Transforming growth factor-β-induced CUX1 isoforms are associated with fibrosis in systemic sclerosis lung fibroblasts

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In the enhancer region of the human type I collagen alpha 2 (COL1A2) gene, we identified cis-elements for the transcription factor CUX1. However, the role of CUX1 in fibrosis remains unclear. Here we investigated the role of CUX1 in the regulation of COL1 expression and delineated the mechanisms underlying the regulation of COL1A2 expression by CUX1 in systemic sclerosis (SSc) lung fibroblasts. The binding of CUX1 to the COL1A2 enhancer region was assessed using electrophoretic mobility shift assays after treatment with transforming growth factor (TGF)-β. Subsequently, the protein expression levels of CUX1 isoforms were determined using Western blotting. Finally, the expression levels of COL1 and fibrosis-related cytokines, including CTGF, ET-1, Wnt1 and β-catenin were determined. The binding of CUX1 isoforms to the COL1A2 enhancer region increased after TGF-β treatment. TGF-β also increased the protein levels of the CUX1 isoforms p200, p150, p110, p75, p30 and p28. Moreover, SSc lung fibroblasts showed higher levels of CUX1 isoforms than normal lung fibroblasts, and treatment of SSc lung fibroblasts with a cathepsin L inhibitor (IW-CHO) decreased COL1 protein expression and reduced cell size, as measured using immunocytochemistry. In SSc and diffuse alveolar damage lung tissue sections, CUX1 localised within α-smooth muscle actin-positive cells. Our results suggested that CUX1 isoforms play vital roles in connective tissue deposition during wound repair and fibrosis.

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1. Introduction

Scleroderma, or systemic sclerosis (SSc), is a connective tissue disease characterised by excessive type I collagen (COL1) deposition in many organs including the lungs, kidneys and skin [1]. Approximately 40% patients with diffuse SSc experience pulmonary fibrosis, a major cause of mortality [1]. Although the aetiology of this disease is poorly understood, tissue scarring occurs after injury following the release of several mediators including TGF-β, PDGF, CTGF, Wnt1 and ET-1 [2–6]. These factors stimulate fibroblasts to differentiate into myofibroblasts, which are characterised by the expression of α-SMA and excessive production of extra- cellular matrix molecules such as COL1 [7,8]. Overexpression of these cytokines results in chronic fibroblast stimulation (fibrosis). Moreover, genetically modified mice with altered TGF-β signalling have been reported to exhibit fibrogenic pathology similar to that observed in patients with SSc, indicating a key role of this cytokine in the pathogenesis of fibrosis [9–11]. Elucidation of pivotal mediators or key signalling pathways that are overactive in fibrosis is crucial for designing better therapeutic strategies for SSc and related disorders.

TGF-β is a potent pro-fibrotic cytokine that promotes myofibroblast differentiation, migration, extracellular matrix synthesis and apoptosis resistance [12–14]. TGF-β induces the expression of the gene encoding human collagen type I alpha 2 (COL1A2) via a Smad-dependent pathway that acts on a TGF-β responsive element (TbRE) in the human COL1A2 proximal promoter [15,16]. COL1 expression in vivo is exclusively controlled by an enhancer sequence that contains several DNase I hypersensitive sites (HSs). These encompass pivotal regulatory sites conferring tissue-, temporal-, cell- and growth factor-specific expression of COL1 [15,16].
The results of our recent study illustrated that TGF-β activates COL1A2 via a non-canonical Smad-independent pathway, which requires enhancer/promoter cooperation. Moreover, we identified a novel TbRE in the human COL1A2 enhancer region and found that it is necessary for COL1A2 activation [16]. Further, we reported that high doses of TGF-β increased CUX1 binding in the proximal promoter and suppressed COL1 expression [17].

In this study, we identified CUX1 binding sites near this TbRE of the COL1A2 enhancer region using in silico analyses. These sites exist at identity island 4 (IS4) near HS4 in the enhancer region [18].

In addition, this study demonstrated that CUX1 responds to TGF-β stimulation and is a potential activator of COL1. Based on these findings, we characterised the role of human CUX1 in the regulation of COL1 expression and subsequent release of pro-fibrotic cytokines. We demonstrated that some isoforms of human CUX1 are strongly induced after TGF-β stimulation, which results in the expression of pro-fibrotic cytokines.

We investigated the expression pattern of human CUX1 isoforms in lung fibroblasts derived from normal cells and patients with SSC. In lung fibroblasts derived from patients with SSC, protein levels of the CUX1 isoforms p200, p150, p110, p75, p30 and p28 were increased compared with those in normal cells. Using electrophoretic mobility shift assays (EMSA) with nuclear extracts from these cells, we demonstrated that CUX1 binding to the COL1A2 enhancer region was increased. Cleavage sites for cathepsin L have been found within CR1 and CR2 and those for caspases have been identified between CR3 and HR of CUX1 [19].

Therefore, we confirmed whether cathepsin L inhibitor (IW-CHO) can regulate COL1. IW-CHO inhibited COL1 in both normal and SSC lung fibroblasts. Taken together, our data strongly suggest that CUX1 isoforms up-regulate COL1 and regulate key pro-fibrotic activities of TGF-β. Our results could provide novel insights into CUX1-mediated regulation of COL1A2 in human lung fibroblasts. These results are an important contribution to the understanding of processes involved in SSC-associated fibrosis.

2. Materials and methods

2.1. Cell culture

Cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin and cultured in a humidified atmosphere of 5% CO₂. We isolated lung fibroblasts as previously described [20]. The cells were cultured under standard conditions in DMEM containing 10% FBS. Pulmonary fibroblasts were obtained from patients who fulfilled the criteria of the American College of Rheumatology for the diagnosis of SSC with lung involvement. Informed consent and ethical approval were obtained. None of the patients was receiving immunosuppressive medication or corticosteroids at the time of biopsy.

2.2. Western blotting

Nuclear extracts and cytosolic fractions were prepared as previously described [16]. The cells were washed with phosphate-buffered saline (PBS) and treated with Laemmli sample buffer. To analyse collagen, the medium was removed and adjusted to 20% (v/v) ammonium sulphate, followed by incubation at 4 °C overnight. The samples were centrifuged, and the pellet was resuspended in Laemmli sample buffer with β-mercaptoethanol for SDS-PAGE, followed by Western blotting. The following antibodies were used: lamin A/C (sc20681, Santa Cruz), COL1 (rabbit antimouse collagen 1, ref 20151, Novotec), ET-1 (T4049, Peninsula), CUX1 (M-222, sc-13024, Santa Cruz), CTGF (L-20, sc14939, Santa Cruz), GAPDH (ab8245-100, Abcam), β-catenin (ab6302-100, Abcam) and Wnt1 (anti-human WNT1 S#500-p250, PeproTech, Inc.).

N-(1-naphthalenesulfonyl)-Ile-Trp-aldehyde (IW-CHO, 0.2 μM, Alexis Biochemicals) was used as IW-CHO. TGF-β (R&D Systems) was used at 4 ng/ml. The cells were serum-starved for 12 h and incubated with or without TGF-β at 4 ng/ml for a further 24 h.

2.3. Plasmid transfection

Cells were seeded in six-well plates before transfection and 24 h later were transfected using FuGENE 6 (Roche, Basel, Switzerland), according to the manufacturer's instructions; this method was used to transfect sh-CUX1 vector as described previously [17].

2.4. EMSAs

Nuclear extracts were prepared from fibroblasts obtained from patients with SSC using a previously described method [16]. Briefly, double-stranded oligonucleotides were synthesised, end-labelled with γ³P-gamma-ATP using T4 kinase and used in binding reactions with nuclear extracts from the cells. Competition was performed with unlabelled oligonucleotides (1.75 pmol/μl). Oligonucleotides for the CUX1-19.51 kb probe (5′-agtggcagtgtaatta-gtga-3′) were used. We employed the standard conditions recommended by the kit manufacturer (Promega) and separated the reaction on a 4% polyacrylamide gel at 4 °C and 160 V. Consensus oligonucleotides for CUX1 (CDP, sc2593, Santa Cruz) and SP1 (sc2502, Santa Cruz) were used.

2.5. Immunocytochemistry

The cells were washed in ice-cold PBS twice and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. The cells were blocked with 3% BSA for 30 min at 25 °C, followed by incubation with CUX1 antibody (0.2 mg/ml) and monoclonal anti-α-SMA clone 1a Cy3-conjugated antibody (0.7 mg/ml) (Sigma-Aldrich) overnight at 4 °C. Subsequently, the cells were washed with ice-cold PBS twice, incubated with an Alexa 488 secondary antibody overnight at 4 °C, washed with ice-cold PBS twice and incubated with DAPI (Roche) for 30 min at room temperature. Slides were viewed and photographed using a Zeiss Axioscope light microscope (Carl Zeiss, Göttingen, Germany) with Axiovision software.

2.6. Immunohistochemistry

For formalin-fixed paraffin-embedded specimens, tissues were fixed and hydrated as previously described [10]. For immunohistochemistry, sections were pre-treated with methanol (VWR, Lutterworth, UK), followed by antigen retrieval in heated 10 mM citrate buffer (pH 6). The primary antibodies were CUX1 antibody (0.2 mg/ml) and monoclonal anti-α-SMA clone 1a Cy3-conjugated antibody (0.7 mg/ml). Sections were sequentially incubated with a 1/200 dilution of an Alexa 488 secondary antibody. Slides were viewed and photographed using a Zeiss Axioscope light microscope with Axiovision software.

2.7. Data analysis

Data are expressed as the mean ± S.E of at least three independent experiments. Statistical analysis was performed using Student's t-test. Values of P < 0.05 were considered significant.
3. Results

3.1. CUX1 binds to the enhancer region of human COL1A2

The CUX1 cis-element CCAAT (Fig. 1A) was identified at IS4 near enhancer-containing DNase I HS4 using in silico analyses. Cut repeats and homeodomains bind to this cis-element (Fig. 1A) [21]. To determine whether CUX1 interacts with the enhancer region of human COL1A2, we performed EMSAs using transcription factor-binding consensus oligonucleotides, CUX1 antibody and sh-CUX1. In these experiments, we not only verified the binding of CUX1 at CUX1 (−19.51 kb) probe sites but also demonstrated the binding of several other transcription factors, including JunD (data not shown). Nuclear extracts from normal lung fibroblasts incubated with or without TGF-β were used, and CUX1 consensus oligonucleotides, CUX1 antibody and sh-CUX1 decreased the intensity of CUX1 bands (Fig. 1B). Furthermore, the binding of CUX1 at CUX1 (−19.51 kb) probe sites increased post-TGF-β treatment in both normal and SSC lung fibroblasts (Fig. 2A).

3.2. TGF-β up-regulates the expression of CUX1 isoforms

CUX1, also known as CCAAT displacement protein (CDP), CUTL1, CUT or Cux, belongs to a family of homeobox transcription factors that are involved in the regulation of cell growth and differentiation [22]. It is evolutionarily conserved and comprises four DNA binding domains, three of which are known as Cut repeats and one is known as the Cut homeodomain [21]. CUX1 mainly acts as a transcriptional repressor, but it can also act as an activator [23–25]. Transgenic mice with CUX1 overexpression have been reported to exhibit multi-organ hyperplasia and organomegaly [26]. Moreover, the CUX1 isoforms p200 and p110 can regulate motility and invasiveness through a TGF-β-dependent mechanism [27,28]. However, the role of CUX1 isoforms in the development of fibrosis remains unclear. We performed Western blotting to determine whether the expression levels of CUX1 isoforms were induced by TGF-β. We found that the expression levels of CUX1 isoforms significantly increased in both normal and SSC lung fibroblasts post-TGF-β treatment (Fig. 2B, C). TGF-β treatment resulted in the induction of p200 and p150 in normal lung fibroblasts. Moreover, TGF-β treatment resulted in the induction of p200, p150, p110, p75, p30 and p28 in SSC lung fibroblasts. These results suggested that TGF-β stimulate the expression of CUX1 isoforms. We also verified whether the expression levels of profibrotic proteins were up-regulated. ET-1, COL1, Wnt1, CTGF and β-catenin levels increased post-TGF-β treatment in normal and SSC lung fibroblasts (Fig. 2D).

3.3. Effects of cathepsin L inhibition on lung fibroblasts derived from patients with SSC

Several studies have illustrated that CUX1 is cleaved into p150, p110, p75 and other subunits by nuclear cathepsin L. To confirm the effects of cathepsin L inhibition on SSC lung fibroblasts, we measured the expression levels of COL1 in both normal and SSC lung fibroblasts. IW-CHO (0.2 μM) was used because its IC50 is 1.9 nM. IW-CHO decreased the expression levels of COL1 in normal and SSC lung fibroblasts (Fig. 2E). To further confirm the effects of cathepsin L on SSC lung fibroblasts, we used immunocytochemistry to study CUX1 localization in these

![Fig. 1](image-url). Electrophoretic mobility shift assays (EMSAs) revealed that CUX1 binds to the human type I collagen alpha 2 gene (COL1A2) enhancer. A. Schematic diagrams of the far upstream enhancer region (FUE) with arrows indicating known DNase hypersensitive sites; the white box indicates the known identity islands of human COL1A2. CUX1 cis-elements are indicated by bold letters. B. EMSA using the CUX1 (−19.51 kb) probe. NE, nuclear extract from normal lung fibroblasts. The right-hand graph of Fig. 1B shows several bands detected using P32-labelled oligos in Lanes 2 and 3. Bands were confirmed using a cold competitor. Unlabelled competing oligo probes are shown in Lanes 4 and 5. Consensus oligo for CUX1, Lanes 8–9. Consensus oligo for SP1, Lanes 6–7. Antibody for CUX1, Lanes 11–13. Sh-CUX1, Lanes 15. FP, free probe.
As shown in Fig. 3, immunofluorescence analysis indicated that SSc lung fibroblasts were CUX1- and α-SMA-positive, with positivity increasing post-TGF-β treatment. CUX1 localised to the nucleus and cytosol. In addition, SSc lung fibroblasts were larger than normal lung fibroblasts (data not shown). IW-CHO decreased the CUX1 localization in the nucleus and reduced the large size of SSc lung fibroblasts with or without TGF-β treatment.

3.4. Immunohistochemical analyses of SSc and diffuse alveolar damage (DAD) lung tissue sections stained with antibodies against CUX1 and α-SMA

Immunocytochemistry revealed the presence of α-SMA-positive fibrotic loci (Fig. 4), which is characteristic of patients with SSc and DAD. CUX1 localised at alveolar cells and fibrotic loci. In addition, CUX1 localised within α-SMA-positive cells (Fig. 4, merged).

4. Discussion

This study demonstrated the binding of CUX1 to the IS4 domain of the COL1A2 enhancer region in both normal and SSc lung fibroblasts. Further, our results demonstrated that TGF-β-induced CUX1 isoforms function as transcriptional activators that increase the expression of COL1 proteins. Previous studies implicated CUX1 in both the activation and suppression of several genes [23–25].

Our previous study found that CUX1 bound to the proximal promoter of COL1A2 after treatment with high doses of TGF-β [22]. In this study, we provide evidence that CUX1 also plays a major role in COL1A2 activation both in vitro and ex vivo via the IS4 domain of the COL1A2 enhancer.

Our data clearly demonstrated that TGF-β induces the expression of CUX1 isoforms, some of which were also up-regulated in SSc lung fibroblasts. In association with this change, the binding of CUX1 to the enhancer region of human COL1A2 increased in response to TGF-β treatment in normal and SSc lung fibroblasts. These results suggested that TGF-β up-regulates CUX1 protein expression, resulting in an increased binding of CUX1 to the human COL1A2 enhancer and COL1 up-regulation.
Another important finding of this study was that TGF-β induces the expression of the CUX1 isoforms p200, p150, p110, p75, p30 and p28 in SSc lung fibroblasts. Cleavage sites have been found for cathepsin L between CR1 and CR2 and for caspase between CR3 and HR of CUX1 [19]. Previous studies have revealed the induction of HA-tagged CUX-1 p200 and p110 in certain cell lines [19,29]. The CUX-1 antibody M-222 simultaneously recognises several CUX1 isoforms in normal and SSc lung fibroblasts following TGF-β treatment. As the molecular weight patterns observed in our study are similar to those previously reported, we believe that all bands observed in this study were specific for CUX1 isoforms.

Our data provide further evidence that CUX1 isoforms may play crucial roles in COL1A2 regulation. First, the levels of all CUX1 isoforms increased in SSc lung fibroblasts compared with the levels in normal lung fibroblasts. Moreover, compared with the findings in normal lung fibroblasts, data from EMSA revealed significantly increased binding of CUX1 at CUX1 (∼ 19.51 kb) probe sites of human COL1A2 in SSc lung fibroblasts. Several reports studies have illustrated that CUX1 isoforms have different DNA binding affinities and functions [19,23–25,30]. Second, COL1 expression decreased by cathepsin L inhibition in normal and SSc lung fibroblasts, highlighting the importance of CUX1 isoforms in fibrosis. Third, CUX1 localised at the nucleus and cytosol (Fig. 3). CUX1 and α-SMA co-localised in the cytosol in SSc lung fibroblasts. IW-CHO decreased the CUX localization in the nucleus and repressed the large size of SSc lung fibroblasts with or without TGF-β treatment (Fig. 3). In addition, in SSc and DAD lung sections, CUX1 localised around fibrotic loci and alveolar cells. Some of these cells were stained by both CUX1 and α-SMA (Fig. 4). Furthermore, in the SSc skin and idiopathic pulmonary fibrosis (IPF) sections, CUX1 localised within and around α-SMA positive cells (See Figs. 1 and 2, reference [31]). These results indicated that CUX1 isoforms are necessary for fibrosis.

In future, investigations on the mutagenesis of CUX1 cleavage sites are required to determine which CUX1 isoforms are crucial. In addition, we need to verify the effects of CUX1 isoforms on the promoter region of α-SMA and pro-fibrotic cytokines. We speculate that some specific features of CUX1 isoforms are important for the activation of human COL1A2 via the IS4 domain, which is located in the COL1A2 enhancer.

In summary, our study demonstrated that the up-regulation of CUX1 isoforms is associated with fibrosis in vitro in SSc lung fibroblasts as well as in tissue sections from patients with SSc and DAD. Hence, these data indicate that the expression of CUX1 isoforms is possibly important for fibrosis and could be relevant to the pathogenesis of SSc.

Fig. 3. Effects of IW-CHO on systemic sclerosis (SSc) lung fibroblasts. Immunofluorescence analysis performed using SSc lung fibroblasts treated with or without TGF-β. Cells were stained using CUX1 antibody, DAPI and α-SMA antibody. IW-CHO treatment decreased the CUX localization in the nucleus and reduced the size of lung fibroblasts in patients with SSc. In addition, in the presence of TGF-β, IW-CHO treatment reduced the size of lung fibroblasts in patients.
Fig. 4. Systemic sclerosis (SSc) and diffuse alveolar damage (DAD) lung tissue sections stained with antibodies against CUX1 and α-smooth muscle actin (α-SMA). The figure shows fibrotic loci that were stained by CUX1 and α-SMA antibodies. Alveolar cells around the loci were positive for CUX1 and α-SMA. CUX1 localised within α-SMA-positive cells.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.06.022.

References


