. siCOL7 cells displayed increased invasion into the gel when compared with siC cells. The lower panel shows quantification of the depth of invasion. ***P<0.001. (B) Upper and lower panels show a WB and a gelatin zymogram, respectively, of MMP2 from conditioned media of siC and siCOL7 MET1 (left panel) as well as SCC-IC1 (right panel) cells. siCOL7 cells showed increased expression and activity of MMP2. A duplicate blot was probed with a Lam332 antibody. The position of the bands corresponding to 92 kDa pro-MMP9, 72 kDa pro-MMP2 and 64 kDa MMP2, as well as the 160 kDa reduced α3 chain (α3 chain-r) of Lam332, is indicated. (C) Detail of siC and siCOL7 MET1 cells at the migrating front in an in vitro scratch assay after staining with a MMP2 antibody (green). (D) Immunofluorescence staining of sections of siC and siCOL7 MET1 organotypic cultures on DED with antibodies to ColVII (red) and the EMT markers, E-cadherin (red), MMP2 (green) and vimentin (green) showing decreased expression of membranous E-cadherin and increased expression of MMP2 and vimentin-positive cells in the papillary dermis in siCOL7 sections. Experiments were performed at day 4 post-transfection in B and C. DAPI (blue) was used as a nuclear stain. Scale bars: 50 μm.
of the early epithelial differentiation marker, keratin 10, was significantly decreased, accompanied by increased expression of the hyperproliferation marker, keratin 6. The late differentiation-associated proteins, keratin 2e and involucrin, had an earlier and disorganised pattern of expression, and the cornified envelope component, transglutaminase I (TGM I), was downregulated in siCOL7 sections (Fig. 5). Analysis of RDEB peritumoural skin suggested similar findings in vivo. RDEB skin with reduced ColVII expression showed earlier and decreased expression of involucrin, whereas RDEB skin with absent ColVII demonstrated markedly reduced involucrin staining compared with normal skin (Fig. 6A). In order to correlate expression of ColVII and tumour differentiation in non-RDEB SCC, a tissue array containing 74 cutaneous SCC tumours was double-labelled with antibodies to ColVII and involucrin. Statistical analysis of scored sections revealed a significant positive correlation (Spearman’s rank correlation coefficient \( \rho = 0.5032, n = 74, P = 0.000017 \)) between ColVII and involucrin expression, suggesting that loss of differentiation in SCC is associated with loss of ColVII expression (Fig. 6B; supplementary material Table S1).

**Injection of recombinant ColVII into the papillary dermis generates a normal pattern of involucrin and E-cadherin expression in siCOL7 3D cultures**

In order to confirm whether the loss of epithelial differentiation and EMT phenotypes observed in siCOL7 sections was specifically a result of loss of ColVII, 10 \( \mu \)g of recombinant full-length ColVII protein were injected into siCOL7 and siC MET1 organotypic cultures on Ded on day 7, composites were cultured for a further 7 days and sections were then double-labelled with antibodies either to ColVII and involucrin or to ColVII and E-cadherin. The recombinant ColVII protein is identical to native ColVII and has been shown previously to be incorporated into the BMZ upon intradermal injection (Chen et al., 2002a; Woodley et al., 2004). As previously shown, linear ColVII was detected in some regions of the BMZ in siC sections whereas ColVII expression was absent in siCOL7 sections (Fig. 7A,B). Expression of involucrin was reduced in the cornified layers of the epidermis in siCOL7 when compared with siC sections. Injection of recombinant ColVII into siCOL7 organotypic cultures resulted in ColVII expression in the location of the BMZ and was sufficient to increase and restore expression of involucrin in the upper layers of the epidermis (Fig. 7A). In addition, injection of ColVII increased and restored membranous expression of E-cadherin in siCOL7 sections (Fig. 7B).

**Loss of ColVII in SCC increases expression of the chemokine ligand-receptor CXCL10-CXCR3 and PLC-β4**

RNA from triplicate biological replicates of siCOL7 and siC MET1 cells was isolated to perform whole-genome Illumina expression arrays. A list of genes that showed a minimum of 2-fold change (up- or downregulated) in expression in siCOL7 compared with siC cells is shown in supplementary material Table S2. Expression of MMP2 (1.5-fold change), vimentin (2.2-fold change) and the \( \alpha v \beta3 \) integrin subunit (1.9-fold change) was significantly increased in invading cells (Fig. 8A). Chemokine receptors (CXCRs) can generate lipid-derived second messengers through PLC activation, leading to a rise in intracellular calcium and subsequent directional movement of cells (Bach et al., 2007). Expression of CXCL10, its receptor CXCR3, and PLC-β4 was examined in siCOL7 and siC cells by performing IF staining on sections of collagen:matrigel gels. In siCOL7 sections, expression of these proteins was significantly upregulated in the most deeply invading epithelial cells (Fig. 8B). Upregulation of PLC-β4 was also demonstrated in the migrating siCOL7 epithelial cells in an in vitro scratch assay compared with control cells (supplementary material Fig. S4). In order to analyse the effect of CXCR3 in our model, a CXCR3 neutralising antibody
demonstrated that ColVII-null invading SCC cells have increased
et al., 1988). IF staining on sections of collagen:matrigel gels
carbohydrates on the plasma membrane of keratinocytes (Morrison
agglutinin (PNA). PNA has been shown previously to bind to
collagen:matrigel gels were IF double-labelled with an antibody
cXCL10 and CXCL2, CXCL3, CXCL6, CXCL8 (also known as IL8) and
and increased vimentin. Increased expression of the
towards a more aggressive phenotype.
SCC cells without ColVII showed increased invasion into
collagen:matrigel gels, accompanied by altered expression of
markers of EMT including loss of E-cadherin, increased MMP2,
expression of membranous CXCR3 was detectable particularly in
epithelial differentiation. IF staining of sections of siCOL7
and siCOL7 MET1
cellular gene expression through
was used in an in vitro scratch assay with siCOL7 and siC cells.
Addition of the CXCR3 neutralising antibody to siCOL7 cells
reduced migration to the same level as control cells (Fig. 8C). In
vivo, in normal skin or RDEB patient skin with ColVII expression,
CXCR3 expression in the epidermis was cytoplasmic and restricted
to the upper layers. Interestingly, in the ColVII-null RDEB patient,
expression of membranous CXCR3 was detectable particularly in
basal and immediate suprabasal layers of the epidermis (Fig. 8D).
The whole-genome Illumina expression array data also
demonstrated significant changes in genes known to be involved in
tGFβ signalling (supplementary material Table S3) in siCOL7
compared with siC MET1 cells (Leivonen and Kahari, 2007; Levy
and Hill, 2005; Valcourt et al., 2005). By using Ingenuity Pathways
Analysis, a signalling network including genes from
supplementary material Table S3 was generated (Fig. 9A). This
network connects COL7A1 directly with the chemokine ligands
[CXCL1, CXCL2, CXCL3, CXCL6, CXCL8 (also known as IL8) and
and NFκB. Sections of collagen:matrigel gels were IF double-labelled with an antibody
to phosphorylated Smad 2/3 (p-Smad 2/3) and the lectin, peanut
agglutinin (PNA). PNA has been shown previously to bind to
carbohydrates on the plasma membrane of keratinocytes (Morrison
et al., 1988). IF staining on sections of collagen:matrigel gels
demonstrated that ColVII-null invading SCC cells have increased
expression of p-Smad2/3 compared with control cells with
56.8±2.5% of invading siCOL7 cells containing cytoplasmic
aggregates compared to 19.06±3.8% of invading siC cells,
confirming involvement of the TGFβ signalling pathway in
invasion of siCOL7 cells (Fig. 9B).

Discussion
Previous studies have suggested an inverse correlation between the
severity of RDEB and ColVII expression (Christiano et al., 1994;
McGrath et al., 1993). This hypothesis was challenged by work
demonstrating that ColVII, specifically its NC1 domain, is required
for Ras-driven human skin tumourigenesis and invasion using
RDEB keratinocytes co-expressing activated Ha-Ras and the NFκB
inhibitor, IκBα, xenotransplanted on immunodeficient mice (Ortiz-
Urda et al., 2005). Ha-Ras mutations have a frequency of less than
10% in sporadic SCCs (Campbell et al., 1993; van der Schroeff et
al., 1990b) and so far have not been detected in RDEB-associated
SCCs (Pourreyron et al., 2007). Hence, it is not known whether
NC1 expression is required for tumourigenesis in models of RDEB
SCC other than the Ha-Ras/IκBα model. In fact, RDEB patients
who lack ColVII expression can develop SCC (Pourreyron et al.,
2007) and patients with dominant dystrophic epidermolysis bullosa
with one normal COL7A1 allele rarely develop SCC (Fine et al.,
2009), arguing against the idea that the NC1 domain of ColVII is
necessary for tumour formation in RDEB.

Reduced expression of ColVII in RDEB keratinocytes has been
associated with increased keratinocyte motility and increased MMP
expression (Bodemer et al., 2003; Chen et al., 2002b). Here, using
siRNA, we provide evidence for the first time that loss of ColVII
in SCC increases cell migration and invasion as well as expression
and activity of MMP2. Furthermore, using a 3D organotypic
culture model it was possible to demonstrate that loss of ColVII in
SCC results in disordered epithelial terminal differentiation. A
positive correlation between loss of ColVII and involucrin was also
demonstrated in peritumoural RDEB skin as well as in a series of
sporadic SCCs in vivo. An abnormal and premature pattern of
keratinocyte differentiation markers, including involucrin,
was previously described in RDEB skin and the SCC precursors, actinic
keratosis and Bowen’s disease (Said et al., 1984; Smoller et al.,
1990b). Failure of the cells to differentiate in a normal fashion might
be one of the first stages of SCC development and progression
through a more aggressive phenotype.

SCC cells without ColVII showed increased invasion into
organotypic cultures with knockdown of ColVII, increased
expression of CXCR3 and its ligand CXCL10 was observed in the
most invasive cells. A differential pattern of CXCR3 expression
was also confirmed in vivo in RDEB peritumoural skin. The
chemokine SDF-1 (also known as CXCL12) and its receptor,
CXCR4, are mediators of invasion and EMT in melanoma, and head
and neck SCC (Bartolome et al., 2004; Onoue et al., 2006; Yoon
et al., 2007). CXCL10 and its receptor CXCR3 have been shown

Fig. 5. Loss of ColVII in cutaneous SCC is associated with disorganised
epithelial differentiation. IF staining of sections of siC and siCOL7 MET1
organotypic cultures on DED with antibodies to the early differentiation
marker, keratin 10 (red), the hyperproliferation marker, keratin 6 (green), and
the later differentiation markers, keratin 2e (red), involucrin (red) and
transglutaminase I (TGM I, red). There was a significant downregulation of
keratin 10 and upregulation of keratin 6 in siCOL7 sections. Keratin 2e and
involucrin showed an earlier and disorganised pattern of expression, and TGM
I was downregulated in siCOL7 sections. DAPI (blue) was used as a nuclear
stain. Scale bars: 50 μm.

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to be involved in actin reorganisation, migration and invasion of melanoma and breast cancer (Kawada et al., 2004; Walser et al., 2006). Moreover, it was recently demonstrated that CXCR3 plays a crucial role in colon cancer cell metastasis to lymph nodes by increasing intracellular calcium concentration, which results in increased migration, invasion and MMP2/9 expression (Kawada et al., 2007). It is possible that the increase in CXCL10 and CXCR3 expression observed in ColVII-null SCC cells may also be contributing to EMT and increased invasion, perhaps through an autocrine regulatory loop, as suggested previously in breast adenocarcinoma (Goldberg-Bittman et al., 2004). In addition, increased CXCL10 expression by the tumour cells may also contribute to invasion by attracting stromal (fibroblast) cells to the tumour-stromal interface. An increase in fibroblasts around invading epithelial cells was observed in organotypic cultures with siCOL7 SCC cells.

Expression of ColVII is regulated by TGFβ at the transcriptional level (Ryynanen et al., 1991; Vindevoghel et al., 1998). Whole-genome Illumina expression array data revealed changes in several genes related to TGFβ signalling (supplementary material Table S3), and Ingenuity Pathways Analysis of the data suggested a central role for the TGFβ pathway connected to NFκB signalling, linking COL7A1 to the chemokine pathways. It is possible that loss of the ColVII protein alters TGFβ signalling as a feedback mechanism to increase COL7A1 gene transcription. Interestingly, in a hypomorphic mouse model of RDEB that expresses ColVII at about 10% of normal levels and shows a close phenotype to severe human RDEB, expression of active TGFβ1 is enhanced in the dermis when compared with wild-type mice. Expression of connective tissue

Fig. 6. Expression of ColVII positively correlates with involucrin expression in cutaneous SCC in vivo. (A) IF staining with an antibody to involucrin (green) in normal skin and in peritumoural skin of two unrelated RDEB patients (patient A and B). Reduced and disorganised expression of involucrin was observed in vivo in skin sections from RDEB patients who have decreased (patient A) or absent (patient B) ColVII expression, when compared to sections of normal skin. (B) IF double-labelling with antibodies to ColVII (green) and the differentiation marker, involucrin (red), in a skin SCC tissue array (SK802). Co-expression of the two proteins is shown in orange. Pictures are representative of a panel of 74 skin SCC sections where a positive correlation between ColVII and involucrin expression was observed. Tumour cells from a well-differentiated SCC (SCCI) show strong involucrin expression, which co-localises with ColVII (orange). Tumour cells at the edge of islands of the SCC show moderate expression of ColVII. In a moderately differentiated SCC (SCCII), there is reduced involucrin and ColVII expression. A poorly differentiated SCC (SCCIII) shows absent expression of both proteins. DAPI (blue) was used as a nuclear stain. Scale bars: 50 μm.

Fig. 7. Recombinant ColVII protein injection corrects loss of terminal epithelial differentiation and EMT. IF double-staining with antibodies to ColVII (green) and involucrin (red) (A), and to ColVII (green) and E-cadherin (red) (B), in siC and siCOL7 MET1 sections, with (+ColVII, bottom panel) and without (top panel) injection of recombinant ColVII protein. Organotypic cultures were incubated for a total period of 14 days, and injected with recombinant full-length ColVII (10 μg in PBS) 7 days after starting the experiment. Control organotypic cultures were injected with PBS only. DAPI (blue) was used as a nuclear stain. Scale bars: 50 μm.
growth factor (CTGF), which was upregulated in siCOL7 cells (+3.7-fold change) compared with control, was also increased in both epidermis and dermis of the hypomorphic mouse (Fritsch et al., 2008).

In normal adult tissues, TGFβ is generally seen as a tumour suppressor, inhibiting cell proliferation. However, depending on dose, cellular context and stage of tumourogenesis, TGFβ can contribute to cancer progression, inducing angiogenesis, suppressing apoptotic activity or enhancing invasion/EMT (Bierie and Moses, 2006; Glick et al., 2008; Han et al., 2005; Li et al., 2006). TGFβ1 is increased in about 80% of human head and neck SCCs, ranging from a 1.5- to 7.5-fold increase compared with endogenous TGFβ1 in normal epithelia (Lu et al., 2004a). Transgenic mice overexpressing wild-type TGFβ1 exhibit increased proliferation associated with increased expression of CTGF, MMP2 and CXCL10 as well as nuclear translocation of the NFκB subunits, p50 and p65 (Li et al., 2004b). Furthermore, Smad3 knockout mice, which are deficient in TGFβ signalling, show decreased susceptibility to the development of skin cancer associated with reduced NFκB signalling (Li et al., 2004a). In human prostate and colon cancer, TGFβ is a potent activator of NFκB (Grau et al., 2006; Lu et al., 2004b) and there are also data supporting a link between TGFβ and CXC signalling during tumour progression (Ao et al., 2007). The mechanism by which loss of ColVII promotes tumourigenesis needs further investigation. Nevertheless, based on our data and the literature, we speculate that in ColVII-null SCCs, TGFβ plays a key role in promoting invasion/EMT, through the cooperation of Smad and non-Smad pathways. TGFβ is known to activate and interact with several different pathways including those of the CXC chemokine ligand-receptor CXCL10-CXCR3 and PLC-β4. (A) Summary of the CXCL and PLC genes, which were differentially expressed (+, upregulated; –, downregulated) in siCOL10 compared with siC MET1 cells in the whole-genome Illumina expression arrays. (B) IF staining with antibodies to CXCL10, its CXCR3 receptor and PLC-β4, in sections of siCOL7 and siC MET1 organotypic cultures on collagen:matrigel gels. There was a significant increase in expression of CXCL10 and CXCR3, as well as PLC-β4, in the invading siCOL7 cells compared with siC cells, suggesting that loss of ColVII induces a chemokine gradient. (C) Computer-generated migration values of an in vitro scratch assay for 12 hours of siC and siCOL7/MET1 cells in the presence of a CXCR3 neutralising antibody or an isotype control (mouse IgG). Addition of a CXCR3 neutralising antibody (10 μg/ml) reduced siCOL7 cell migration to control levels. The experiment was performed at day 4 post-transfection. Data represent the mean ± s.d. (n=6). *P<0.05. (D) IF staining with a CXCR3 (green) antibody in peritumoural skin of two unrelated RDEB patients. In normal skin, expression of CXCR3 is mainly cytoplasmic and restricted to the upper spinous and granular layers of the epidermis. Patient A, with reduced expression of ColVII, showed a pattern of CXCR3 expression similar to the one observed in normal skin. In a patient with absent ColVII (patient B), CXCR3 expression was membranous and present in the cells of the basal and immediate suprabasal layers of the epidermis. DAPI (blue) was used as a nuclear stain. Scale bars: 50 μm.
organotypic model of invasion and differentiation that is similar to human epidermis and confirmation of the findings, in vivo, in human SCC tumours and RDEB skin. One might argue that in RDEB, inherited mutations in COL7A1 cause blistering and scarring that subsequently leads to SCC. However, a salient point is that the major difference between severe RDEB patients and normal individuals is loss of ColVII expression, with reduced or absent anchoring fibril formation.

This work is the first to show that loss of basement-membrane ColVII increases SCC invasion, through a mechanism that involves disorganised keratinocyte differentiation and EMT linked to CXCL10–CXCR3–PLC-β4 signalling, providing functional evidence of the role of COL7A1 as a tumour suppressor gene in skin. Recent work (Chan et al., 2008; Wood et al., 2007) showed that the COL7A1 gene is mutated and a candidate tumour suppressor gene in breast cancer, adding further importance to our findings. Furthermore, hypermethylation of COL7A1 in breast cancer resulted in loss of ColVII expression in tumours correlating with a poor prognosis. Our data may therefore also be relevant to tumours from other organs in which ColVII is expressed, including breast, bowel, lung, oesophageal and head and neck cancers.

Materials and Methods

Cell culture

The human non-RDEB cutaneous SCC keratinocyte cell lines MET1 (Proby et al., 2000), SCC-IC1 and the RDEB (severe generalised) SCC keratinocytes were cultured in DMEM:Ham’s F12 (3:1) supplemented with 10% FCS, 1% L-glutamine (200 mM) and Ready Mix Plus (0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 10 ng/ml EGF, 5 μg/ml transferrin, 8.4 ng/ml cholera toxin and 13 ng/ml isothyrosonine). Human foreskin fibroblasts (HFFs) were cultured in DMEM supplemented with 10% FCS and 1% L-glutamine (200 mM). All cells were cultured at 37°C and 10% CO2. Both cell lines were derived from primary cutaneous invasive SCCs and spontaneously immortalised in culture. MET1 has wild-type p53 and neither cell line has Ras-activating mutations in codons 12, 13 or 61 of the N and K ras genes (Popp et al., 2000; Pourreyron et al., 2007).

Human RDEB and non-RDEB SCC skin

Archival sections were obtained of formalin-fixed and paraffin-embedded tumours and peritumoral skin from two unrelated RDEB (severe generalised) patients (patient A, approximately 50% of ColVII expression compared with normal skin; patient B, absent ColVII expression). The ‘SK802. Skin cell carcinoma tissue array’ was obtained from US Biomax (Insight Biotechnology, UK). This study was conducted according to the Declaration of Helsinki Principles and was approved by the East London and City Health Authority Research Ethics Committee and cooperating centres.

Antibodies and recombinant proteins

The rabbit polyclonal anti-NC1 antibody, recombinant full-length ColVII and recombinant NC1 domain were purified as described (Chen et al., 2002a; Chen et al., 1997). The pKal rabbit polyclonal antibody recognising all three chains of laminin 332 was the generous gift of Dr M. Peter Marinkovich (Stanford University School of Medicine, USA) (Veitch et al., 2003). The E-cadherin (H-108) rabbit polyclonal and p-Smad2/3 (Ser 423/425) goat polyclonal antibodies were obtained from Santa Cruz Biotechnology, UK. Mouse pan-cytokeratin (AE1/AE3) and vimentin (V9) monoclonal antibodies were purchased from DakoCytomation, UK. Mouse monoclonal transglutaminase type I (B.C.1), and αv/β3 integrin (LM609) antibodies were obtained from Biogenesis, UK and Chemicon, UK, respectively. Goat polyclonal CXCL10 and mouse monoclonal CXCR3 (49801) antibodies were obtained from R&D Systems Europe Ltd, UK. The mouse monoclonal PLC-β4 (56) antibody was purchased from BD Biosciences, UK. The following in-house mouse monoclonal antibodies were used: HECDC-1 for E-cadherin (Shimoyama et al., 1989), SY5 for involucrin, LHK10 for keratin 10, LHK2e for involucrin, anti-keratin 6 (supernatant diluted 1:2 for IF staining). Antibodies were used at the following dilutions for WB and IF: anti-ColVII (1:2000 for WB and 1:400 for IF), anti-pKal (1:2000 for WB); anti-E-cadherin (H-108 and HECDC-1), anti-vimentin, anti-MMP2 (Ab-3), anti-transglutaminase type I, anti-involucrin, anti-keratin 10 and p-Smad2/3 (1:100 for IF staining); anti-pan-cytokeratin (1:200 for DAB staining); anti-αv/β3 integrin, anti-CXCL10, anti-CXCR3 and anti-PLC-β4 (1:50 for IF staining); anti-keratin 2e (neat supernatant for IF staining); and anti-keratin 6 (supernatant diluted 1:2 for IF staining).

siRNA transfection

For ColVII knockdown, MET1 and SCC-IC1 cell lines were transfected with a SMARTpool of four synthetic siRNAs (Dharmacon, UK), targeting ColVII (#M-011017-00). Transfection was performed according to the manufacturer’s protocol and optimised for a six-well plate. Briefly, cells were plated at 50% confluence and

Fig. 9. Ingenuity-generated network suggests that loss of ColVII affects TGFβ and/or NFκB signalling. (A) Network showing connections between ColVII and CXCL10–CXCR3 through TGFβ and NFκB signalling in siCOL7 cells. The differentially upregulated (red) and downregulated (green) genes in siCOL7 MET1 cells shown in the whole-genome Illumina expression arrays were mapped in the Ingenuity database. A signalling network generated by the Ingenuity Pathway Analysis software showed that relevant genes such as MMPs (including MMP2), integrin αv (ITGAV), vimentin (VIM) and CXCLs, as well as TGFβ-related genes, were linked through TGFβ and NFκB. The network shows direct (full arrows) and indirect (dotted arrows) interactions of TGFβ and NFκB with the up- and downregulated genes, which include CXCL10, CXCR3 and PLCB4. The colour intensity in the genes relates to the degree of fold change, with darker red/green corresponding to a higher-fold change. (B) IF staining with an antibody for p-Smad2/3 (green) in sections of siCOL7 and siC MET1 organotypic cultures on collagen:matrigel gels. The plasma membrane was stained with biotin-labelled lectin PNA (red). Top and bottom panels (confocal microscopy) show different magnifications of the same section. There was a significant increase in expression of p-Smad2/3 in the invading siCOL7 cells compared to siC cells. DAPI (blue) was used as a nuclear stain. Scale bars: 50 μm in the top panels, B; 15 μm in the bottom panels, B.
subjected to transfection the following day using 4 μg of DharmaFECT1 (Dharmacon, UK) transfection reagent and 12.5 nM final concentration of each siRNA (apart from the assay where siRNA concentration was titrated). Transfection on media was replaced with complete DMEM:Ham’s F12 media after 16 hours. CoVII protein expression was analysed by WB on cell extract and conditioned media, 2-10 days post-transfection. Cells incubated with the transfection reagent only (Mock) as well as cells transfected with a pool of non-targeting siRNAs (siCONTROL Non-Targeting siRNA Pool) were used as negative controls.

**Protein analysis**

For cell extract immunoblot analysis, keratinocytes were lysed in 1× Triton-lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA pH 7.4, 1% Triton X-100 and 10% glycerol) containing a protease-inhibitor cocktail (Roche, UK) and 10 mM EDTA (for CoVII and Laminin 332 analyses). For conditioned media immunoblot analysis, 90% confluent cells were incubated in serum-free media, and the cultures were maintained for an additional 24 hours. The media were collected, a protease- inhibitor cocktail, 2 mM N-ethylmaleimide (NEM), 1 mM PMSF and 10 mM EDTA were added, and concentrated 15-fold using a centrifugal filter device (Amicon, UK). For CoVII and laminin 332 analyses, reduced cell extract or conditioned media were subjected to 6% SDS-PAGE. Otherwise, lysates were subjected to 10% SDS-PAGE. Proteins were electrotransferred onto a nitrocellulose membrane. The presence of ColVII protein was detected by WB analysis using a primary antibody at 4°C overnight, and an HP-coupled anti-mouse or -rabbit secondary antibody for 1 hour at room temperature, and developed using ECL. For densitometry analysis, the image analysis program ImageJ, version 1.5.4s was used.

**Gelatin zymography**

For gelatin zymography, conditioned media from keratinocytes were produced and collected as described above. Non-reduced samples were subjected to SDS-PAGE on a 10% acrylamide gel containing 1 mg/ml of gelatin (Invitrogen, CA). After electrophoresis, gels were washed with 2.5% Triton X-100 for 2× 30 minutes, and incubated in assay buffer (15 mM Tris pH 7.4, 5 mM CaCl2, 1 mM PMSF and 2 mM NEM) for 48 hours at room temperature to induce gelatin lysis by gelatinases. The reaction was stopped by staining the gel in a solution of 0.1% Cooomassie Brilliant Blue G-25, 30% methanol and 10% acetic acid for 1 hour, and destaining in a solution of 30% methanol and 10% acetic acid.

**In vitro epidermal models**

Organotypic cultures on collagen:matrigel gels were performed as previously described with some modifications (Nyström et al., 2005). Collagen:matrigel gels were prepared by mixing 3.5 volumes of type I collagen (First Link, UK), 3.5 volumes of Matrigel (BD Biosciences, UK), 1 volume of 10% DMEM, 1 volume of FCS and 1 volume of DMEM with 10% FCS/HFF (resuspended at a density of 5×10³/ml). One ml of the gel mixture was placed into each well of a 24-well plate and allowed to polymerise at 37°C for 1 hour. After polymerisation, 1 ml of DMEM was added per well and gels were incubated for 18 hours to equilibrate. After that, siRNA-transfected non-RDEB SCC keratinocytes were seeded into a plastic ring placed on the reticular dermal surface of the DED, and incubated for 24 hours. After 24 hours, the rings were removed and gels were raised to the air-liquid interface on stainless steel grids. The gels were harvested at day 10, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin.

For organotypic cultures on DED, siRNA-transfected non-RDEB SCC keratinocytes were seeded into a plastic ring placed on the reticular dermal surface of the DED, and incubated for 4 days. After that, the ring was removed, the DED was inverted and transfected ECC keratinocytes (5×10⁶) were seeded into a steel ring placed on the papillary dermal surface. Twenty-four hours later, the cultures were raised to the air-liquid interface on stainless steel grids. The DED cultures were harvested at day 14, and either fixed in 4% PFA for paraffin embedding or frozen at −80°C.

**Injection of recombinant full-length CoVII into organotypic cultures on DED**

Organotypic cultures on DED were performed as described earlier with the difference that recombinant full-length CoVII protein was injected into the DED cultures 7 days after raising the cultures to the air-liquid interface. Ten μg of recombinant CoVII protein diluted in PBS to a total volume of 100 μl was injected intradermally into the centre of the DED using a 30-gauge needle. DED injected with 100 μl of PBS only was included as a negative control. The DEDs were cultured for 7 days more and subsequently harvested, fixed and embedded as described earlier.

**IF staining and microscopy**

Five μm-thick paraffin sections were fixed and paraffin-embedded organotypic cultures were cut using a microtome, and air-dried at room temperature overnight. Cryosections (5 μm thick) from organotypic cultures were cut in a cryostat, fixed in 4% PFA and air-dried. Paraffin sections were de-paraffinised using xylene and hydrated in descending grades of ethanol to distilled water, and antigen retrieval was performed by heating samples in 10 mM citrate buffer, pH 6, for 10 minutes. Immunohistochemistry was performed as follows: sections from organotypic cultures or patient’s skin were permeabilised and blocked at room temperature by incubating in PBS with 0.1% Triton X-100 for 10 minutes and in PBS with 0.1% Triton X-100 and 0.3% BSA for 10 minutes, respectively. Incubation with the primary antibody diluted in 0.1% Triton X-100 and 0.3% BSA was performed at 4°C overnight, unless otherwise stated. The secondary Alexa Fluor 568-red or 488-green, goat anti-rabbit or goat anti-mouse (Invitrogen, UK) or Alexa Fluor 488-green donkey anti-goat (Molecular Probes, UK) antibodies were added at 1:800 dilution for 45 minutes at room temperature. The biotin-labelled lectin PNA (Vector Laboratories, UK) was used at a concentration of 10 μg/ml for 30 minutes followed by Texas Red Avidin DCS (1:200) for 10 minutes at room temperature. DAPI (1:10 000) was used as a nuclear stain. Sections were examined and photographed using either a Leica DMRB00 fluorochrome microscope (Leica Germany) or a Zeiss LSM 510 confocal microscope (Zeiss Germany). Cells were photographed using a Nikon Eclipse TE2000-S time-lapse microscope (Nikon Japan). Images were analysed using Photoshop (Adobe) software. Invading cells containing p-Smad2/3 aggregates were counted by eye from three 400× fields from each condition.

**Cell migration assay**

Keratinocyte migration was assessed by an in vitro scratch assay as previously described with some modifications (Cha et al., 1996). Briefly, cells were plated densely in a six-well tissue culture plate. Confluent cells were then treated with mitomycin C (10 μg/ml) for 1 hour at 37°C to inhibit cell proliferation, and a standardised scratch was made using a P1000 plastic tip. Cells were incubated in DMEM:Ham’s F12 with 15 μg/ml of type I collagen to promote migration (First Link, UK). When added, purified recombinant full-length CoVII, purified CoVII NC1 domain or BSA were used at a concentration of 5 μg/ml. The CXCR3-neutralising antibody and isotype control were used at a concentration of 10 μg/ml. The MMP2-neutralising antibody and isotype control were used at a concentration of 2 μg/ml. The extent of scratch closure was quantified by measuring the area of the scratch before and 36 hours after migration, using ImageJ and expressed as percentage change. Confirmation of a difference in migration as statistically significant was calculated using an unpaired Student’s t-test with a two-tailed distribution. For MMP2 and PLC-B4 IF staining, siRNA-transfected SCC cells were grown to confluency, scratched using a P1000 plastic tip and incubated in complete HK growth media at 37°C in 10% CO₂ for 12 hours. Cells were then fixed in cold 4% PFA for 10 minutes and washed three times in 1× PBS. IF staining was performed as described earlier. The MMP2 or PLC-B4 (diluted 1:50) antibodies were added to the cells at 4°C overnight. Live cell time-lapse microscopy of an in vitro scratch assay was performed as described above, except the cells were incubated in a Nikon Eclipse TE2000-S time-lapse microscope (Nikon Japan) for 60 hours at 37°C in 10% CO₂. Pictures were taken every 30 minutes and data was analysed using MetaMorph version 6.3r2 software.

**Cell invasion assay**

Depth of keratinocyte invasion into collagen:matrigel gels was assessed using AE1/AE3-immunostained sections. For immunostaining, sections were prepared, permeabilised and blocked as described above. Antigen retrieval using 0.1% α-chymotrypsin for 20 minutes at 37°C was performed. Endogenous peroxidases were neutralised with 0.45% hydrogen peroxide in methanol for 15 minutes, and the primary antibody applied for 1 hour at room temperature. An anti-mouse IgG biotinylated secondary antibody (Vectastain EliteSections) and peroxidase-labelled streptavidin (Vectastain Elite ABC Reagent; Vector Laboratories) were applied for 30 minutes each. The peroxidase was visualised using diaminobenzidine (DAB) (DakoCytomation, UK) and counterstained in Meyer’s Hematoxylin. The depth of keratinocyte invasion (vertical distance from the base of the epidermis to the deepest invading keratin-staining cell) into the gel was quantified using ImageJ in five different areas of three separate sections from each cell type.

**Whole-genome Illumina expression arrays**

For whole-genome Illumina expression analysis, RNA was extracted from triplicate biological replicates of siCOL7 and siMET1 cells 4 days after siRNA transfection. Total RNA was extracted from the cells and purified using the RNeasy Kit (Qiagen, UK) according to the manufacturer’s instructions, and hybridised to a HybridiZe 6-Sample BeadChip (whole-genome gene expression for BeadStation). The generated data were analysed using Illumina’s BeadStudio Data Analysis Software and subjected to pathway analysis using Ingenuity Pathways Analysis software (Ingenuity Systems, Redwood City, CA).
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