

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

Journal:	<i>Journal of Cellular and Molecular Medicine</i>
Manuscript ID	Draft
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	<p>Wang, Junfei; Qilu Hospital of Shandong University, Department of Pulmonary Medicine; Woolcock Institute of Medical Research, Respiratory Cell and Molecular Biology</p> <p>Faiz, Alen; University of Groningen, University Medical Center Groningen, Department of Pulmonary Diseases; University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Department of Pathology and Medical Biology</p> <p>Ge, Qi; Woolcock Institute of Medical Research, Respiratory Cell and Molecular Biology; The University of Sydney. Sydney, Discipline of Pharmacology</p> <p>Vermeulen, Cornelis; University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Department of Pathology and Medical Biology</p> <p>vandervelden, Joanne; The University of Melbourne, Centre for Animal Biotechnology</p> <p>Snibson, Kenneth J. ; The University of Melbourne, Centre for Animal Biotechnology</p> <p>van de Velde, Rob; Woolcock Institute of Medical Research, Respiratory Cell and Molecular Biology</p> <p>Swant, Sonia; Woolcock Institute of Medical Research, Respiratory Cell and Molecular Biology</p> <p>Xenaki, Dikaia; Woolcock Institute of Medical Research, Respiratory Cell and Molecular Biology</p> <p>Oliver, Brian; Woolcock Institute of Medical Research, Respiratory Cell and Molecular Biology; The University of Sydney, Discipline of Pharmacology</p> <p>Timens, Wim; University Medical Center Groningen, Pathology and Medical Biology</p> <p>ten Hacken, Nick; University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Department of Pathology and Medical Biology</p> <p>van den Berge, Maarten; University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Department of Pathology and Medical Biology</p> <p>James, Alan; Sir Charles Gairdner Hospital, Department of Pulmonary Physiology and Sleep Medicine</p> <p>Elliot, John; Sir Charles Gairdner Hospital, Department of Pulmonary</p>

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

	Physiology and Sleep Medicine Dong, Liang; Qilu Hospital of Shandong University, Department of Pulmonary Medicine Burgess, Janette; Woolcock Institute of Medical Research, Respiratory Cell and Molecular Biology; The University of Sydney. Sydney, Discipline of Pharmacology; University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Department of Pathology and Medical Biology Ashton, Anthony; Kolling Institute of Medical Research, University of Sydney, Division of Perinatal Research
Keywords:	CTGF, promoter regulation, airway smooth muscle, asthma

SCHOLARONE™  
Manuscripts

For Peer Review

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

Junfei Wang <sup>1,2</sup>, Alen Faiz <sup>3,4,5</sup>, Qi Ge <sup>2,6</sup>, Cornelis J. Vermeulen <sup>3,4</sup>, Joanne Van der  
Velden <sup>7</sup>, Kenneth J Snibson <sup>7</sup>, Rob van de Velde <sup>2</sup>, Sonia Sawant <sup>2</sup>, Dikaia Xenaki <sup>2</sup>,  
Brian Oliver <sup>2,8</sup>, Wim Timens <sup>4,5</sup>, Nick ten Hacken <sup>3,4</sup>, Maarten van den Berge <sup>3,4</sup>, Alan  
James <sup>9,10</sup>, John G Elliot <sup>9</sup>, \*Liang Dong <sup>1</sup>, #Janette K Burgess <sup>2,4,5,6</sup>, \*,#Anthony W  
Ashton <sup>11</sup>

<sup>1</sup>Department of Pulmonary Medicine, Qilu Hospital of Shandong University, Jinan,  
Shandong, China

<sup>2</sup>Woolcock Institute of Medical Research, University of Sydney, NSW, Australia;

<sup>3</sup>University of Groningen, University Medical Center Groningen, Department of  
Pulmonary Diseases, Groningen, The Netherlands

<sup>4</sup>University of Groningen, University Medical Center Groningen, GRIAC (Groningen  
Research Institute for Asthma and COPD), Groningen, The Netherlands

<sup>5</sup> University of Groningen, University Medical Center Groningen, Department of

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Pathology & Medical Biology, Groningen, The Netherlands

<sup>6</sup> Discipline of Pharmacology, The University of Sydney, NSW, Australia;

<sup>7</sup> Melbourne Veterinary School, Faculty of Veterinary and Agricultural Science,  
University of Melbourne, Parkville, Australia;

<sup>8</sup>. School of Life Sciences, University of Technology, Sydney, Australia

<sup>9</sup> Department of Pulmonary Physiology and Sleep Medicine, Sir Charles Gairdner  
Hospital.

<sup>10</sup> School of Medicine and Pharmacology, The University of Western Australia, WA,  
6009.

<sup>11</sup> Division of Perinatal Research, Kolling Institute of Medical Research, Sydney,  
NSW, Australia

# These authors contributed equally.

\*Corresponding Authors:

Anthony Ashton

[anthony.ashton@sydney.edu.au](mailto:anthony.ashton@sydney.edu.au)

Liang Dong

[dl5506@126.com](mailto:dl5506@126.com)

For Peer Review

**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)- $\beta$  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- $\beta$ . However, *in silico* analysis of the CTGF promoter suggested that distant transcription factor binding sites may influence CTGF promoter activation by TGF- $\beta$  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,

1  
2  
3  
4 higher CTGF gene expression in bronchial biopsies was correlated with increased  
5  
6  
7  
8 basement membrane thickness indicating that the enhanced CTGF expression in  
9  
10  
11 A-ASM may contribute to airway remodelling in asthma.  
12  
13  
14  
15  
16  
17

18 **Key words:** Asthma, airway smooth muscle, connective tissue growth factor, airway  
19  
20  
21 remodelling  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**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 way 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



1  
2  
3  
4 paracrine signalling from the ASM in may have as much to do with airway  
5  
6  
7 remodelling as their contractile state (reviewed in [14]). Ultimately, the increased  
8  
9  
10 deposition of extracellular matrix (ECM) proteins by ASM cells in asthma is key to  
11  
12  
13 the airway narrowing that takes place [12,15-18]. Often this is a response to an  
14  
15  
16 imbalance in the cytokines/growth factors present in their local milieu[3].  
17  
18

19  
20 In lung tissue, ASM cells are a potent source of connective tissue growth factor  
21  
22 (CTGF), a member of the Cysteine rich 61, CTGF, Nephroblastoma (CCN) family of  
23  
24  
25 proteins[19]. Our previous studies have shown greater CTGF expression in primary  
26  
27  
28 asthmatic (A)-ASM cells than non-asthmatic (NA)-ASM cells after TGF- $\beta$   
29  
30  
31 treatment[20-22]. CTGF controls ECM deposition and ultimately airway  
32  
33  
34 biomechanics through changes to collagen deposition which increase ECM density  
35  
36  
37 and airway stiffness[23]. Indeed, the increased stiffness of the matrix in which  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

induction by TGF- $\beta$  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- $\beta$  induction of CTGF release from  
A-ASM cells and the potential links to airway remodelling in asthma.

For Peer Review

## 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.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**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 15min. Slides were washed and incubated with serum free protein block (DAKO) at 37°C for 30min followed by incubation with rabbit anti-CTGF primary antibody (2.5µg/ml for human, 5µg/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 10min 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  $1 \times 10^4$  cells/cm<sup>2</sup> 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- $\beta$ 1 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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

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<sub>2</sub>EDTA, 1mM EGTA, 1mM NaF, 20mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2mM Na<sub>3</sub>VO<sub>4</sub>, 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 NA- and A-ASM 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<sup>-ΔΔCt</sup> method after normalization against the 18s rRNA probe (4319413E-1011052, Life Technologies).

**Western blot**

1  
2  
3  
4 Total cellular protein was extracted and separated on a 10% (w/v) SDS-PAGE gel,  
5  
6  
7  
8 and transferred to Polyvinylidene difluoride (PVDF) membrane (Merck-Millipore).  
9  
10  
11 Membranes were blocked in 5% (w/v) skim milk in Tris buffered saline (TBS, 20mM  
12  
13  
14 Tris base, 150mM NaCl, PH7.4) containing 0.05% (v/v) tween 20 for 30 min at room  
15  
16  
17  
18 temperature and incubated with goat polyclonal anti-CTGF antibody (1:1000,  
19  
20  
21 sc-14939, Santa-Cruz Biotechnology, Dallas, TX, USA) or anti-GAPDH (1:10000,  
22  
23  
24 MAB-374, Millipore) at 4°C overnight. Membranes were washed and incubated with  
25  
26  
27  
28 horseradish peroxidase conjugated secondary antibody (1:2000 for CTGF (P0160),  
29  
30  
31 1:50000 for GAPDH (P0161), DAKO). Images were captured using a Kodak Image  
32  
33  
34  
35 station 4000mm and band intensity was quantified with Carestream MI SE software.  
36  
37  
38

### 39 **CTGF promoter constructs**

40  
41  
42  
43 A Gluc-on reporter plasmid containing the full length CTGF promoter (-1500 to +200  
44  
45  
46 bp) driving expression of a secreted Gaussia luciferase was purchased from  
47  
48  
49 GeneCopoeia (Cat. HPRM25713-PG04, Rockville, MD, USA). A series of 5' deleted  
50  
51  
52  
53 constructs were generated by PCR using the primers shown in table 2. The full  
54  
55  
56  
57 length CTGF promoter was excised using the restriction enzymes Hind III and Bgl II  
58  
59  
60

(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<sup>2</sup> 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 (1ng/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- $\beta$ 1 (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**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

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 realting 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,  $\log_2$ (PC<sub>20</sub> to methacholine) and  $\log_2$ (% 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 ( $P < 0.05$ ).

For Peer Review

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**Results**

**Increased CTGF expression in asthmatic lung tissue *in vivo* and exaggerated release of CTGF from asthmatic ASM cells *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- $\beta$ 1 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- $\beta$ 1 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- $\beta$  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- $\beta$  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 phosphatase (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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

transcriptional start site. There was no enhancement of the CTGF promoter activity after TGF- $\beta$  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- $\beta$  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- $\beta$ , 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- $\beta$  [25,27,33,34]. Indeed, treatment of transfected NIH 3T3 fibroblasts with TGF $\beta$  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- $\beta$  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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

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- $\beta$ . 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- $\beta$  induced CTGF mRNA in A-ASM cells was more stable than NA-ASM cells providing a possible explanation for the differential expression of TGF- $\beta$  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 ( $\beta \pm \text{SE}$  0.472 $\pm$ 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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

with lower FEV1 % predicted, more severe bronchial hyperresponsiveness or higher %  
  
eosinophil levels in sputum (Fig 6 C-E).

For Peer Review

## 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- $\beta$  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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

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- $\beta$  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 TGF $\beta$

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 K<sup>27</sup> 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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

smooth muscle cells [37]. However, our previous data conclusively showed this pathway is not involved with CTGF regulation by TGF- $\beta$  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- $\beta$  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- $\beta$  element, Ets1, NF-1-like sequence, TIE-like site amongst others. Some of these factors have previously been implicated in TGF- $\beta$  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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

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

This study was supported by National Health and Medical Research Council of the Australian Government (NHMRC grant #1061712 [JKB, AWA] #454437 [KS], Fellowship #1032695 [JKB]), a research grant from GlaxoSmithKline and the University of Groningen and European Union Rosalind Franklin Fellowship (JKB).

## Conflicts of interests

The authors confirm there are no conflicts of interests in this study.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

REFERENCES.

1. **Global Asthma Network.** 2016 GINA Report, Global strategy for Asthma Management and Prevention. 2016.
2. **Broide DH.** Immunologic and inflammatory mechanisms that drive asthma progression to remodeling. *J Allergy Clin Immunol.* 2008; 121: 560-70; quiz 71-2.
3. **Burgess JK.** The extracellular matrix: Friend or foe in airway disease? *Minerva Pneumologica.* 2010; 49: 219-36.
4. **James AL, Elliot JG, Jones RL, et al.** Airway smooth muscle hypertrophy and hyperplasia in asthma. *Am J Respir Crit Care Med.* 2012; 185: 1058-64.
5. **Salvato G.** Quantitative and morphological analysis of the vascular bed in bronchial biopsy specimens from asthmatic and non-asthmatic subjects. *Thorax.* 2001; 56: 902-6.
6. **Chetta A, Foresi A, Del Donno M, et al.** Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest.* 1997; 111: 852-7.
7. **Niimi A, Matsumoto H, Takemura M, et al.** Relationship of airway wall

- thickness to airway sensitivity and airway reactivity in asthma. *Am J Respir Crit Care Med.* 2003; 168: 983-8.
8. **Grzela K, Litwiniuk M, Krejner A, et al.** Increased angiogenic factors in exhaled breath condensate of children with severe asthma - New markers of disease progression? *Respir Med.* 2016; 118: 119-21.
9. **Suzaki Y, Hamada K, Sho M, et al.** A potent antiangiogenic factor, endostatin prevents the development of asthma in a murine model. *J Allergy Clin Immunol.* 2005; 116: 1220-7.
10. **Burgess JK, Boustany S, Moir LM, et al.** Reduction of tumstatin in asthmatic airways contributes to angiogenesis, inflammation, and hyperresponsiveness. *Am J Respir Crit Care Med.* 2010; 181: 106-15.
11. **Saadeh C, Saadeh C.** Asthma remission in a patient with rheumatoid arthritis while on antiangiogenesis therapy during a rheumatoid arthritis trial demonstrated by forced oscillation and spirometry. *J Asthma.* 2007; 44: 281-3.
12. **Manuyakorn W, Howarth PH, Holgate ST.** Airway remodelling in asthma and novel therapy. *Asian Pac J Allergy Immunol.* 2013; 31: 3-10.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

13. **Elliot JG, Jones RL, Abramson MJ, et al.** Distribution of airway smooth muscle remodelling in asthma: relation to airway inflammation. *Respirology*. 2015; 20: 66-72.

14. **Lauzon AM, Martin JG.** Airway hyperresponsiveness; smooth muscle as the principal actor. *F1000Res*. 2016; 5.

15. **Johnson PR.** Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol*. 2001; 28: 233-6.

16. **Oliver BG, Black JL.** Airway Smooth Muscle and Asthma. *Allergology International*. 2006; 55: 215-23.

17. **Doeing DC, Solway J.** Airway smooth muscle in the pathophysiology and treatment of asthma. *J Appl Physiol (1985)*. 2013; 114: 834-43.

18. **Wright DB, Trian T, Siddiqui S, et al.** Functional phenotype of airway myocytes from asthmatic airways. *Pulm Pharmacol Ther*. 2013; 26: 95-104.

19. **Wells JE, Howlett M, Cole CH, et al.** Deregulated expression of connective tissue growth factor (CTGF/CCN2) is linked to poor outcome in human cancer. *Int J Cancer*. 2015; 137: 504-11.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60
20. **Burgess JK, Johnson PR, Ge Q, et al.** Expression of connective tissue growth factor in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med.* 2003; 167: 71-7.
21. **Xie S, Sukkar MB, Issa R, et al.** Regulation of TGF- $\beta$ 1-induced connective tissue growth factor expression in airway smooth muscle cells. *American Journal of Physiology Lung Cellular & Molecular Physiology.* 2005; 288: L68-L76.
22. **Johnson PR, Burgess JK, Ge Q, et al.** Connective tissue growth factor induces extracellular matrix in asthmatic airway smooth muscle. *Am J Respir Crit Care Med.* 2006; 173: 32-41.
23. **Holgate ST.** Pathogenesis of asthma. *Clin Exp Allergy.* 2008; 38: 872-97.
24. **Karger A, Fitzner B, Brock P, et al.** Molecular insights into connective tissue growth factor action in rat pancreatic stellate cells. *Cell Signal.* 2008; 20: 1865-72.
25. **Ohyama Y, Tanaka T, Shimizu T, et al.** Runx2/Smad3 complex negatively regulates TGF- $\beta$ -induced connective tissue growth factor gene expression

in vascular smooth muscle cells. *J Atheroscler Thromb.* 2012; 19: 23-35.

26. **Tran CM, Markova D, Smith HE, et al.** Regulation of CCN2/connective tissue growth factor expression in the nucleus pulposus of the intervertebral disc: role of Smad and activator protein 1 signaling. *Arthritis Rheum.* 2010; 62: 1983-92.

27. **Grotendorst GR, Okochi H, Hayashi N.** A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. *Cell Growth Differ.* 1996; 7: 469-80.

28. **Johnson PR, Roth M, Tamm M, et al.** Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med.* 2001; 164: 474-7.

29. **Hawker KM, Johnson PR, Hughes JM, et al.** Interleukin-4 inhibits mitogen-induced proliferation of human airway smooth muscle cells in culture. *Am J Physiol.* 1998; 275: L469-77.

30. **Van der Velden J, Barker D, Barcham G, et al.** Increased vascular density is a persistent feature of airway remodeling in a sheep model of chronic asthma.

- Exp Lung Res.* 2012; 38: 307-15.
31. **Broekema M, Volbeda F, Timens W, et al.** Airway eosinophilia in remission and progression of asthma: Accumulation with a fast decline of FEV1. . *Respiratory Medicine.* 2010; 104: 1254-62.
32. **Broekema M, Timens W, Vonk JM, et al.** Persisting remodeling and less airway wall eosinophil activation in complete remission of asthma. *Am J Respir Crit Care Med.* 2011; 183: 310-6.
33. **Leask A, Holmes A, Black CM, et al.** Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. *J Biol Chem.* 2003; 278: 13008-15.
34. **Holmes A, Abraham DJ, Sa S, et al.** CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J Biol Chem.* 2001; 276: 10594-601.
35. **Singh SR, Billington CK, Sayers I, et al.** Can lineage-specific markers be identified to characterize mesenchyme-derived cell populations in the human airways? *Am J Physiol Lung Cell Mol Physiol.* 2010; 299: L169-83.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

36. **Kiwanuka E, Junker JP, Eriksson E.** Transforming growth factor beta1 regulates the expression of CCN2 in human keratinocytes via Smad-ERK signalling. *Int Wound J.* 2017.

37. **Chowdhury I, Chaqour B.** Regulation of connective tissue growth factor (CTGF/CCN2) gene transcription and mRNA stability in smooth muscle cells. Involvement of RhoA GTPase and p38 MAP kinase and sensitivity to actin dynamics. *Eur J Biochem.* 2004; 271: 4436-50.

38. **Lin SC, Chou HC, Chiang BL, et al.** CTGF upregulation correlates with MMP-9 level in airway remodeling in a murine model of asthma. *Arch Med Sci.* 2017; 13: 670-6.

39. **Piao HM, Yamauchi K, Pan L-H, et al.** Increased Levels of CTGF mRNA Expression in a Murine Model of Allergic Airway Inflammation. *Allergology International.* 2005; 54: 107-15.

40. **Brook PO, Perry MM, Adcock IM, et al.** Epigenome-modifying tools in asthma. *Epigenomics.* 2015; 7: 1017-32.

41. **Comer BS, Ba M, Singer CA, et al.** Epigenetic targets for novel therapies of



- lung diseases. *Pharmacol Ther.* 2015; 147: 91-110.
42. **Geisinger MT, Astaiza R, Butler T, et al.** Ets-1 is essential for connective tissue growth factor (CTGF/CCN2) induction by TGF-beta1 in osteoblasts. *PLoS One.* 2012; 7: e35258.
43. **Shi L, Dong N, Fang X, et al.** Regulatory mechanisms of TGF- $\beta$ 1-induced fibrogenesis of human alveolar epithelial cells. *J Cell Mol Med.* 2016; 20: 2183-93.
44. **Xu X, Wan X, Geng J, et al.** Rapamycin regulates connective tissue growth factor expression of lung epithelial cells via phosphoinositide 3-kinase. *Exp Biol Med* 2013; 238: 1082-94.
45. **Yang J, Velikoff M, Canalis E, et al.** Activated alveolar epithelial cells initiate fibrosis through autocrine and paracrine secretion of connective tissue growth factor. *Am J Physiol Lung Cell Mol Physiol.* 2014; 306: L786-96.
46. **Postma DS, Timens W.** Remodeling in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc.* 2006; 3: 434-9.
47. **James AL, Wenzel S.** Clinical relevance of airway remodelling in airway

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

diseases. *Eur Respir J.* 2007; 30: 134-55.

48. **Jeffery PK.** Remodeling and inflammation of bronchi in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc.* 2004; 1: 176-83.

49. **Malmstrom K, Pelkonen AS, Malmberg LP, et al.** Lung function, airway remodelling and inflammation in symptomatic infants: outcome at 3 years. *Thorax.* 2011; 66: 157-62.

50. **Kim ES, Kim SH, Kim KW, et al.** Basement membrane thickening and clinical features of children with asthma. *Allergy.* 2007; 62: 635-40.

51. **Shkumatov A, Thompson M, Choi KM, et al.** Matrix stiffness-modulated proliferation and secretory function of the airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol.* 2015; 308: L1125-35.

52. **Lee PF, Bai Y, Smith RL, et al.** Angiogenic responses are enhanced in mechanically and microscopically characterized, microbial transglutaminase crosslinked collagen matrices with increased stiffness. *Acta Biomater.* 2013; 9: 7178-90.

53. **Bordeleau F, Mason BN, Lollis EM, et al.** Matrix stiffening promotes a

tumor vasculature phenotype. *Proc Natl Acad Sci U S A*. 2017; 114: 492-7.

54. **Jorgenson AJ, Choi KM, Sicard D, et al.** TAZ activation drives fibroblast spheroid growth, expression of profibrotic paracrine signals, and context-dependent ECM gene expression. *Am J Physiol Cell Physiol*. 2017; 312: C277-c85.
55. **Raghunathan VK, Morgan JT, Dreier B, et al.** Role of substratum stiffness in modulating genes associated with extracellular matrix and mechanotransducers YAP and TAZ. *Invest Ophthalmol Vis Sci*. 2013; 54: 378-86.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Table 1. Details of asthmatic and non-asthmatic donors

Patient	Age	Sex	Diagnosis	Sample type
1	67	male	healthy	transplant
2	47	male	healthy	transplant
3	22	female	healthy	biopsy
4	64	male	ILD	transplant
5	76	male	pulmonary cryptococcosis	resection
6	61	female	pulmonary hypertension	transplant
7	41	female	adenocarcinoma	resection
8	29	male	healthy	biopsy
9	16	male	healthy	transplant
10	65	male	cancer	resection
11	27	female	asthmatic	biopsy
12	54	male	asthmatic	biopsy
13	23	male	asthmatic	biopsy
14	58	male	asthmatic	biopsy
15	21	male	asthmatic	biopsy
16	38	male	asthmatic	biopsy
17	61	female	asthmatic	biopsy
18	85	male	asthmatic	biopsy
19	51	male	asthmatic	biopsy
20	63	male	asthmatic	biopsy
21	59	female	asthmatic	biopsy
22	64	male	asthmatic	biopsy
23	NA	NA	healthy	transplant
24	52	male	cancer	resection
25	22	male	healthy	biopsy
26	60	female	cancer	resection
27	27	male	asthmatic	biopsy
28	33	male	asthmatic	biopsy
29	50	male	asthmatic	biopsy

Abbreviations: ILD Interstitial lung disease, NA not available

For Peer Review

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Table 2. primer sequence for CTGF 5’ deletion mutant constructs

Primer Name	Primer sequence (5’ to 3’)
-100 forward	ATC GAG ATC TAA CAA CAT AGA TTC CAA ATG A
-400 forward	ATC GAG ATC TGT AAT GGA ATC AGA CTT CTT A
-700 forward	ATC GAG ATC TAA AAC TAA GCA AGA GTT TTG G
-1000 forward	ATC GAG ATC TCT TCA GCT ACC TAC TTC CTA A
-1300 forward	ATC GAG ATC TAT GCG AGG AAT GTC CCT GTT T
Reverse primer	ATC CGA GCT CGG TAC CAA GCT T

**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- $\beta$  (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-PCR 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- $\beta$ . #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- $\beta$  (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 reporter (&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- $\beta$  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 promotor construct used in this project. **C.** Validated SMAD and TGF- $\beta$  transcription factor binding sites across difference 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- $\beta$  (1 ng/mL) with actinomycin D (10  $\mu$ g/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.  $\beta$ ; correlation co-efficient.  $p$ ; 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 FEV(1) 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 ( $PC_{20} \leq 32$  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 ( $\beta$ -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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

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 ( $2 \times 100$  bp).

#### Gene expression quantification

The trimmed fastQ files were 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.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

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).

For Peer Review

## 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- $\beta$  (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- $\beta$  stimulated and control (\*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001). Data represent mean $\pm$ SEM (n=\*\*\*).

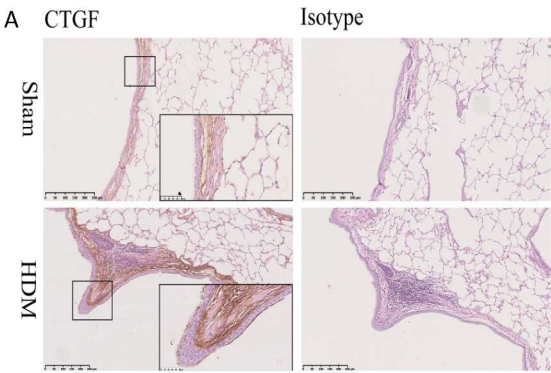


Figure 1

Figure 1

210x297mm (300 x 300 DPI)



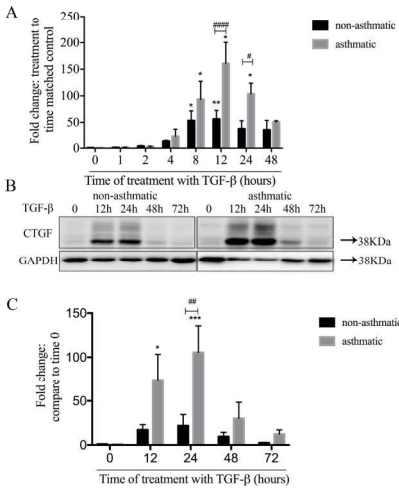


Figure 2

Figure 2

210x297mm (300 x 300 DPI)

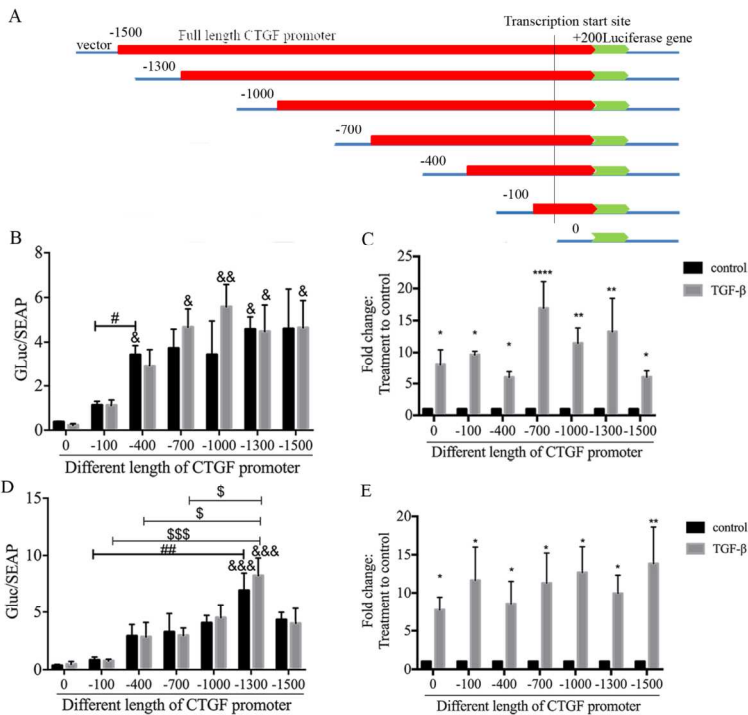


Figure 3

Figure 3

210x297mm (300 x 300 DPI)

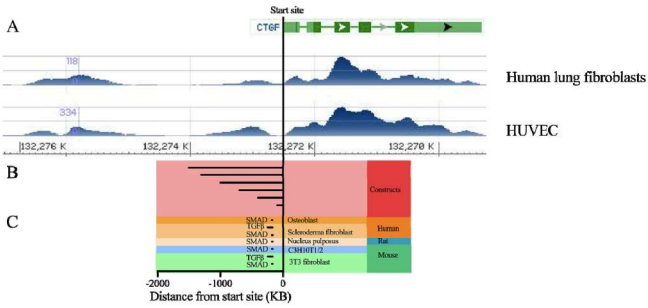


Figure 4

Figure 4

210x297mm (300 x 300 DPI)

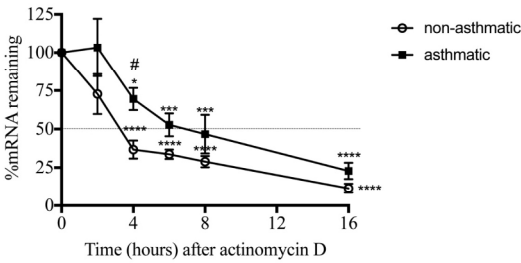


Figure 5

Figure 5

210x297mm (300 x 300 DPI)

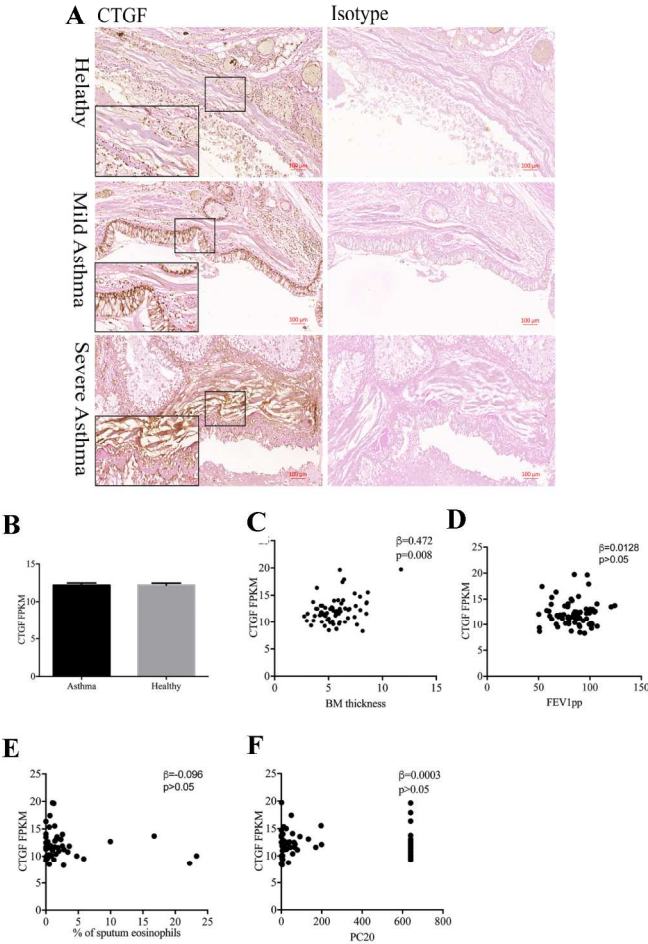


Figure 6

Figure 6

210x297mm (300 x 300 DPI)