

Connective tissue growth factor causes EMT-like cell fate changes *in vivo* and *in vitro*

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Summary

Connective tissue growth factor (CTGF) plays an important role in the pathogenesis of chronic fibrotic diseases. However, the mechanism by which paracrine effects of CTGF control the cell fate of neighboring epithelial cells is not known. In this study, we investigated the paracrine effects of CTGF overexpressed in fibroblasts of Col1a2-CTGF transgenic mice on epithelial cells of skin and lung. The skin and lungs of Col1a2-CTGF transgenic mice were examined for phenotypic markers of epithelial activation and differentiation and stimulation of signal transduction pathways. In addition to an expansion of the dermal compartment in Col1a2-CTGF transgenic mice, the epidermis was characterized by focal hyperplasia, and basal cells stained positive for α SMA, Snail, S100A4 and Sox9, indicating that these cells had undergone a change in their genetic program. Activation of phosphorylated p38 and phosphorylated Erk1/2 was observed in the granular and cornified layers of the skin. Lung fibrosis was associated with a marked increase in cells co-expressing epithelial and mesenchymal markers in the lesional and unaffected lung tissue of Col1a2-CTGF mice. In epithelial cells treated with TGF β , CTGF-specific siRNA-mediated knockdown suppressed Snail, Sox9, S100A4 protein levels and restored E-cadherin levels. Both adenoviral expression of CTGF in epithelial cells and treatment with recombinant CTGF induced EMT-like morphological changes and expression of α -SMA. Our *in vivo* and *in vitro* data supports the notion that CTGF expression in mesenchymal cells in the skin and lungs can cause changes in the differentiation program of adjacent epithelial cells. We speculate that these changes might contribute to fibrogenesis.

Key words: CTGF, Epithelial–mesenchymal transition, Dermal fibrosis, Pulmonary fibrosis

Introduction

Organ fibrosis is the outcome of a complex set of cellular interactions initially caused by a pathological injury followed by a process of dysregulated healing (Wynn, 2008). Tissue or organ fibrosis results in increased morbidity and mortality in humans. Hallmarks of fibrosis are the accumulation of fibroblasts and their contractile partners, the myofibroblasts. In addition to resident fibroblasts, perivascular fibroblasts or pericytes, mesenchymal stem cells, and circulating fibrocytes derived from the bone marrow compartment also contribute to the enhanced numbers of fibroblasts and to increased collagen production (Abraham et al., 2007; Krieg et al., 2007). A certain proportion of fibroblast-like cells in fibrotic lesions originate from epithelial cells that have acquired migratory and proliferative characteristics (Krieg et al., 2007). These cells are stimulated by a variety of cytokines, with TGF β being a key player in fibrosis (Pohlers et al., 2009).

CTGF is a secreted matricellular protein that has been implicated as a regulator of cellular proliferation, angiogenesis and remodeling of the extracellular matrix (Friedrichsen et al., 2003; Ivkovic et al., 2003; Leask and Abraham, 2006). CTGF can act on the same cells that produce it in an autocrine manner elevating extracellular matrix (ECM) synthesis in normal fibroblasts to a level seen in cells of fibrotic lesions (Shi-wen et al., 2000). Our recent mouse model, in which CTGF is

overexpressed in mesenchymal cells *in vivo*, clearly demonstrates that fibrosis of multiple organs can be mediated by CTGF alone independently of TGF β activation (Sonnylal et al., 2010).

During normal development and during wound healing, epithelial mesenchymal transition (EMT) plays a very dynamic role (Kalluri and Neilson, 2003). During EMT, tightly adjoining epithelial cells resting on a basal lamina with apical–basolateral polarization convert to a non-polarized mesenchymal cell phenotype, detach from the epithelial layer, develop migratory properties and express proteins that are typical of mesenchymal cells. In certain pathological situations, such as in kidney and lung fibrosis, epithelial cells acquire mesenchymal characteristics and contribute to enhanced synthesis of collagen. During EMT, epithelial cells acquire the ability to interact with specific interstitial matrices subsequent to traversing the basement membrane. Generally such cell fate switches of cellular identity and function are important for disease progression.

We have reported that transgenic mice expressing high levels of CTGF specifically in mesenchymal cells (Col1a2-CTGF) manifest hallmarks of sustained multi-organ fibrosis recapitulating key aspects of the fibrotic disease systemic sclerosis (SSc, scleroderma) (Sonnylal et al., 2010). In addition to overt changes in the connective tissue compartments, we observed focal hyperplasia in the epidermal layer of the fibrotic skin and in uninvolved areas of fibrotic lung. We hypothesize that

the persistent overexpression of CTGF in mesenchymal cells leads to epithelial cell proliferation and differentiation by a paracrine mechanism.

In the present study, we explored the paracrine effects resulting from the production and secretion of CTGF by mesenchymal cells on neighboring epithelial cells in fibrotic skin and lung of Col1a2-CTGF transgenic mice. We found that some basal keratinocytes in the skin and alveolar epithelial cells in fibrotic foci and normal areas of the lung are morphologically and functionally different from epithelial cells in these tissues in wild-type (wt) mice. Cells that were committed to an epithelial lineage are undergoing a change in their genetic program that is exemplified by expression of EMT marker genes. In the skin these changes are associated with activated MAPK and ERK signaling. *In vitro* studies in lung epithelial cells showed that the onset of EMT marker gene expression due to exogenous TGF β can be blocked by CTGF knockdown suggesting that CTGF mediates TGF β -induced EMT. Furthermore, expression of CTGF in lung epithelial cells or treatment with exogenous CTGF also induced EMT-like changes *in vitro*. Overall, our results provide new evidence that CTGF causes an EMT-like phenotype in skin and lung *in vivo*, and that these changes are likely to occur through a paracrine mechanism.

Results

Alteration in keratin and integrin β gene expression in the skin of Col1a2-CTGF transgenic mice

Mice overexpressing CTGF in mesenchymal cells developed dramatic and progressive thickening of the dermis. In addition, we observed increased hyperplasia and hypertrophy of keratinocytes in focal areas of the dermal–epidermal junction. Evidence for hyper-proliferation of epidermal cells was shown by the increase in the multilayer structure of the epidermis of Col1a2-CTGF mice. Previous experiments had also shown a threefold increase in BrdU incorporation in the areas of epidermal hyperplasia (data not shown). These results were confirmed by increased expression of the K16 proliferation marker in the interfollicular epidermis of transgenic mice compared to wt littermate controls (Fig. 1A,B, green), with an increase in the number of positive cells to nearly 10% of the total cell frequency.

Since the epidermis of Col1a2-CTGF mice showed increased hyperplasia, we wanted to determine if the apical–basal polarity was maintained by the basal cells in the epithelium. To address this, we analyzed the expression of integrins. Integrins are transmembrane receptors that mediate cell–cell adhesion and terminal differentiation of keratinocytes. In wt skin the distribution of integrin β 4 was, as expected, mainly confined to the basal side of the basal epithelial cells. In contrast, the expression of integrin β 4 in the epidermis of Col1a2-CTGF transgenic mice was irregularly distributed all around the hyperproliferative epithelial cells (Fig. 1A,B, red). Another epithelial specific integrin, α v β 6, that is barely detectable in quiescent epithelial cells is rapidly induced upon epithelial injury and imparts increased migratory properties to these cells (Huang et al., 1996). Interestingly, we observed a marked enhancement in the expression of integrin β 6 in the epidermis of Col1a2-CTGF transgenic mice compared to littermate controls (Fig. 1C,D) suggesting that these cells might have increased migratory properties. The redistribution of integrin β 4 suggests that the basal cells in the epidermis had lost their apical–basal polarity.

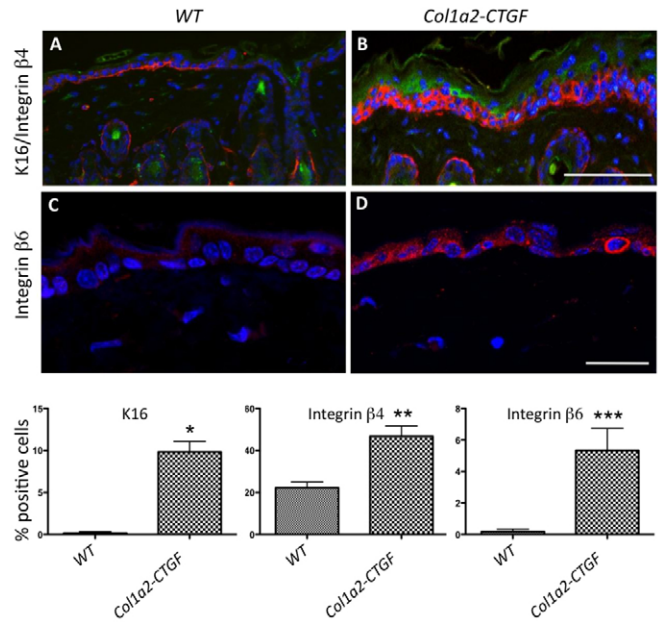


Fig. 1. Abnormal expression of hyperproliferative epidermal markers in Col1a2-CTGF transgenic mice. The expression of keratin 16 (a hyperproliferative epidermal marker), integrin β 6, and integrin β 4 in the skin of wt and Col1a2-CTGF transgenic mice was examined by immunofluorescence. Increased expression of keratin 16 (green) was observed suprabasally in the epidermis in Col1a2-CTGF transgenic mice (B) compared to wt controls (A). Increased expression of integrin β 4 (red) was observed in the hyperproliferative epidermis of Col1a2-CTGF transgenic mice (B) whereas in wt skin integrin β 4 expression was restricted to the basal side of the basal layer and seemed reduced (A). Epidermal cells also showed a marked increase in the level of integrin β 6 in Col1a2-CTGF transgenic mice (D), whereas no staining was observed in control skin (C). Scale bars: A, B=100 μ m (A,B), 50 μ m (C,D). Quantification of the expression of these proteins is shown below. Data are means \pm s.d.; $n=6$ skin sections. * $P<0.0001$, ** $P<0.001$, *** $P<0.0005$, Student's *t*-test.

Based on the marked increase in integrin β 6 expression, we hypothesize that these cells had acquired increased migratory properties. Both changes are likely the result of changes in the genetic program of these cells.

Col1a2-CTGF mice exhibit focal hyperplasia of the epidermis associated with cell fate changes in epithelial cells

The increase in collagen in the dermis of Col1a2-CTGF transgenic mice was associated with increased numbers of myofibroblasts (Sonnylal et al., 2010). Immunostaining of skin for α SMA revealed large numbers of cells positive for α SMA in both the papillary and the reticular dermis of Col1a2-CTGF transgenic mice (Sonnylal et al., 2010). Intriguingly, we have observed that α SMA-positive cells are intermingled with basal cells in the hypertrophic epidermis of Col1a2-CTGF transgenic mice (Fig. 2A,C and corresponding DIC images B,D). We explored this finding further and examined the origin of these cells. Immunostaining for Snail, a marker for EMT (Kalluri and Neilson, 2003), revealed significant Snail levels in areas in which there was focal hyperplasia of the epidermis in Col1a2-CTGF transgenic mice. Intense cytoplasmic staining for Snail was seen in the basal cells adjacent to the dermis and clear Snail

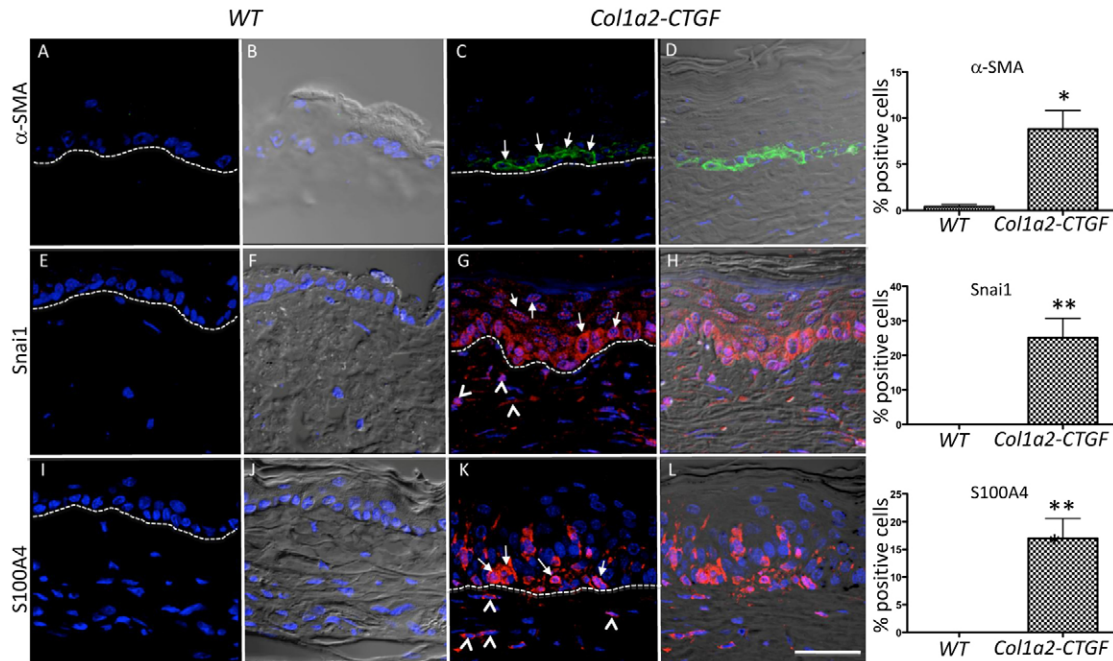


Fig. 2. Changes in the genetic program of the basal cells in the epidermis. Immunofluorescence was performed on skin sections of Col1a2-CTGF transgenic mice and wt littermate controls with antibodies to α SMA, Snai1 and S100A4. *De novo* expression of α SMA was increased in the epidermis of Col1a2-CTGF fibrotic skin (C; corresponding DIC overlay in D), indicated by white arrows, compared with wt littermate controls (A; corresponding DIC overlay in B). Cells in the basal layer also stained positive for Snai1 (G; corresponding DIC overlay in H) indicated by white arrows, whereas no Snai1 staining was observed in controls (E; corresponding DIC overlay in F). Similarly, S100A4 staining was observed in basal cells of the epidermis of Col1a2-CTGF transgenic mice (K; corresponding DIC overlay in L), and absent in control sections (I; corresponding DIC overlay in J). Arrowheads indicate the cells expressing Snai1 and S100A4 in the dermis. The horizontal dashed line marks the dermal–epidermal junction. Scale bar: 50 μ m. Quantification of the expression of these proteins is shown on the right. Data are means \pm s.d., $n=6$ skin sections. * $P<0.005$, ** $P<0.001$, *** $P<0.001$, Student's *t*-test.

staining primarily confined to the nucleus was seen in the upper layers of the epidermis in Col1a2-CTGF transgenic mice. Snai1 staining was absent in wt controls (Fig. 2E,G and corresponding DIC images F,H). To determine if the epidermal cells of Col1a2-CTGF transgenic mice displayed properties of newly formed mesenchymal cells, immunofluorescence was performed with antibodies against S100A4 or FSP-1, a Ca^{2+} binding protein closely associated with EMT. We observed that cells in both the basal and suprabasal layer of the Col1a2-CTGF epidermis showed robust staining for S100A4, unlike the skin epidermis of wt mice where staining for S100A4 was absent (Fig. 2I,K and corresponding DIC images J,L). Marked staining for α SMA, Snai1 and S100A4 in the epidermal cells within focal areas of hyperplasia in the epidermis of Col1a2-CTGF transgenic mice strongly suggest that these cells were undergoing an EMT-like process.

We also observed staining for S100A4, a marker of newly formed fibroblasts, in cells of the papillary dermis suggesting that these cells either arose from resident fibroblasts or originated from epithelial cells by EMT (Fig. 2I,K). Taken together these results strongly suggest that overexpression of CTGF in the dermal compartment is able to induce EMT-like changes in the adjacent epithelial cells of the epidermis most likely by paracrine mechanisms.

Aberrant Sox9 expression in the epidermis of adult Col1a2-CTGF transgenic mice

Sox9, which belongs to the HMG box super-family of DNA binding proteins, is a key transcription factor for chondrocytes and several other lineages (Pritchett et al., 2011; Bi et al., 1999).

In the skin, and particularly in the hair follicle, Sox9 has essential roles in the development of the outer root sheath (ORS) and of the stem cell compartment (the bulge) (Vidal et al., 2005). Recent evidence in the literature has implicated Sox9 expression in diseases that affect the extracellular matrix such as in skin keloids (Naitoh et al., 2005), glomerular sclerosis of the kidney (Bennett et al., 2007), and activated stellate cells in the liver (Hanley et al., 2008). We wanted therefore to investigate whether the distribution of Sox9 expression in the skin of Col1a2-CTGF transgenic mice was altered. Using immunofluorescence, we showed abundant expression of nuclear Sox9 in the basal cells of the epidermis in Col1a2-CTGF transgenic mice but not in wt mice (Fig. 3A,B and corresponding DIC images C,D). Increased expression of Sox9 (over threefold) was also observed in the ORS and in the bulge of hair follicles (Fig. 3E,F and corresponding DIC images G,H).

Similar to the abnormal expression of α SMA, Snai1 and S100A4, the anomalous Sox9 expression patterns strongly suggest that overexpression of CTGF in mesenchymal cells results in major cell fate changes in the basal layer of the epidermis.

Multiple signaling pathways are activated in the epidermis of Col1a2-CTGF transgenic mice

We have previously provided evidence that increased expression of CTGF in mesenchymal cells causes constitutive activation of multiple signaling molecules including phosphorylated p38 (p-p38), Erk1/2 (pErk1/2), Akt (pAkt), and PI3K (Sonnylal et al.,

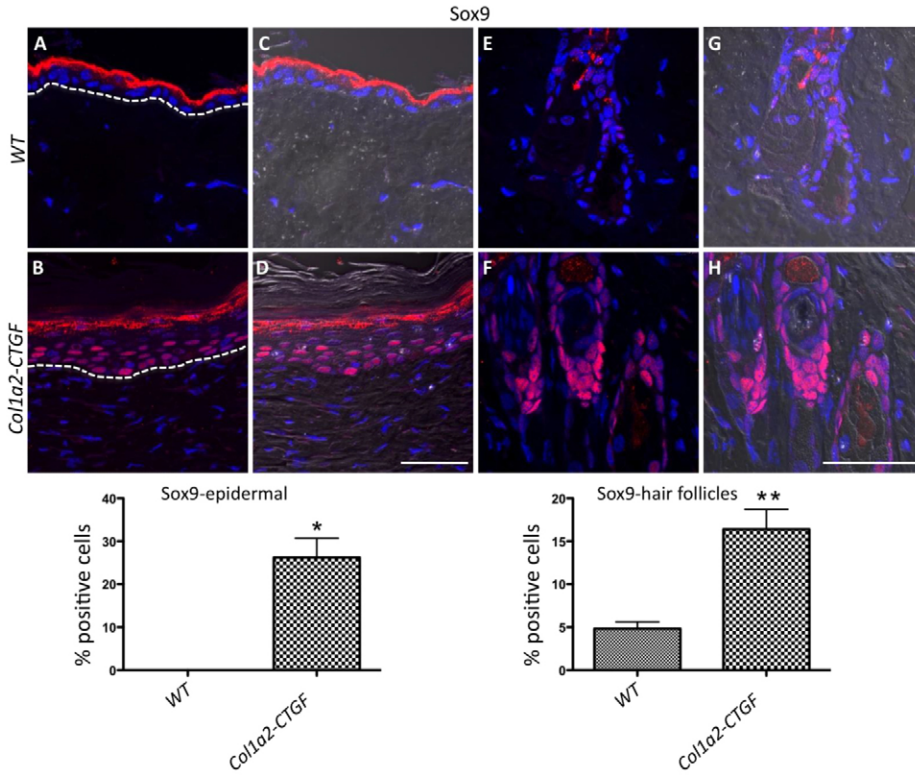


Fig. 3. Abnormal expression of Sox9 in the epidermis of Col1a2-CTGF transgenic mice. Immunofluorescence of skin sections showed abundant Sox9 expression in the basal cells of the epidermis of Col1a2-CTGF mice (B; corresponding DIC overlay in D) compared to wt controls (A; corresponding DIC overlay in C). Increased Sox9 expression was also observed in the ORS and the bulge of hair follicles in Col1a2-CTGF transgenic mice (F; corresponding DIC overlay in H) compared to wt controls (E, corresponding DIC overlay in G). Autofluorescence in the cornified layer of the skin is observed in both transgenic mice and controls. Scale bar: 50 μ m. Quantification of the expression is shown below. Data are means \pm s.d., $n=6$ skin sections. * $P<0.0004$, ** $P<0.002$; Student's *t*-test.

2010). Because integrin $\beta 6$ causes the activation of Erk1/2 and p38 signaling molecules in epithelial cells (Sullivan et al., 2011; Ahmed et al., 2002), and because these molecules are known to

stimulate the proliferation of these cells, we examined the skin using routine histology (Fig. 4A,B) and the phosphorylation status of Erk1/2 and p38 in the skin of Col1a2-CTGF transgenic

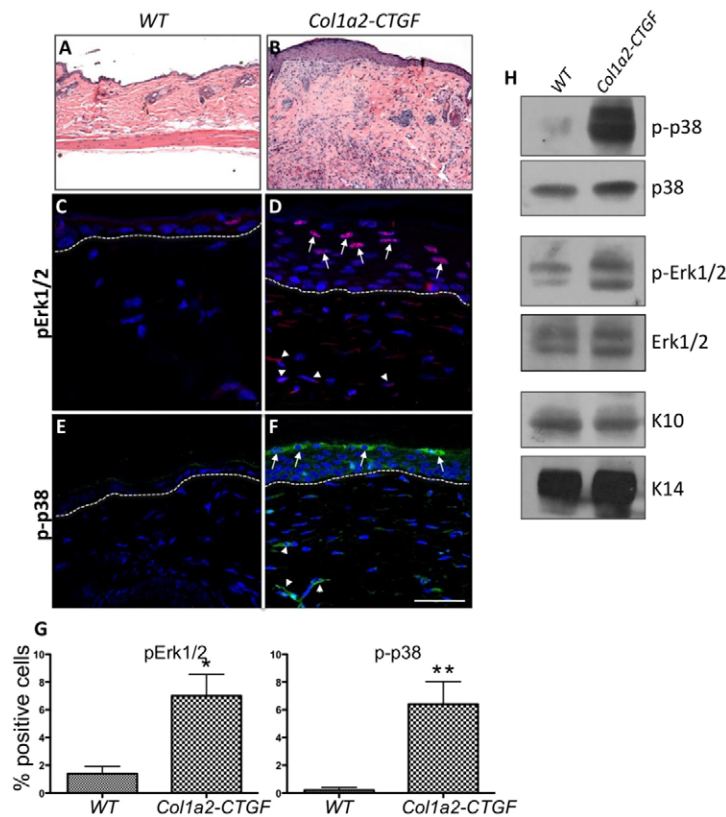


Fig. 4. Constitutive activation of MAPK and ERK signaling in the epidermis of Col1a2-CTGF transgenic mice. (A,B) Compared with wt mice, Col1a2-CTGF transgenic mice showed pronounced and progressive dermal fibrosis with focal thickening of the epidermis (Hematoxylin and Eosin staining). (C–F) Skin sections from Col1a2-CTGF transgenic mice and wt littermate controls were stained with antibodies against p-ERK1/2 and p-p38. Increased nuclear localization of p-ERK1/2 and p-p38 was observed in transgenic mice (D,F) compared to wt controls (C,E). Increased p-ERK1/2 and p-p38 was also observed in the dermis (white arrowheads). (H) Western blot analysis of keratinocyte cell extracts from Col1a2-CTGF transgenic mice also revealed increased p-p38 and pERK1/2 compared to control samples. Total p38 and ERK1/2 were unchanged in both samples and keratins 10 and 14 were used as loading controls. (G) Quantification of p-ERK1/2 and p-p38 expression. Data are means \pm s.d., $n=6$ skin sections. * $P<0.01$, ** $P<0.005$, Student's *t*-test.

mice and wt littermate controls. These experiments would help us gain insights into the mechanism that could contribute to increased proliferation and dedifferentiation of keratinocytes. Immunofluorescence with antibodies against phosphorylated p38 (p-p38) and ERK1/2 (p-ERK1/2) showed three- to sevenfold increases in the levels of both pErk1/2 (Fig. 4C,D,G) and p-p38 (Fig. 4E,F,G) in the granular and cornified layers of the epidermis. We hypothesize that the increased expression of integrin $\beta 6$ and the increased activation of p38 and Erk1/2 could account for the hyper-proliferation of keratinocytes in Col1a2-CTGF transgenic mice.

Western blot analysis of protein extracts from primary keratinocytes also showed constitutive activation of ERK1/2 and p38 in cells from Col1a2-CTGF transgenic mice (Fig. 4H). Levels of total p38 and ERK1/2 were the same in cells derived from both Col1a2-CTGF and wt mice (Fig. 4H).

Contribution of epithelial cells to fibrotic lung lesions in Col1a2-CTGF transgenic mice

Col1a2-CTGF transgenic mice died early from extensive focal fibrotic lesions within the lung parenchyma. The fibrosis was characteristic with markedly increased Masson's trichrome staining and increased collagen type I, CTGF expression compared to wt control mice (Sonnylal et al., 2010) (supplementary material Fig. S1A–D). We also observed a three to fourfold increase in BrdU-positive cells in the fibrotic lesions suggesting increased numbers of cells undergoing cell division (data not shown). To determine the proportion of cells that are myofibroblasts and to distinguish them from alveolar

epithelial cells we performed co-immunostaining of lung sections from Col1a2-CTGF transgenic mice and wt mice for α SMA and TTF-1, markers for myofibroblasts and alveolar epithelial cells, respectively. The immunofluorescence analysis revealed a marked increase in α SMA-positive cells in the lesional areas of the lungs of Col1a2-CTGF transgenic mice, whereas α SMA-positive cells were lacking in the lungs of wt control mice (Fig. 5A–D,G). A significant proportion of cells in the fibrotic lesions were also positive for TTF-1 (Fig. 5A,C and the corresponding DIC images in B,D) consistent with the BrdU staining suggesting an increased proliferation of epithelial cells within the fibrotic lesions. Most interesting was the observation of cells within the fibrotic foci that were expressing both α SMA and TTF-1 (Fig. 5C,D) suggesting that these cells that were once committed to an epithelial lineage are in an intermediate stage between an epithelial and a mesenchymal cell type. In other areas of the lung in which the overall alveolar architecture was preserved, co-immunostaining for α SMA and TTF-1 revealed abnormal expression of α SMA in epithelial cells lining the alveoli that were also TTF-1 positive (Fig. 5E and corresponding DIC image Fig. 5F,H), similar to the staining pattern in the fibrotic foci. In lung sections from wt control mice cells lining the alveolar wall only expressed TTF-1 (Fig. 5A and corresponding DIC image Fig. 5B). This suggests that at least some of the α SMA expressing myofibroblasts in the lungs of the Col1a2-CTGF transgenic mice are likely to be derived from epithelial cells. Thus, in non-fibrotic areas of the lung α SMA-positive cells are in an intermediate stage, expressing both epithelial and mesenchymal markers. In addition cells in the

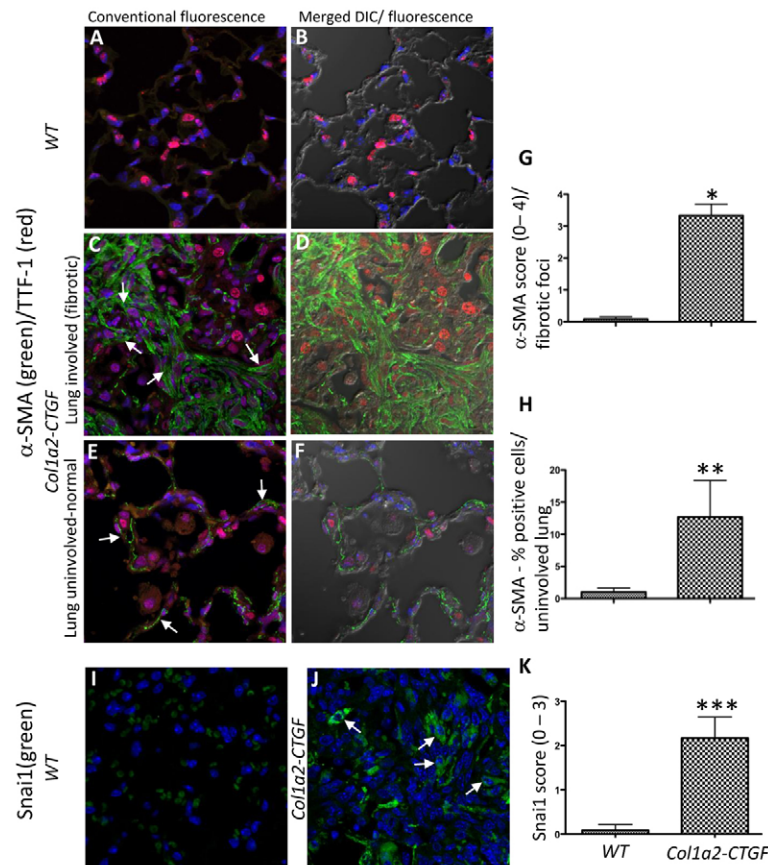


Fig. 5. Alveolar epithelial abnormality in the lungs of Col1a2-CTGF mice. (A–F) Immunofluorescence of lung sections from Col1a2-CTGF mice and wt controls co-immunostained for α SMA and TTF-1 showed increased numbers of α SMA-expressing myofibroblasts in fibrotic lesions of Col1a2-CTGF transgenic lungs (C, arrows; corresponding DIC overlay in D) compared to wt controls (A; corresponding DIC overlay in B). In epithelial cells lining the alveoli in normal areas of lungs from Col1a2-CTGF mice abnormal expression of α SMA was evident (E; corresponding DIC overlay in F) compared to wt control mice (A; corresponding DIC overlay in B). (I, J) Increased expression of Snai1 was observed in fibrotic lesions in lungs from Col1a2-CTGF transgenic mice (J, arrows), compared to wt controls (I). Autofluorescence of red blood cells occurred in wt lung section stained for Snai1 (I). Scale bar: 50 μ m. (G, H, K) Quantification of expression was performed on samples from six mice per group. Data are means \pm s.d. * P <0.0001, ** P <0.0006, *** P <0.0001, Student's t -test.

fibrotic foci also stained for Snail suggesting that these cells have undergone EMT (Fig. 5I,J,K). These results strongly suggest that overexpression of CTGF in lung fibroblasts can induce EMT-like changes in alveolar epithelial cells as assessed by co-expression of epithelial and acquisition of mesenchymal cell markers.

EMT in lung fibrosis is associated with increased TGF β signaling

To further understand the mechanisms of lung fibrosis in Col1a2-CTGF transgenic mice, the expression levels of potential CTGF target genes were measured by qRT-PCR. Since the fibrotic lung had an abundance of macrophages, a known source of TGF β (Khalil et al., 1996), we also measured the mRNA levels of *Tgfb1* and *Tgfb1*. In addition to the expected increase in *Ctgf* mRNA, we observed a marked enhancement of the levels of *Tgfb1* and *Tgfb1* mRNAs. Genes that are specific targets of the TGF β signaling pathway such as *Timp1*, *Serpine1* (*PAI-1*), *Col1a1*, *Fbn1* (fibronectin), *Acta1* (alpha smooth muscle actin, α SMA) and *Sox9* were also overexpressed (Fig. 6). These alterations in mRNA expression levels suggest that TGF β may be an important participant in the fibrotic phenotype of the CTGF-induced lung fibrosis and may also play a role in EMT of alveolar epithelial cells described above.

In vitro TGF β -mediated EMT in lung epithelial cells is inhibited by specific CTGF knockdown

There is evidence in the literature that TGF β can induce EMT in lung epithelial cells *in vitro* (Xu et al., 2009). We wished to determine whether this TGF β -induced EMT is mediated by CTGF. Lung epithelial cells were treated *in vitro* with recombinant TGF β 1. We observed changes in cell morphology from a rounded epithelial structure to a more spindle shaped fibroblast-like structure (data not shown). In addition, western blot analysis showed that the epithelial marker e-cadherin was downregulated and other mesenchymal markers, including α SMA, S100A4, SOX9, were upregulated in TGF β 1-treated cells (Fig. 7, lanes 5 and 6), indicating that EMT was occurring. This evidence of EMT-like changes was accompanied by increased expression of CTGF (Fig. 7, lanes 5 and 6). To directly assess the role of increased CTGF in cellular changes in epithelial cells, we used *Ctgf* siRNA to inhibit CTGF expression after TGF β 1 treatment. Specific CTGF knockdown significantly decreased expression of SNAIL1, α SMA and SOX9 expression

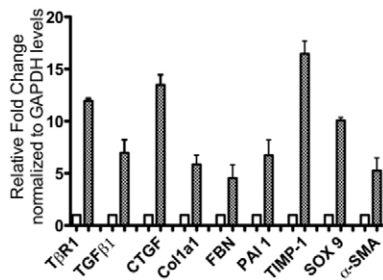


Fig. 6. Persistent activation of fibrotic and EMT marker genes in the lungs of Col1a2-CTGF transgenic mice. qRT-PCR revealed a marked increase in the expression of CTGF, TGF β , their target genes, and the EMT markers Sox9 and α SMA in Col1a2-CTGF transgenic mice compared to wt controls. Results are expressed as average fold change compared to wt control mice; $n=4$ mice per group.

and increased expression of e-cadherin following TGF β 1 stimulation (Fig. 7, lanes 7 and 8). No effect was observed on SNAIL1, α SMA or SOX9 expression when cells were transfected with a non-specific scrambled siRNA (Fig. 7, lane 6). Hence, knockdown of CTGF blocks TGF β 1-induced EMT in epithelial cells *in vitro* suggesting that CTGF mediates expression of EMT markers in these TGF β 1 treated cells.

CTGF treatment induces EMT-like changes in lung epithelial cells *in vitro*

As the TGF β 1-induced EMT-like changes seen in lung epithelial cells are, at least in part, mediated by CTGF we investigated whether treatment of cells with CTGF alone could induce EMT-like changes. Lung epithelial cells were transduced with an adenovirus expressing CTGF from the CMV promoter (Ad.CTGF). Phase contrast microscopy images of control cells transduced with a GFP-expressing adenovirus (Ad.gfp) showed a typically epithelial cuboidal morphology (Fig. 8A,a), whereas cells transduced with Ad.CTGF showed a change in morphology to a spread, spindle morphology characteristic of mesenchymal cells (Fig. 8A,b-c). In addition, epithelial cells transduced with Ad.CTGF show expression of the mesenchymal marker α SMA (Fig. 8B,f), whereas this marker is absent in control cells transduced with Ad.gfp (Fig. 8B,e). Deconvoluted z-stack images (Fig. 8B,g) demonstrate that the expressed α SMA forms stress fibers. These experiments showed EMT-like changes occur when CTGF is expressed by the epithelial cells themselves. In order to more closely mimic the *in vivo* situation in the Col1a2-CTGF mice, in which the CTGF is expressed by fibroblast cells neighboring the epithelial cells, we treated epithelial cells *in vitro* with recombinant human CTGF (rhCTGF). Treatment of cells with rhCTGF in the absence of serum induced expression of α SMA (Fig. 8B,b,c) with no expression seen in untreated cells (Fig. 8B,a), and deconvoluted z-stack images (Fig. 8B,d) demonstrate that the expressed α SMA forms stress fibers. The induced expression seen by immunofluorescence was confirmed by western blot analysis (Fig. 8C) which showed a significant ($P=0.004$) induction of α -SMA in treated cells. In order to assess any morphological changes, cells were stained with Rhodamine-phalloidin and co-stained for α SMA (Fig. 8D). Untreated cells (Fig. 8D,a-c) show peripheral actin filaments (Fig. 8D,a) with no α SMA (Fig. 8D,b,c merge). Cells treated with 100 ng/ml rhCTGF (Fig. 8D,d-f) show an increase in phalloidin staining (Fig. 8D,d) and an induction of α SMA (Fig. 8D,e,f merge). Furthermore, deconvoluted z-stack images (Fig. 8D,g-i) show that the induced α SMA is incorporated into the actin filament stress fibers.

As the fibrotic lungs of the Col1a2-CTGF mice show increased TGF β signaling (Fig. 6), we investigated whether the EMT-like changes induced by CTGF *in vitro* may be mediated by increased TGF β signaling in a positive feedback loop. Epithelial cells stimulated with rhCTGF were exposed to 1d11, a neutralizing antibody to TGF β , or IgG control. Cells treated with rhCTGF alone induced expression of α SMA (Fig. 8E,b) compared to untreated cells (Fig. 8E,a). Cell counts showed a significantly ($P<0.00005$) higher percentage of α SMA-positive cells following treatment with 50 or 100 ng/ml rhCTGF (Fig. 8E; Panel e, lanes 2,3). Antibody blockade of TGF β with 1d11 prevented the CTGF-induced expression of α SMA (Fig. 8E,c). Cell counts showed a significant ($P<0.00005$) reduction in the percentage of α SMA-positive cells with 10 or 25 μ g/ml 1d11

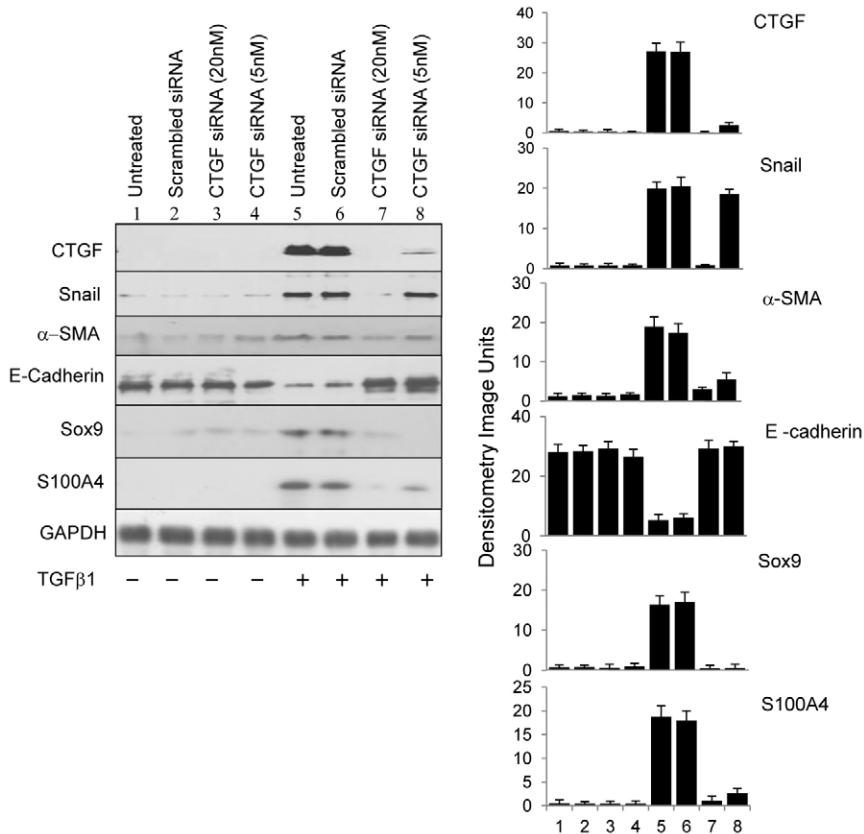


Fig. 7. *In vitro* TGFβ1-mediated EMT in lung epithelial cells is dependent on CTGF expression. Western blot analysis of lung epithelial cells treated with TGFβ1 (lanes 5–8) shows increased expression of CTGF, αSMA, Sox9, S100A4 and reduced expression of E-cadherin (lanes 5 and 6). Specific knockdown using CTGF siRNA significantly inhibited Snail, αSMA, Sox9, S100A4 overexpression and rescued the downregulation of E-cadherin (lane 7). No effect was observed on Snail, αSMA, Sox9, S100A4, or E-cadherin expression when cells were either un-transfected or transfected with a non-specific scrambled siRNA (lanes 5, 6). Representative blots are shown. Bar graphs show relative expression of the proteins after the different treatments (normalized to GAPDH loading control; means ± s.d. of three independent experiments).

(Panel e, lanes 4,5) compared to rhCTGF alone (Panel e, lanes 2,3), and with 25 μg/ml 1d11 (Panel e, lane 5) the percentage of αSMA-positive cells was not significantly ($P>0.9$) different from unstimulated cells (Fig. 8E, Panel e, lane 1). Treatment of rhCTGF stimulated cells with 10 or 25 μg/ml IgG control did not result in a reduction ($P>0.3$) in the percentage of αSMA-positive cells (Fig. 8E, Panel e, lanes 6, 7). In addition LY-364947, a TGFβRI inhibitor, also prevented the CTGF-induced expression of αSMA (Fig. 8E,d). Cell counts showed a significant ($P<0.00005$) reduction in the percentage of αSMA-positive cells with 50, 300 or 600 nM LY-364947 (Panel e, lanes 8,9,10) compared to rhCTGF alone (Panel e, lanes 2,3), and with 300 or 600 nM LY-364947 the percentage of αSMA-positive cells was not significantly ($P>0.2$) different from unstimulated cells (Fig. 8E, Panel e, lanes 9,10 compared to lane 1). Interestingly, stimulation of epithelial cells with 2 ng/ml TGFβ induced significantly ($P<0.003$) more αSMA-positive cells than stimulation with 100 ng/ml rhCTGF (Panel e, lane 11 compared to lane 3). These results demonstrate that CTGF, either endogenously expressed by epithelial cells or given exogenously as a recombinant protein, is able to induce EMT-like changes *in vitro*. These results also indicate that CTGF-induced expression of αSMA is mediated by activation of TGFβ signaling. Taken together our results show that CTGF is able to induce EMT-like changes in epithelial cells and is able to induce αSMA expression in stress fibers.

Discussion

Studies of different fibrotic diseases, as well as the characterization of Col1a2-CTGF transgenic mice, point to

CTGF as a key player in the fibrotic disease process. Indeed, the relative levels of CTGF found in fibroblasts from these mice are similar to levels observed in fibroblasts from systemic sclerosis patients (Shi-wen et al., 2000). However, the paracrine effects of CTGF on cellular targets outside of fibroblasts is largely unknown as is the cell and molecular mechanism underlying CTGF action on keratinocytes in skin, alveolar epithelial cells in lung or tubulointerstitial cells in kidney. The current study focuses primarily on the dermal and pulmonary compartment, the most profoundly affected tissues in the Col1a2-CTGF transgenic mice (Sonnylal et al., 2010). In most organs Col1a2 expression is located in cells of mesenchymal origin (Bou-Gharios et al., 1996), however in kidney, expression has also been observed in tubular epithelial cells upon injury (Fragiadaki et al., 2011). Interestingly, in a study using administration of aristolochic acid (AA) to induce renal fibrosis, the Col1a2-CTGF transgenic mice did not exhibit more fibrosis compared with wt AA-treated mice (Fragiadaki et al., 2011). This is however perhaps not surprising as AA-administration at the similar dose range (5–10 mg/kg) has been shown to induce as severe nephropathy resulting in florid interstitial inflammation with immune cell infiltrates and severe tissue damage and necrosis (Pozdzik et al., 2008; Zhou et al., 2010). This pathology was shown to be associated with a profound induction of TGFβ expression and Smad signaling which was shown to be responsible for the collagen matrix accumulation (Wang, Y. et al., 2008; Li et al., 2009). In this study we show that increased expression of CTGF in fibroblasts of Col1a2-CTGF transgenic mice results in impaired or loss of epithelial characteristics in adjacent epithelial structures and

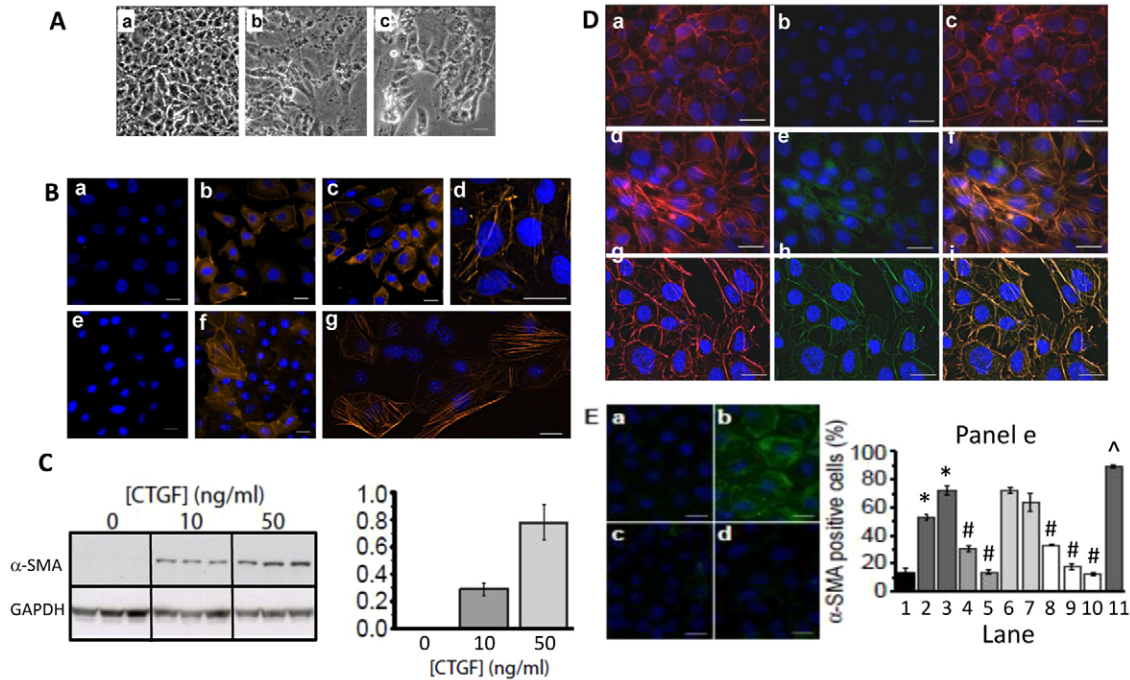


Fig. 8. CTGF induces EMT-like changes *in vitro*, which are dependent on TGF β signaling. (A) CTGF expression induces a change in morphology *in vitro*. Phase-contrast images of epithelial cells transduced with Ad.GFP (a) show a typically epithelial cobblestone morphology, whereas epithelial cells transduced with Ad.CTGF (b,c) show a more spread, spindly morphology typical of mesenchymal cells. Scale bars: 20 μ m. (B,C) CTGF induces expression of the mesenchymal marker α SMA in epithelial cells *in vitro*. Epithelial cells were stained with a Cy3-conjugated anti- α SMA antibody (orange) and nuclei were counter-stained with DAPI (blue). Cells were either exposed to exogenous rhCTGF (b–d) or transduced with adenovirus expressing CTGF (f,g) or GFP control (e). Untreated (a) or Ad.gfp-transduced (e) epithelial cells show no α SMA expression, whereas cells treated with 50 ng/ml (b), 100 ng/ml (c), or 500 ng/ml (d) rhCTGF, or transduced with Ad.CTGF (f,g) express α SMA. Deconvolution of z-stack images (d,g) shows that the expressed α SMA forms stress fibers. Scale bars: 20 μ m. In C, western blot analysis of epithelial cells treated with 10 or 50 ng/ml rhCTGF shows a significant ($P=0.004$) induction of α SMA compared to controls. Representative blots for control and 50 ng/ml rhCTGF are shown. Graph shows densitometry of α SMA (normalized to β -tubulin loading control). Data are means \pm s.e.m. of three independent experiments. P -values were determined using Student's t -test. (D) Exogenous CTGF induces a change in actin filaments *in vitro*. Epithelial cells were co-stained with Rhodamine-conjugated phalloidin (red; a,d,g) and an anti- α SMA antibody (green; b,e,h), and images were merged (c,f,i). Nuclei were counter-stained with DAPI (blue). Untreated cells (a–c) show mainly peripheral actin filaments that do not contain α SMA. Cells treated with 100 ng/ml rhCTGF (d–i) show an increase in actin filaments across the cells and these filaments include α SMA. Deconvoluted z-stack images (g–i) show the formation of actin filaments and α SMA into stress fibers. Scale bars: 20 μ m. (E) α SMA induction by CTGF is prevented by neutralization of TGF β or inhibition of TGF β RI. Epithelial cells were stained with an anti- α SMA antibody (green), and nuclei were counter-stained with DAPI (blue). Cells were untreated (a), exposed to rhCTGF (100 ng/ml; b), rhCTGF and a neutralizing anti-TGF β antibody, 1d11 (100 ng/ml and 25 μ g/ml, respectively; c); or rhCTGF and the small molecule TGF β RI inhibitor LY-364947 (100 ng/ml and 300 nM, respectively; d). Panel e, shows a graphical representation of the frequency of α SMA-positive cells in treated and untreated cultures (as a percentage of total cell number) as means \pm s.e.m. of four fields of view at 20 \times magnification. Lane 1, control cultures; lanes 2 and 3, cells treated with rhCTGF at 50 and 100 ng/ml respectively; lanes 4 and 5, cells treated with rhCTGF at 100 ng/ml in the presence of 1d11 at 10 or 25 μ g/ml, respectively; lanes 6 and 7, cells treated with rhCTGF at 100 ng/ml in the presence of a control IgG at 10 or 25 μ g/ml, respectively; lanes 8, 9 and 10, cells treated with rhCTGF at 100 ng/ml in the presence of the TGF β RI inhibitor LY-364947 at 50, 300 or 600 nM, respectively; lane 11, cells treated with TGF β at 2 ng/ml. * $P<0.00005$ compared to untreated; # $P<0.00005$ compared to 100 ng/ml rhCTGF alone; ^not significant ($P>0.3$) compared to 100 ng/ml rhCTGF alone; Student's t -test.

acquisition by these cells of mesenchymal properties *in vivo* in the skin and lung presumably by paracrine mechanisms.

In addition to the fibrotic phenotype, Col1a2-CTGF transgenic mice develop hair loss early on associated with hair follicle abnormalities and focal hyperplasia of the epidermis. This hyperproliferation was associated with changes in cell morphology and marked changes in gene expression including increased expression of keratin K16. In the normal skin epidermis keratins K5 and K14 are expressed in the basal cells and keratins K1 and K10 are expressed in the suprabasal layers (Koch and Roop, 2004). In some diseased states such as in psoriasis where the epithelial cells undergo hyperproliferation and/or aberrant differentiation, expression of keratins K6 and its partner K16 are induced in the inter-follicular epidermis (Bernot et al., 2002). Recent, proteomic

analysis of whole skin lesions from SSc patients shows abnormal expression of K6 and K16 suggesting hyperproliferation of the epidermal component in SSc (Aden et al., 2008).

In the skin of Col1a2-CTGF transgenic mice expression of the epithelial-specific integrin β 6 is also markedly increased in focal areas of the epidermis. We speculate that the increase in β 6 expression might eventually favor enhanced signaling by CTGF since integrins have previously been shown to be involved in such signaling. Mice in which integrin β 6 overexpression is targeted to the basal keratinocytes, showed loss of hair in the lower back associated with fibrosis and chronic ulceration characterized by hyperplasia of epithelial cells and thick connective tissue (Häkkinen et al., 2004), a phenotype that is analogous to that of Col1a2-CTGF transgenic mice.

Furthermore the focal changes in the morphology of epidermal cells and the abnormal expression of α SMA, Snail and S100A4 by these cells in Col1a2-CTGF transgenic mice strongly suggests that the increased levels of CTGF that are secreted by the dermal fibroblasts resulting in an EMT-like process in neighboring epithelial cells presumably through a paracrine mechanism. EMT is a process by which epithelial cells undergo a transition into a mesenchymal cell-type giving rise to fibroblasts and myofibroblasts. EMT is characterized by loss of epithelial markers, loss of epithelial cell polarity, acquisition of mesenchymal markers and an invasive phenotype (Kalluri and Neilson, 2003). This process is now being recognized to play an important role in tissue repair and fibrosis. One recent report provides suggestive evidence consistent with EMT in post-menopausal frontal fibrosing alopecia (Nakamura and Tokura, 2011). This disease is characterized by a recession of the hairline, a reduction or loss of terminal hair follicles and fibrosis around the affected hair follicles. Cells expressing Snail were present in the fibrotic dermis raising the possibility that these cells might have been derived from epithelial cells.

Emerging evidence from studies of liver fibrosis showed that TGF β -induced expression of Sox9 in hepatic stellate cells (HSC) in culture correlated with increased type I collagen production (Hanley et al., 2008). This increase can be inhibited by knockdown of Sox9 by a specific siRNA suggesting a role of Sox9 in ECM synthesis (Hanley et al., 2008).

In the Col1a2-CTGF transgenic mice the production of excess CTGF in dermal fibroblasts results in the abundant expression of Sox9 in the basal cells of the epidermis and in the outer root sheath and bulge of hair follicles. We speculate that this occurs through a paracrine mechanism. Interestingly embryonic ablation of Sox9 in K14 expressing epithelial cells of the skin results in defects in the stem cell compartment and subsequent structures that arise from the stem cells. These mice also displayed impaired wound repair (Nowak et al., 2008). Our *in vitro* experiments in lung A549 epithelial cells also show that knockdown of CTGF in these cells is associated with markedly decreased levels of Sox9. Thus both *in vivo* and *in vitro* experiments establish a clear correlation between levels of CTGF and those of Sox9. Increased expression of Sox9 is also observed in different cancers (Wang et al., 2007; Schaeffer et al., 2008). Inhibition of endogenous Sox9 in CWR22Rv1, a human prostate cancer cell line, resulted in decreased cellular proliferation (Wang et al., 2007). Transplantation of these cells in immuno-compromised mice resulted in decreased tumor growth correlating with its role in cellular proliferation (Wang, H. et al., 2008). In *in vitro* experiments, Sox9 activates the expression of the EMT marker Snail2 (Sakai et al., 2006) and co-expression of Sox9 and Snail2 can induce EMT in neural epithelial cells (Cheung et al., 2005). These lines of evidence would suggest the hypothesis that in our model system increase in expression of Sox9 in the skin might promote proliferation of progenitor cells and changes in the genetic program of existing epithelial cells or of newly formed epithelial cells undergoing EMT. In the epidermis of Col1a2-CTGF transgenic mice skin there was a marked increase in phosphorylated p38 and ERK. We speculate that ERK and p38 activation could mediate the paracrine effects of CTGF and together with the increased levels of β 6 integrin have a role in cell proliferation and migration. Previous *in vivo* studies of BLM-induced lung fibrosis have shown by fate mapping experiments that one third of cells expressing the fibroblast specific marker

S100A4 are derived from alveolar epithelial cells indicative of EMT (Tanjore et al., 2009). Similar EMT-like event is also observed in endoplasmic reticulum stress-induced lung fibrosis (Tanjore et al., 2011). Other studies have suggested a link between CTGF and lung fibrosis. Intratracheal bleomycin treatment induces lung fibrosis with an increase in CTGF expression in a bleomycin-sensitive mouse strain (C57BL/6) but not in a bleomycin-resistant mouse strain (BALB/c) (Lasky et al., 1998). Adenoviral-mediated CTGF expression in rat lungs induces a transient fibrosis (Bonniaud et al., 2003). Additionally, adenoviral expression of CTGF in the lungs of bleomycin-resistant BALB/c mice leads to significant lung fibrosis after treatment with bleomycin (Bonniaud et al., 2004). The lung fibrosis seen in Col1a2-CTGF transgenic mice was associated with increased proliferation of epithelial cells in the fibrotic lesions exemplified by the increased number of cells that expresses the epithelial specific transcription factor TTF-1. These fibrotic lesions also contained a large number of myofibroblasts expressing α SMA and a large percentage of these cells were co-expressing both α SMA and TTF-1 suggesting that the myofibroblast population arose from alveolar epithelial cells. This hypothesis was also supported by evidence that in the uninvolved areas of the transgenic mouse lung, which seemingly displayed a normal architecture, epithelial cells lining the alveoli co-expressed α SMA and TTF-1. Fibrotic lesions also displayed cells positive for Snail suggesting that EMT is associated with lung fibrosis. We propose that in the lung of Col1a2-CTGF transgenic mice EMT is a consequence of the paracrine effects of the abnormal high levels of secreted CTGF.

The results of the real-time PCR experiments on whole lung RNA of Col1a2-CTGF transgenic mice showed markedly increased RNA levels of TGF β and its downstream signature targets. This is likely to be a consequence of the abundance of macrophages in the fibrotic lung. In contrast, in the skin of Col1a2-CTGF transgenic mice no macrophages were observed. Microarrays of total skin RNA did not reveal any significant increase in TGF β and TBR1 RNA levels (data not shown). We propose that the combined increase in TGF β and CTGF in the fibrotic lung not only causes increased myofibroblast transformation and excess ECM deposition but could also contribute to EMT in the lung epithelial cells. To understand the role of CTGF in mediating EMT in the lung, we have used lung epithelial cells to show that the activation of EMT marker genes by TGF β can be inhibited by CTGF siRNA. We have also demonstrated that CTGF expression by epithelial cells *in vitro* causes EMT-like changes. Treatment of lung epithelial cells with recombinant CTGF also induces EMT-like changes. Additionally, we have demonstrated that the EMT-like changes induced by recombinant CTGF *in vitro* can be prevented by immunoinhibition of TGF β or by inhibition of TGF β RI suggesting that TGF β signaling plays a central role in the EMT process in lung epithelial cells. These *in vitro* findings strongly suggest that paracrine CTGF is likely to have a direct effect on inducing EMT *in vivo*. We speculate that these CTGF-induced EMT-like changes may be a contributor to the development of lung fibrosis. The results also suggest that the induction of TGF β and TGF β RI by CTGF may establish a positive feedback loop driving increased expression of endogenous CTGF and further contributing to the EMT-like and fibrotic changes seen in the lungs of Col1a2-CTGF mice.

In summary our *in vivo* observations strongly suggest that paracrine effects of secreted CTGF produced in fibroblastic cells of Col1a2-CTGF mice stimulate epithelial cells to modify their genetic program in order to undergo changes that mimic features of EMT. The results of our *in vitro* experiments support these conclusions. Thus CTGF plays a crucial role not only in promoting a profibrotic program in fibroblastic cells but also in the EMT-like transition of epithelial cells into mesenchymal cells. We speculate that the increase in Sox9 expression in skin and lung epithelial cells may be an important factor in the EMT-like phenotype.

Materials and Methods

Transgenic mice

The Col1a2-CTGF transgenic mice used in this study have been described previously (Sonnlyal et al., 2010). Briefly, the murine homolog of CTGF (Fisp-12) was cloned into a vector containing the 6-kb enhancer and minimal promoter of the murine Col1a2 gene, an IRES-*lacZ* reporter, and the murine protamine polyA signal. Transgenic mice were produced by the standard method of pronuclear injection of linear DNA into a fertilized mouse egg. All experiments performed with the mice were in compliance with the standards of care approved by the M. D. Anderson Cancer Center Institutional Animal Care and Use Committee. Genotyping was performed by PCR for the *lacZ* reporter on DNA extracted from ear biopsies.

Immunofluorescence of tissue sections and quantification

Skin and lung tissues from Col1a2-CTGF transgenic mice and wild-type (wt) littermate controls were fixed overnight in neutral buffered 4% paraformaldehyde at 4°C, processed through graded alcohols, and paraffin embedded. Immunofluorescence was performed on 5 µm sections with the following primary antibodies: Keratin 16 (Novus Biologicals, Littleton, CO, USA), integrin β4 (Southern Biotech, Birmingham, AL, USA), integrin β6 (R&D Systems, Minneapolis, MN, USA), αSMA (Sigma-Aldrich, St. Louis, MO, USA), Snail and CTGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), S100A4 (Abcam, Cambridge, MA, USA), Sox9 (Millipore, Billerica, MA, USA), TTF-1, p-p38, pERK (Cell Signaling, Lake Placid, NY, USA), and type I collagen (Novotec, Lyon, France). The secondary antibodies used were Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 555 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA); nuclei were counterstained with Topro (Life Technologies, Carlsbad, CA, USA) and sections were mounted in ProLong Gold (Life Technologies, Carlsbad, CA, USA). Image stacks were collected using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, USA). For type I collagen, sections were stained using the Vectastain ABC kit as previously described (Sonnlyal et al., 2010). Quantitation for K16, integrin β4, integrin β6, αSMA, Snail, S100A4, Sox9, pErk1/2 and p-p38 staining of skin sections was performed using MetaMorph software by counting the number of positive cells within a 100 µm² box in at least six different tissue sections. The expression of αSMA and Snail in lung sections was graded on a scale of 0 to 4 with 0 signifying no staining, 2 signifying moderate staining and 3 or 4 signifying extensive staining. The number of αSMA-positive cells in uninvolved areas of lung was quantified as described above.

Cell culture

The lung epithelial cell lines A549 (ATCC: CCL-185) (Giard et al., 1973) and SV40-T2 (Clement et al., 1991) were maintained in DMEM medium (Life Technologies, Carlsbad, CA, USA) containing 10% FBS (BioSera, Ringmer, UK). For all treatments cells were serum starved overnight (cells were washed twice in PBS to remove serum then grown in DMEM without serum) and treatments were performed in DMEM without serum.

Keratinocytes were isolated from Col1a2-CTGF transgenic mice and wt littermate controls as described previously (Flores et al., 2000). Briefly, the skin biopsies were incubated in dispase II (Roche, Basel, Switzerland) overnight at 4°C to separate the epidermis from the dermis. The epidermis was carefully peeled off the following day, chopped into small pieces, and incubated in 0.25% trypsin-EDTA (Life Technologies, Carlsbad, CA, USA) for 20 minutes at 37°C. Cells were plated on J2-3T3 feeder cells and cultured in F medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.4 µg/ml hydrocortisone, 24 ng/ml adenine, 8.4 ng/ml cholera toxin, 5 µg/ml insulin, 13 ng/ml 3,3,5-triiodo-L-thyronine and 10 ng/ml EGF.

Transfection with siRNA

Epithelial cells were transfected with ON-TARGETplus CTGF siRNA or non-targeting control siRNA (Dharmacon) using Fugene6 transfection reagent (Roche) in serum-free medium according to the manufacturers' instructions. After 48 hours

transfected cells were treated with 4 ng/ml recombinant TGFβ for a further 24 hours before proteins were extracted for western blot analysis.

Adenoviral transduction

Epithelial cells were grown in T25 flasks (Corning Inc., Corning, NY, USA) to 70% confluence then transduced at a multiplicity of infection of 100 with either Ad.CTGF (Ad5 serotype adenovirus expressing human CTGF from the CMV promoter) or Ad.gfp (Ad5 serotype adenovirus expressing GFP from the CMV promoter, negative control). Briefly, the cell monolayer was washed in PBS to remove all serum, incubated for 2 hours with adenoviral particles in a minimal volume of DMEM without serum, washed twice in PBS to remove any remaining virus particles, then normal growth media was replaced. After viral transduction the cells were allowed to recover for 24 hours then seeded into chamberslides (BD Bioscience, Franklin Lakes, NJ, USA) at 70% confluence and allowed to attach for 24 hours before fixing and staining.

Treatment with recombinant proteins, neutralizing antibodies and inhibitors

Recombinant human CTGF (rhCTGF) produced in HEK293 cells was bought from EMP Genetech (Ingolstadt, Germany). Recombinant TGFβ was from R&D Systems (Minneapolis, MN, USA). 1d11 (a mouse IgG₁ neutralizing antibody against TGFβ) was from Abcam (Cambridge, MA, USA), purified mouse IgG (negative control) was from Vector Labs (Burlingame, CA, USA). LY-364947 (a selective TGFβRI inhibitor) was from Calbiochem (Merck KGaA, Darmstadt, Germany). For immunofluorescence, epithelial cells were seeded at 60% confluence in chamberslides, allowed to attach for 24 hours, serum-starved overnight, then treated with rhCTGF, with and without 1d11, IgG or LY-364947, in DMEM without serum for 48 hours before fixing and staining. For western blot analysis epithelial cells were seeded in six-well plates, allowed to attach for 24 hours, serum-starved overnight, then treated with rhCTGF, with and without 1d11, IgG or LY-364947, in DMEM without serum for 24 hours before protein extraction.

Immunofluorescence of epithelial cell culture

Cells in chamber slides were fixed in ice-cold methanol (for staining with anti-αSMA antibody) or 2% PFA (for anti-αSMA and phalloidin co-staining). Cy3-conjugated anti-αSMA (clone 1A4, Sigma-Aldrich, St. Louis, MO, USA), unconjugated anti-αSMA (clone 1A4, Dako, Glostrup, Denmark), and Rhodamine-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO, USA) were used at 1:100 dilution. Purified mouse IgG (Vector Labs, Burlingame, CA, USA) was used as negative control. Unconjugated anti-αSMA was detected with Alexa Fluor 488 chicken anti-mouse IgG (Life Technologies, Carlsbad, CA, USA) at 1:1000 dilution. Nuclei were counterstained with DAPI and mounted in Vectashield (Vector Labs, Burlingame, CA, USA). Stained cells were imaged on an Axioskop Mot2 plus microscope with a monochrome camera and Axiovision microscope control software (Carl Zeiss, Oberkochen, Germany). The IgG control was used to set the exposure time. Z-stack images were taken using automated stage movement and inter-slice distances were optimised to fulfill the Nyquist criterion. Z-stacks were deconvoluted using an iterative point spread function in Axiovision. Cell counts were performed using the cell counter plugin for ImageJ (US National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

Cell extracts for western blot analysis has been described earlier (Sonnlyal et al., 2007). Briefly, cells were cultured as monolayers, washed with ice-cold PBS, and proteins were extracted with RIPA buffer with protease inhibitors (50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin). Cell extracts were resolved on 10% SDS-PAGE and immunoblotted as described earlier (Sonnlyal et al., 2007). In addition to the antibodies described above, the following antibodies were also used in western blotting: CTGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), p38, ERK (Cell Signaling, Lake Placid, NY, USA), e-cadherin (BD Biosciences, Franklin Lakes, NJ, USA), GAPDH, β-tubulin (Abcam, Cambridge, MA, USA). Blots were quantified with respect to the expression of GAPDH by densitometric analysis using the UVP Biospectrum AC image system with VisionWorksLS software. The images present are representative of three independent experiments.

Real-time PCR

Standard RT-PCR was performed with the following primers: TGFβ1 forward, 5'-GAAGGCCGGCTTCATGTCATG-3'; TGFβ1 reverse, 5'-TGTGACAGCAAAGTAAACGAACTCCAC-3'; TβR1 forward, 5'-ATTGCTGGTCCAGTCTGTCT-3'; TβR1-reverse, 5'-CCTGATCCAGACCCTGATGT-3'; Col1a1 forward, 5'-CCCCTGCAT GTCGCTAT-3'; Col1a1 reverse, 5'-GCTACGCTT TCTTTCAGATGTAT-3'; Fibronectin forward, 5'-GGTCTGCAGAGTTGACAGTG-3'; Fibronectin reverse, 5'-GGAGAAGTTTGTGCATGGTGTCC-3'; CTGF forward, 5'-TTGTAATGGCAGGCACAGGTC-3'; CTGF reverse, 5'-CGCA-

CAAGAACCACCTCTG-3' PAI 1 forward, 5'-ATGCGGGCTGAGATGAC-AAAG-3'; PAI 1 reverse, 5'-ACTGCAAAAAGTCAGGATCGA-3'; α SMA forward, 5'-GCCAAGTCCAGACGCATGATG-3'; α SMA reverse, 5'-TATGCTCTGGACGTACAAC-3'; TIMP 1 forward, 5'-CACAAGCCTGGATTCCG-TGG-3'; TIMP 1 reverse, 5'-TCCCTTGCAAATGGAGAGTGAC-3'; Sox9 forward, 5'-GACGTGGGCAAGCTCT-3'; Sox9 reverse, 5'-GTAATCCGGG-TGGTCTCT-3'.

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D.A. and B.de.C. are joint senior authors.

Author contributions

S.S., D.A., S.X., J.N. and B.d.C. developed the hypothesis, and conceived and designed the experiments. S.S., S.X., H.J., A.T., V.R.S. P.A. and M.P. performed the experiments and contributed to writing the paper. S.S., H.J., D.A., J.N., M.P. and B.d.C. contributed to the supervision of the study, and helped in the analysis and interpretation of data from experimental results. S.S., D.A. and B.d.C. wrote the paper. All authors provided critical comments on drafts of the paper, and all read and approved the final manuscript.

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