Unique mechanisms of connective tissue growth factor regulation in airway smooth muscle in asthma: relationship with disease severity

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**Keywords:** CTGF, promoter regulation, airway smooth muscle, asthma
Unique mechanisms of connective tissue growth factor regulation in airway smooth muscle in asthma: relationship with airway remodelling

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**Abstract:** Neovascularisation, increased basal membrane thickness and increased airway smooth muscle (ASM) bulk are hallmarks of airway remodelling in asthma. In this study, we examined CTGF dysregulation in human lung tissue and animal models of allergic airway disease. Immunohistochemistry revealed that ASM cells from patients with severe asthma (A) exhibited high expression of CTGF, compared to mild and non-asthmatic (NA) tissues. This finding was replicated in a sheep model of allergic airways disease. *In vitro*, transforming growth factor (TGF)-β increased CTGF expression both in NA- and A-ASM cells but the expression was higher in A-ASM at both the mRNA and protein level as assessed by PCR and western blot. Transfection of CTGF promoter-luciferase reporter constructs into NA- and A-ASM cells indicated that no region of the CTGF promoter (-1500 to +200 bp) displayed enhanced activity in the presence of TGF-β. However, *in silico* analysis of the CTGF promoter suggested that distant transcription factor binding sites may influence CTGF promoter activation by TGF-β in ASM cells. The discord between promoter activity and mRNA expression was also explained, in part, by differential post-transcriptional regulation in A-ASM cells due to enhanced mRNA stability for CTGF. In patients,
higher CTGF gene expression in bronchial biopsies was correlated with increased basement membrane thickness indicating that the enhanced CTGF expression in A-ASM may contribute to airway remodelling in asthma.

**Key words:** Asthma, airway smooth muscle, connective tissue growth factor, airway remodelling
INTRODUCTION

Asthma is a common, chronic respiratory disease affecting more than 300 million people worldwide[1]. The main characteristics of asthma are airway inflammation, airway hyperresponsiveness and airway remodelling[2]. The structural changes in the airways, termed airway remodelling, include increased airway smooth muscle (ASM) bulk, increased basal membrane thickness and vascular expansion[3,4]. The extent of airway remodelling correlates with several clinical features of asthma [5-8] and agents that normalise the remodelling response potentially improve asthma symptoms[9-11]. Once considered a manifestation of chronic inflammation recent studies have identified remodelling is a separate but parallel component of the asthmatic process[12].

In asthma the observed increase in ASM cell bulk and contractility directly mediates airway narrowing and is central to the process of airway remodelling. Increased ASM number correlates with increased reticular basement membrane thickness and eosinophilia, but not neutrophilia [13]. Further, the secretory profiles of ASM from asthmatic patients differ significantly from those of non-asthmatics suggesting that
paracrine signalling from the ASM in may have as much to do with airway remodelling as their contractile state (reviewed in [14]). Ultimately, the increased deposition of extracellular matrix (ECM) proteins by ASM cells in asthma is key to the airway narrowing that takes place [12,15-18]. Often this is a response to an imbalance in the cytokines/growth factors present in their local milieu[3].

In lung tissue, ASM cells are a potent source of connective tissue growth factor (CTGF), a member of the Cysteine rich 61, CTGF, Nephroblastoma (CCN) family of proteins[19]. Our previous studies have shown greater CTGF expression in primary asthmatic (A)-ASM cells than non-asthmatic (NA)-ASM cells after TGF-β treatment[20-22]. CTGF controls ECM deposition and ultimately airway biomechanics through changes to collagen deposition which increase ECM density and airway stiffness[23]. Indeed, the increased stiffness of the matrix in which asthmatic ASM cells are embedded promotes a more proliferative and pro-inflammatory ASM phenotype [14].

The mechanisms underlying the differential regulation of CTGF expression in A-ASM are not currently known. Studies in other systems have reported that CTGF
induction by TGF-β is regulated through interactions of transcription factors with promoter elements directly upstream of the promoter start site[24-27]. In this study, we investigated the mechanisms that enhance TGF-β induction of CTGF release from A-ASM cells and the potential links to airway remodelling in asthma.
MATERIALS AND METHODS

Primary Airway Smooth Muscle Cell Isolation and culture

Approval for experiments with human lung tissue was provided by the Ethics Review Committee of the South West Sydney Area Health Service, St Vincent’s Hospital Sydney, Strathfield Private Hospital, Royal Prince Alfred Hospital and the University of Sydney Human Research Ethics Committee. Primary human ASM cells were obtained through dissection of donated lung tissue following transplantation and from endobronchial biopsies from volunteers who provided written informed consent, as described previously [28,29]. The patients’ details are described in table 1.

Cells were grown in 10% (v/v) foetal bovine serum (FBS) (JRH Biosciences, Brooklyn, VIC, Australia) /high glucose Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) containing 100 units/mL of penicillin, 100 µg/mL of streptomycin, 0.25 µg/mL of Amphotericin B (Thermofisher, Waltham, MA USA) and 25 mM HEPES (Sigma-Aldrich). Use of primary ASM cells was restricted to passage numbers 2 and 8.
Detection of CTGF by Immunohistochemistry

Sections from archived paraffin-embedded lung tissue were obtained from non-, mild and severely asthmatic patients [3,4] as well as a sheep model of allergic airways disease that had been previously described [30]. Slides were de-paraffinised in xylene and rehydrated through graded ethanol solutions. Peroxidase-Blocking solution (DAKO, Agilent Pathology, Foster City, CA, USA) was used to block endogenous peroxidases at 37 °C for 15 min. Slides were washed and incubated with serum free protein block (DAKO) at 37 °C for 30 min followed by incubation with rabbit anti-CTGF primary antibody (2.5 µg/ml for human, 5 µg/ml for sheep lung tissue, ab6922; Abcam, Cambridge, UK) or Rabbit IgG (same concentration as CTGF antibody, X0903, DAKO) overnight at 4 °C. Primary antibodies were diluted in REAL antibody diluent (DAKO) to minimise non-specific binding. After washing, slides were incubated with EnVision+ system HRP labelled polymer anti-rabbit secondary antibody (K4003, DAKO) for 45 minutes at 37 °C. Liquid Diaminobenzidine+ (DAB) substrate chromogen system (DAKO) was added and incubated for 10 min at room temperature. All slides were counterstained with haematoxylin (with eosin for human
sections) before dehydration through graded ethanol and mounted with Dibutyl Phthalate in Xylene (DPX, Tingalpa, QLD, Australia) mounting medium (VWR BDH Prolabo® Chemicals). Human slides were scanned using a Wide-field FL and TL microscope ZEISS Axio Scan.Z1 SlideScanner (Zeiss, Oberkochen, Germany) and the sheep slides were scanned by a NDP scanner (HAMAMATSU, Hamamatsu, Japan).

**ASM cell stimulation**

For all experiments, unless otherwise indicated, ASM cells were seeded into 6-well plates at a concentration of 1x10^4 cells/cm^2 in 5% (v/v) FBS/DMEM and grown for 3 days before being made quiescent in 0.1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, United States)/DMEM for 24 hours. Cells were treated with recombinant human TGF-β1 protein (1ng/ml, R&D systems, Minneapolis, MN, USA) for the indicated durations. Cells were washed twice with ice cold phosphate-buffered saline (PBS, Sigma-Aldrich) and lysed in lysis buffer prior to total RNA being extracted using the ISOLATE RNA mini kit (Bioline, London, UK) according to the manufacturer’s instructions, quantified with a Nanodrop 2000.
Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored in -20°C for further use. Alternately, cells were scraped into protein lysis buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM Na$_2$EDTA, 1mM EGTA, 1mM NaF, 20mM Na$_4$P$_2$O$_7$, 2mM Na$_3$VO$_4$, 1% (v/v) Triton X-100, 10% (v/v) Glycerol, 0.1% (w/v) SDS, 0.5% (w/v) Sodium Deoxycholate, 1mM Phenylmethylsulfonyl fluoride (PMSF) and 1:100 Protease Inhibitor Cocktail Set III (Merck-Millipore, Billerica, MA, USA)) and stored at -20°C until analysis.

**Real time reverse transcription polymerase chain reaction**

To investigate CTGF mRNA expression, real time PCR was conducted on NAO and AOASM cells with primers and probe specific to CTGF (Hs01026927_g1, Life Technologies, Carlsbad, CA, United States) and BioSense SensiFast™ Probe Hi-ROX Mastermix (Bioline) using a StepOne Plus detection system (Thermo Fisher). Relative gene expression between treatments were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization against the 18s rRNA probe (4319413E-1011052, Life Technologies).

**Western blot**


Total cellular protein was extracted and separated on a 10% (w/v) SDS-PAGE gel, and transferred to Polyvinylidene difluoride (PVDF) membrane (Merck-Millipore). Membranes were blocked in 5% (w/v) skim milk in Tris buffered saline (TBS, 20mM Tris base, 150mM NaCl, PH7.4) containing 0.05% (v/v) tween 20 for 30 min at room temperature and incubated with goat polyclonal anti-CTGF antibody (1:1000, sc-14939, Santa-Cruz Biotechnology, Dallas, TX, USA) or anti-GAPDH (1:10000, MAB-374, Millipore) at 4°C overnight. Membranes were washed and incubated with horseradish peroxidase conjugated secondary antibody (1:2000 for CTGF (P0160), 1:50000 for GAPDH (P0161), DAKO). Images were captured using a Kodak Image station 4000mm and band intensity was quantified with Carestream MI SE software.

CTGF promoter constructs

A Gluc-on reporter plasmid containing the full length CTGF promoter (-1500 to +200 bp) driving expression of a secreted Gaussia luciferase was purchased from GeneCopoeia (Cat. HPRM25713-PG04, Rockville, MD, USA). A series of 5’ deleted constructs were generated by PCR using the primers shown in table 2. The full length CTGF promoter was excised using the restriction enzymes Hind III and Bgl II
(New England Labs, Ipswich, MA, United States) and the generated PCR products ligated into the vector using T4 DNA ligase (Promega, Madison, WI, United States).

All constructs were sequence verified at the Australian Genome Research Facility (Melbourne, VIC, Australia) prior to expansion and transfection.

**Transfection and luciferase assay**

ASM cells and NIH 3T3 fibroblasts were seeded in 12-well plates at densities of $1 \times 10^4$ cells/cm$^2$ and $8 \times 10^4$ cells/well, respectively, for 24 hours in 10% (v/v) FBS/DMEM. The full length CTGF promoter, or the 5’ deleted constructs, were transfected into the indicated cells using lipofectamine 3000 (L-3000075, Invitrogen, Carlsbad, CA, United States) transfection reagent according to the manufacturer’s instructions using either 1 or 1.6 µg of plasmid DNA for ASM and NIH3T3 cells, respectively. Cells were stimulated with TGF-β1 (1 ng/ml for ASM and 10 ng/mL for NIH3T3) 24 hours after transfection, for a further 24 hours. The supernatant was collected for luciferase detection by Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia) and total RNA isolated from the cell monolayers using the ISOLATE RNA mini kit (Bioline) for CTGF mRNA detection.
Analysis of Distant Transcription factor binding sites with potential to regulate CTGF promoter.

To investigate other potential transcription factor binding sites in close proximity to the CTGF promoter, H3K27Ac binding (a marker of transcription factor binding) was investigated. This analysis was conducted using chromatin immunoprecipitation sequence (ChIP-Seq) data on human umbilical vein endothelial cells (HUVECs) and normal human lung fibroblasts (NHLF) generated as part of the Encyclopedia of DNA Elements (ENCODE) Project (GSE29611).

Assessment of mRNA stability in ASM cells.

To measure CTGF mRNA stability, ASM cells rendered quiescent by incubation in 0.1% (w/v) BSA/DMEM for 24 hours were treated with TGF-β1 (1ng/ml) for 8 hours. After washing with PBS, actinomycin D (10 mg/mL, Sigma-Aldrich) was added to the media for a further 0-16 hours as indicated. Total RNA was isolated and CTGF mRNA was quantified by PCR as described above.

CTGF gene expression relationship with clinical factors
We obtained high quality RNAseq data from 184 biopsies. Biopsies were derived from 77 healthy subjects and 107 current or former asthma patients. All patients originated from cohorts investigated earlier by our research group, and a set of previously acquired clinical and genotype data is available [31,32](Vonk et al. submitted). The study protocol was approved by the University Medical Center Groningen medical ethics committee. All subjects gave their written informed consent. For full patient information and details relating to RNA isolation and sequencing refer to the online supplement.

A linear model was fitted to CTGF gene expression derived from RNA sequencing in bronchial biopsies (expressed as FKPM) as a function of forced expiratory volume in 1 second percentage predicted (FEV1% predicted), basement membrane (BM) thickness, \( \log_2(\text{PC}_{20} \text{ to methacholine}) \) and \( \log_2(\% \text{ of eosinophils}) \) in sputum of asthmatics (n=69). Age, gender and smoking status were used as correction factors. All analyses were conducted using R (version 3.3.2).
Statistical Analysis

Data were tested for normal distribution and analysed via a two-way analysis of variance (ANOVA) with Bonferroni post-test. A p-value less than 0.05 was considered to be statistically significant (P<0.05).
Results

Increased CTGF expression in asthmatic lung tissue \textit{in vivo} and exaggerated release of CTGF from asthmatic ASM cells \textit{in vitro}

Immunohistochemistry showed that CTGF was detected in both sham and house dust mite (HDM)-sensitised (n=6 for both) sheep lung tissues and the staining was concentrated in the airway smooth muscle layer (Fig 1). Having established that asthmatic ASM shows dysregulation of CTGF expression we sought to understand the mechanism responsible. Treatment of both asthmatic (A-) (n=5) and non-asthmatic (NA-) (n=7) ASM cells with TGF-β1 induced CTGF expression (Fig 2. A), confirming our previous observations [20,22]. As before, the increase of CTGF mRNA in A-ASM cells was 3- and 2.5-fold greater than in NA-ASM cells at 12 (p<0.0001) and 24 hours (p<0.05) after TGF-β1 treatment, respectively. Similar kinetics were observed in CTGF protein expression (Fig 2B and C); however, the magnitude of induction was greater (5- and 4-fold at 12 and 24 hours respectively) in A- (n=7) than NA- (n=4) ASM cells (p<0.01).
Transcriptional regulation of CTGF promoter (-1500 to +200 bp) is the same in asthmatic and non-asthmatic ASM cells

Other studies have reported that CTGF expression induced by TGF-β is regulated by an interaction between transcription factors and CTGF promoter binding sites immediately upstream of the promoter start site. To investigate mechanisms underlying the greater CTGF release from A-ASM cells, compared to NA-ASM cells, after TGF-β treatment we transfected a full length (-1500 to + 200 bp) CTGF promoter Luciferase reporter construct and 5’ truncated promoter constructs (Fig 3A) into both A- and NA-ASM cells. The secretion of alkaline phosphotase (SEAP), driven by a CMV promoter within the constructs, was used to normalise for transfection efficiency. Basal promoter activity was observed with the (-400 to +200 bp) construct, compared to the promoter-less reporter and the (-100 to +200 bp) construct, and this activity was equivalent in A- and NA-ASM, consistent with basal CTGF expression. The longer promoter constructs did not have greater luciferase activity compared to the -400bp construct in either A- or NA-ASM cells (Fig 3B & D respectively) suggesting basal expression was regulated proximal to the
transcriptional start site. There was no enhancement of the CTGF promoter activity after TGF-β treatment in either NA- or A- ASM cells nor did a new regulatory element emerge in one of the truncated mutants (Fig 3B & D). This was in direct contrast to CTGF mRNA expression which increased in both A- (Fig 3C) and NA-(Fig 3E) ASM cells after TGF-β treatment in the presence of all transfections.

**Tissue specific genetic elements indicate CTGF regulation in lung tissue is unique.**

To assess whether our promoter construct was indeed inducible by TGF-β, and to determine whether CTGF regulation in ASM was different to other tissues, we transfected NIH-3T3 cells and examined luciferase activity. Previous reports have shown that CTGF promoter-luciferase reporter constructs increase activity 2-4 fold when NIH-3T3 cells are stimulated with TGF-β [25,27,33,34]. Indeed, treatment of transfected NIH 3T3 fibroblasts with TGFβ induced a 2 fold increase in luciferase expression compared to unstimulated cells (supplementary Fig 1) indicating our CTGF promoter construct (-400 to +200) was indeed inducible but just not in human
ASM cells.

Having found that the CTGF promoter regulation in human ASM cells differed from that reported in other cell lines [25,27,33,34] we investigated regions of transcription factor binding activity surrounding the CTGF transcriptional start site to look for additional regulatory elements. This analysis was conducted by investigating H3K27Ac binding (a marker of transcription factor binding). For this analysis we used human lung fibroblasts as previously no differences in gene expression was detected between lung fibroblasts and ASM cells, indicating highly similar gene expression regulation[35]. There was a strong region of activity immediately upstream of the CTGF transcriptional start site in HUVECs (-1300 to -200 bp), which was less active in human lung fibroblasts (Fig 4A). This region spanned the -1500bp promoter construct we had analysed (Fig 4B), and contained several validated SMAD and TGF-β response elements previously reported to drive CTGF expression in other species (Fig 4C) [25,27,36]. However, an alternate genomic region 5’ to the (-1300 to -200bp) site (-4200 to –2400 bp) showed robust H3K27Ac binding in lung cells but
relatively low activity in HUVECs (Fig 4A). These findings suggest that this region may be responsible for the alternative regulation of CTGF expression in human lung cells.

**CTGF mRNA stability is enhanced in asthmatic ASM cells.**

Ibrul Chowdhury et al previously reported that FBS induced CTGF mRNA expression was regulated by both new transcription and mRNA stabilization in primary bladder smooth muscle cells [37]. To further characterise the regulation of CTGF in A- and NA-ASM cells we compared the kinetics of CTGF RNA turnover induced by TGF-β. In NA-ASM cells CTGF transcripts were rapidly degraded, with a half-life of 3 hours (Fig 5). In contrast, the half-life of CTGF mRNA in A-ASM cells was prolonged (7 hours) suggesting that the mRNA stability was enhanced in the A-ASM cells. Linear regression showed that the degradation rate after 6 hours between A- (k=-2.99) and NA- (k=-2.42) ASM cells was similar. However, in NA-ASM cells the degradation of CTGF mRNA in the first 6 hours was, 20.3% higher than in the A-ASM cells (k=-15.82 versus k=-12.6, respectively) suggesting
enhanced stability and slower degradation are the source of the longer mRNA half-life in A-ASM cells. These data suggested that TGF-β induced CTGF mRNA in A-ASM cells was more stable than NA-ASM cells providing a possible explanation for the differential expression of TGF-β induced CTGF between A- and NA-ASM cells.

CTGF gene expression is unchanged in mild asthmatic patients but relates to basement membrane thickness

Immunohistochemical staining showed CTGF protein expression was concentrated in the ASM area in human lung tissue, with enhanced detection visible in asthmatic tissues, particularly from severe asthmatics (Fig 6A). No difference in CTGF mRNA expression (Fig 6B) was detected between bronchial biopsies derived from mild to moderately severe asthmatics (n=69) and healthy controls (n=77). In addition, within asthmatic subjects, we found a significant relationship between CTGF expression and basement membrane (BM) thickness (beta±SE 0.472±0.174, p=0.008, Fig 6C) suggesting ASM-derived CTGF expression may influence airway narrowing and remodelling in asthma. In contrast, higher CTGF expression was not associated
with lower FEV1 % predicted, more severe bronchial hyperresponsiveness or higher %
eosinophil levels in sputum (Fig 6 C-E).
Discussion

This is the first study that has focused on the regulation of CTGF mRNA in primary ASM cells. Tissue localisation in asthmatic airways indicated that the ASM cells were the primary location of CTGF expression. We have shown that, unlike other cell types, regulation of CTGF mRNA expression in primary ASM cells may not be located in the first 1500 bases in the CTGF promoter, but rather lie some distance upstream. In addition, post-transcriptional regulation of mRNA stability plays a role in the differential TGF-β induced CTGF mRNA expression in A- and NA-ASM cells. CTGF expression in asthmatic patients correlated with the degree of basement membrane thickening, suggesting CTGF may contribute to the mechanisms driving airway remodelling.

In our study, we observed greater CTGF expression in airway tissues taken from lung segments of sheep chronically exposed to HDM allergen. These findings were consistent with other in vivo studies that have reported that CTGF mRNA and protein are upregulated in murine models of allergic airways disease[38,39]. CTGF was also
enhanced in lung tissue from severe asthmatic patients, compared to non-asthmatic controls. The ASM cells, as the primary source of CTGF in lung tissue, are uniquely positioned to drive remodelling (new blood vessel formation and basement membrane thickening) as they lie immediately below the lamina propria, a major location of vascular expansion and ECM deposition in remodelling airways.

To illuminate the cause of the difference in regulation of CTGF mRNA between A- and NA-ASM cells, we investigated transcriptional and post-transcriptional regulation of CTGF in primary ASM cells. The well characterised TGF-β responsive elements in the CTGF promoter [25,27,33,34] appeared to not be involved in the regulation of CTGF in ASM cells. Our discovery of a novel CTGF transcriptional regulatory region, upstream of what is considered to be the core promoter region, in lung fibroblasts opens new realms in the tissue specific regulation of CTGF expression.

Given the similarities in gene expression we speculate that this mechanism is likely to play a role in the unique regulation of CTGF in ASM; however, the absence of ASM data precluded our validation in ASM directly. We have previously shown TGFβ
induced CTGF through activation of the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) signalling pathways in ASM cells. ERK is known to be linked to SMAD2/3 activation [36] and likely targets the traditional core promoter region (-1300 to -200bp) of CTGF which appears to be SMAD sensitive (Fig 4). However, PI3K has not been associated with SMAD 2/3 signalling to date and therefore may target the alternative promoter regulatory region we have identified in this study. Moreover, how these distant genomic elements are recruited to the core promoter to modulate CTGF expression is unknown and may be influenced by epigenetic modification of histones (particularly K27 acetylation) which is readily acknowledged to be different in asthmatic and healthy airways[40,41].

Further research is necessary to identify the transcriptional regulatory elements, potentially within the -4200 to –2400 bp region, activated by these alternative signalling pathways for driving CTGF expression.

We also showed that CTGF mRNA stability was enhanced in A-ASM cells. Chowdhury and colleagues previously showed this to be mediated by p38 in bladder
smooth muscle cells [37]. However, our previous data conclusively showed this pathway is not involved with CTGF regulation by TGF-β in A-ASM [22]. The mechanism underlying this enhanced CTGF mRNA stability is currently unknown.

The similarities in basal promoter activity in A- and NA-ASM were just as surprising as the lack of TGF-β responsiveness in the CTGF promoter. We identified that the basal promoter in ASM is located between -100 and -400 bp. This region contains predicted binding sites for SMAD, AP1, TGF-β element, Ets1, NF-1-like sequence, TIE-like site amongst others. Some of these factors have previously been implicated in TGF-β signalling [26,27,42] whilst others have not. The similarity of basal CTGF promoter activity in A- and NA-ASM underscores the importance of the inflammatory milieu in the asthmatic airway in directing the phenotype of ASM during disease.

The dysregulation of CTGF in asthmatic airways may have profound consequences for disease progression, as suggested by the association of CTGF gene expression levels with basement membrane thickening in our patient cohort. One limitation in
our study is that the biopsies from which we obtained the gene signal were of a mixed cell population and we have no information about the ASM content in each biopsy. This may have altered the CTGF gene signal as CTGF is also expressed by airway epithelial cells [43-45] and it is not known if these levels are also altered in asthma. If the epithelial gene expression of CTGF is not increased in asthma this may have reduced the strength of the association we observed with basement membrane thickness. The basement membrane is 2-3 fold thicker in asthmatic compared to healthy airways and is associated with increased airway resistance, limitations to airflow and decreased lung function[46,47]. Association of basement membrane thickening with poor clinical outcomes is somewhat controversial with adults [32,48] but shows better correlation in children[48-50]. The thicker basement membrane of asthmatic airways also has an altered elastic modulus compared to healthy airways[51]. The increased extracellular matrix (ECM) stiffness that accompanies such a change is likely to contribute to the pro-remodelling environment found in asthmatic airways as stiffer matrices promote angiogenesis [52,53] and ASM cell proliferation [51]. Indeed, stiffer matrices may also enhance CTGF expression
through Taz activation [54,55], completing a positive feedback loop in the asthmatic airway that would co-ordinate all aspects of airway remodelling (ASM bulk, neovascularisation and basement membrane thickening).

In conclusion, our data strongly suggests that the unique regulatory mechanisms that underpin the enhanced CTGF expression in A-ASM are pivotal for the development of airway remodelling. Thus, CTGF represents an underappreciated target for future therapeutic intervention addressing an aspect of disease pathogenesis currently not effectively treated by existing approaches.
Acknowledgements

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Conflicts of interests

The authors confirm there are no conflicts of interests in this study.
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Table 1. Details of asthmatic and non-asthmatic donors

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Abbreviations: ILD Interstitial lung disease, NA not available
Table 2. Primer sequence for CTGF 5’ deletion mutant constructs

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<td>Reverse primer</td>
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FIGURE LEGENDS

Fig1. CTGF expression is increased in asthmatic lung tissue. CTGF expression was assessed by immunohistochemistry in HDM-exposed and saline-exposed (sham control) lung segments from the same sheep [30] (n=5). Isotype-matched negative control antibody on serial sections shown for comparison. Representative images shown for each group.

Fig2. Asthmatic airway smooth muscle (ASM) cells have different kinetics of CTGF induction. ASM cells from A- and NA-donors were stimulated with TGF-β (1ng/ml) for up to 72hours and CTGF transcript (A; NA-ASM (n=7) and A-ASM (n=5)) and protein (B; NA-ASM (n=4) and A-ASM (n=7)) levels examined by Q-PRC and western blot, respectively. Representative images of western blots are shown. Changes in CTGF expression by western blot were quantified using image J software (C). *p<0.05, **p<0.01, ***p<0.001 denotes significance between BSA and TGF-β. #p<0.05, ##p<0.01, ####p<0.0001# indicates significant difference between NA- and A-ASM cells.
Fig3. Basal regulation of the CTGF promoter is the same in NA- and A-ASM cells. A. Schematic of the 5’ deleted CTGF promoter constructs used to examine regulation in NA- and A-ASM cells. Different lengths of the human CTGF promoter (-1500 to +200; ■) were placed upstream of a Luciferase reporter construct (■). SEAP expression was driven by CMV promoter in the same construct and was used as a control for transfection efficiency. Luciferase activity in conditioned media was detected after stimulation of transfected ASM +/- TGF-β (1ng/ml; NA- (B, n=5) and A-ASM (D, n=5)). CTGF mRNA in NA- (C) and A-ASM (E) was measured by Q-PCR in the same cells used for luciferase assays to determine the effectiveness of induction for the endogenous gene. “&” denotes significance between promoterless (0) and luciferase reporte (&p<0.05, &&p<0.01, &&&p<0.001). #means significant difference between different length of CTGF reporter in BSA treated cells, #p<0.05, ##p<0.01. “$”indicates difference between different length of CTGF reporter in TGF treated cells. * indicates significant difference of CTGF mRNA expression between TGF-β and BSA, *p<0.05, **p<0.01, ****p<0.0001.
Fig4. Differential usage of promoter elements endows cell-type specific regulation of CTGF in ASM. **A.** H3K27ac profiling from human lung fibroblasts and HUVECs surrounding the CTGF gene by ChIP-Seq. **B.** Plasmid CTGF promoter construct used in this project. **C.** Validated SMAD and TGF-β transcription factor binding sites across different species. HUVECs, human umbilical vein endothelial cells; SMAD, Similar to Mothers Against Decapentaplegic. Analysis was performed using the ENCODE database.

Fig5. CTGF mRNA stability is enhanced in A-ASM cells. NA- (n=4) and A-ASM cells (n=5) were treated with TGF-β (1 ng/mL) with actinomycin D (10 µg/ml) added after 8 hours for up to 16 hours. CTGF mRNA expression was measured by Q-PCR to assess the rate of turnover. * means significant difference in CTGF mRNA expression to time 0, *p<0.05, ***p<0.001, ****p<0.0001. # p<0.05 indicates a significant difference between NA- and A-ASM.
Fig6. CTGF expression and correlations with clinical indices in asthmatic patients. A. CTGF expression was assessed by immunohistochemistry in human lung tissue (n=5 for healthy control, mild asthma and severe asthma). Representative images shown for each group. B-E. CTGF mRNA expression was detected in bronchial biopsies from healthy controls and mild asthmatic patients (B). A linear model comparing the association between CTGF expression in bronchial biopsies and BM thickness (C), FEV1pp (D), % of sputum eosinophils (E) and PC20 (F) was conducted correcting for age, gender and smoking status. β; correlation co-efficient. ρ; significance value of the correlation. BM basement membrane, FEV1pp forced expiratory volume in 1 second percentage predicted, PC20 the concentration of methacholine needed to produce a 20% fall in FEV1 from baseline. See [32] (table 1) for lung function on this cohort.
SUPPLEMENTAL INFORMATION

MATERIALS AND METHODS

Subjects

We obtained high quality RNAseq data from 184 biopsies. Biopsies were derived from 77 healthy subjects and 107 current or former asthma patients. All patients originated from cohorts investigated earlier by our research group, and a set of previously acquired clinical and genotype data is available (Broekema et al. 2010, 2011, Vonk et al. submitted).

All 107 patients had a previous doctors diagnosis of asthma, documented reversibility and AHR to histamine (PC20 =< 32 mg/mL). We split the persistent asthmatics on the basis of use of inhaled corticosteroids (ICS), since corticosteroids are known to have strong impact on cell physiology and thus potentially act as a confounder in our analysis (ref). We considered subjects to be in clinical remission if they had not had an asthma attack or wheeze in the last 3 years, and did not use asthma medication (β-agonists and ICS) for the past 3 months. If individuals, in addition to the previous criteria did not have AHR to histamine and AMP (> 32 mg/mL in 30 s tidal breathing and > 320 mg/mL in 2 min tidal breathing respectively) and had no signs of airflow...
obstruction (FEV1 %predicted > 80% pre bronchodilator or >90% post bronchodilator), they were considered to be in complete remission.

The control cohort of 77 respiratory healthy subjects was derived from the NORM study (Boudewijn et al. 2015). In this study, current smokers and never smokers older than 18 years were recruited. Subjects were considered respiratory healthy if they had no respiratory symptoms, no history of respiratory disease and normal pulmonary function. Normal pulmonary function was defined as a post bronchodilator FEV1/FVC higher than lower limit of normal, absence of AHR to methacholine (PC20 <16mg/mL) and absence of reversibility (FEV1 %predicted to salbutamol < 10%). Subjects were excluded if they used inhaled or oral corticosteroids within the last 5 years, or during 5 years of their lives.

The study protocol was approved by the local medical ethics committee. All subjects gave their written informed consent.

**RNA extraction, Sample preparation and High-throughput sequencing**

Bronchial biopsies were taken from segmental divisions of the main bronchi. Biopsies frozen in Tissuetek at -80°C were thawed at room temperature and cut from the
blocks when they were semi-solid. Total RNA was extracted using AllPrep DNA/RNA Mini kit (Qiagen). Samples were lysed in 600 µl RLT-plus buffer using an IKA Ultra Turrax T10 Homogenizer, and RNA was purified according to the manufacturer’s instructions. RNA samples were dissolved in 30 µl RNase free water. Concentrations and quality of RNA were checked using a Nanodrop-1000 and run on a Labchip GX (Perkin Elmer).

RNA samples were further processed using the TruSeq Stranded Total RNA Sample Preparation Kit (Illumina), using an automated procedure in a Caliper Sciclone NGS Workstation (Perkin Elmer). In this procedure, all cytoplasmic and mitochondria rRNA was removed (RiboZero Gold kit). The obtained cDNA fragment libraries were loaded in pools of multiple samples unto an Illumina HiSeq2500 sequencer using default parameters for paired-end sequencing (2 × 100 bp).

**Gene expression quantification**

The trimmed fastQ files where aligned to build b37 of the human reference genome using HISAT (version 0.1.5) allowing for 2 mismatches (Kim et al. 2015). Before gene quantification SAMtools (version 1.2) was used to sort the aligned reads (Li et al.
2009). The gene level quantification was performed by HTSeq (version 0.6.1p1) using –mode=union --stranded=no and using Ensembl version 75 as gene annotation database (Anders et al. 2015).
**SUPPLEMENTAL FIGURE LEGENDS**

**SuppFig1. Induction of the CTGF promoter in NIH3T3 fibroblasts.** Luciferase activity in conditioned media was assessed after stimulation of transfected NIH3T3 fibroblasts with either TGF-β (1ng/ml, □) or control media (■). SEAP expression was driven by CMV promoter in the same construct and was used as a control for transfection efficiency. “&” denotes significance between promoterless (0) and luciferase reporter (&p<0.05, &&p<0.01, &&&p<0.001). * indicates significant difference between TGF-β stimulated and control (*p<0.05, **p<0.01, ****p<0.0001). Data represent mean±SEM (n=***).
Figure 1

210x297mm (300 x 300 DPI)
Figure 2

210x297mm (300 x 300 DPI)
Figure 3

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Figure 4

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Figure 5

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Figure 6

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