IN VITRO INVESTIGATIONS OF TRANSFORMING GROWTH FACTOR-β2 INDUCED AIRWAY WALL REMODELLING

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Gaynor Anne Campbell

School of Medicine
Division of Translational Medicine

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Abbreviations

AHR	Airway hyperresponsiveness
ALI	Air-liquid interface
ANOVA	Analysis of variance
ASM	Airway smooth muscle
ATP5B	ATP synthase
AWR	Airway wall remodelling
BAL	Bronchoalveolar lavage
BEBM	Bronchial epithelial basal medium
BEDM	Bronchial epithelial differentiation medium
BSA	Bovine serum albumin
CM	Conditioned medium
COPD	Chronic obstructive pulmonary disease
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBEC	Human bronchial epithelial cells
HDM	House dust mite
IL	Interleukin
LABA	Long-acting β2-agonist
LAP	Latency associated peptide
LLC	Large latent complex
LPS	Lipopolysaccharide
LTBP	Latent TGF-β binding protein
MMP	Matrix metalloproteinase
NHBEC	Normal human bronchial epithelial cells
NHLF	Normal human lung fibroblasts
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid-Schiff's
PBS	Phosphate buffered saline

q-PCR	Quantitative polymerase chain reaction
RBM	Reticular basement membrane
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
SABA	Short acting β2-agonist
SLC	Small latent complex
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TIMP	Tissue inhibitor of metalloprotease
TLR	Toll-like receptor
TβR	TGF-β receptor
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of matrix metalloproteinase
TnC	Tenascin C
TNF-α	Tumour necrosis factor-α
vWF	von Willebrand factor

Abstract

Airway wall remodelling contributes to decreased lung function in asthma. Key features of the remodelling process are thickening of the reticular basement membrane, differentiation of fibroblast-like cells with contractile properties termed myofibroblasts and sub-epithelial deposition of extracellular matrix.The pro-fibrogenic cytokine transforming growth factor-β2 (TGF-β2) is purported to drive remodelling responses. TGF-β2 may be upregulated in asthmatic epithelium, and is secreted by bronchial epithelial cells following injury.

In this study significant increases in reticular basement membrane thickening and myofibroblast differentiation were identified by histology and immunohistochemistry of mild asthmatic and healthy human bronchial biopsy tissue, although no significant differences in TGF-β2 expression were identified.

It was hypothesised that the proteolytic action of house dust mite (HDM) allergens would lead to increased activation of latent TGF-β2 secreted by bronchial epithelial cells. A transformed cell line, 16HBE14o-, did not show increased activation or expression following HDM extract challenge, however TGF-β2 activation and expression was increased following exposure of primary human bronchial epithelial cells to a HDM extract.

Myofibroblast differentiation and matrix deposition by healthy and mild asthmatic- derived primary bronchial fibroblasts were assessed by α-smooth muscle actin expression and soluble collagen production, following challenge with exogenous TGF-β2. Results presented here show asthmatic bronchial fibroblasts are more sensitive to the myofibroblast priming effects of TGF-β2. Bronchial epithelial cell conditioned media challenge of healthy fibroblasts led to greater increases in matrix deposition and myofibroblast differentiation than was attributable to TGF-β2, with greatest increases seen following asthmatic epithelial cell conditioned media exposure. Responses were greater than suggested by the epithelial TGF-β2 levels, so it is suggested that additional soluble mediators play a part in airway wall remodelling responses. Further work is required to identify the soluble mediators secreted by bronchial epithelial cells that control the responses of the underlying fibroblasts.

Gaynor Anne Campbell
The University of Manchester
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Declaration

No portion of the work referred to has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

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Dedication

For Dad Campbell, who taught me how to eat the whale…

Chapter 1: Introduction

1.1 Asthma

Asthma is a disorder of the lung characterised by inflammation, over-production of mucus and bronchial smooth muscle contraction. It was previously thought that asthma was a condition of episodic broncho-constriction with a remitting pathology, however it is clear that both acute and chronic inflammatory responses occur, alongside extensive structural changes in the conducting airway wall. The structural changes are collectively termed airway wall remodelling (AWR), and evidence suggests AWR may precede the development, and thereby diagnosis, of asthma (Bush, 2008; Cohn *et al.*, 2004).

Investigations of disease heterogeneity show asthma is not a single entity differing in severity, but an overlapping range of phenotypes with divergent measures of inflammation, allergic stimulation, cell-type involvement and airway remodelling. These features contribute to airway hyperresponsiveness (AHR) and decreases in lung function (Holgate and Polosa, 2006).

Pharmacological intervention focuses on relief of asthma symptoms and reduction of underlying inflammation. In recent years new treatments have been developed directed at reducing or blockading known originators of acute asthmatic episodes such as IgE and IL-5. Treatments do not target airway wall remodelling, and much remains unknown regarding how remodelling phenomena are initiated and maintained, and their contribution to disease morbidity.

1.1.1 Asthma prevalence and symptoms

Asthma affects 300 million people worldwide, including 5.4 million sufferers in the UK requiring NHS treatment costing an estimated £996 million in 2009 (AsthmaUK, 2010). Asthma can be defined as widespread variable airflow obstruction that is often reversible (GINA, 2010). Asthma symptoms include,

but are not limited to, cough, wheeze and shortness of breath, which can be both acute and chronic. Often symptoms occur with diurnal variation, and severity of symptoms is increased in the early hours of the morning. Airway or bronchial hyper-responsiveness (AHR/BHR), whereby an abnormal degree of airway narrowing occurs following inhaled exposure to a bronchoconstrictor, is an important indicator of asthma. Pathological hallmarks of asthma are limited to regions of the conducting airways: the bronchi and bronchioles.

1.1.2 Asthma diagnosis and treatment

A diagnosis of asthma is made by a clinician following consideration of numerous factors including patient history, symptoms and triggers, in conjunction with pulmonary function tests. Measurements of forced expiratory volume in one second (FEV_1) and peak expiratory flow (PEF), typically show decreased values in comparison to healthy individuals and are characteristically variable (BTS, 2008). Variability can indicate poor disease management or difficult disease. Analysis of severe asthma patient characteristics has identified four subclinical phenotypes of asthma: eosinophilic inflammation positive and eosinophilic inflammation negative, subdivided into Th2-cytokine high and Th2-cytokine low (Bradding and Green, 2009).

Disease management can require a number of treatments. For the majority of asthmatics the condition is well controlled by a combination of β_2-adrenoreceptor antagonists and inhaled glucocorticoids. However, only 70% of asthmatics respond to glucocorticoids (Mjaanes *et al.*, 2006). This variation in response is possibly due to differences in disease phenotype and genetic background. A brief description of commonly used treatments follows.

1.1.2.1 β_2-adrenoreceptor agonists

Delivered by inhalation and commonly referred to as β_2-agonists, these bind β_2-adrenergic receptors (β_2AR) on airway smooth muscle cells, activating downstream signalling pathways that result in relaxation of the muscle

thereby reducing bronchoconstriction. β2-agonists can be broadly split into long-acting (LABA- up to 12 hours) and short-acting (SABA- 1-2 hours). LABAs or SABAs can be used alone as "rescue therapy" during asthma exacerbations. The *B_2AR* gene is encoded on chromosomal region 5q31-32 in the IL-4 gene cluster, and can be downregulated by repeated exposure to β2-agonists (Bhatnagar *et al.*, 2006). LABAs plus inhaled glucocorticoids act synergistically in relief of asthma symtoms.

1.1.2.2 Glucocorticoids

Inhaled glucocorticoids (GC), also referred to as glucocorticosteroids, downregulate inflammation in the asthmatic airway. In severe asthma, a course of oral steroids may also be used. Glucocorticoids enter cells by diffusion across the cell membrane and bind glucocorticoid receptors (GR) in the cytoplasm. The receptor is activated and released from a chaperone protein, allowing the GC-GR complex to enter the nucleus and bind to glucocorticoid response elements (GRE) and co-activator complexes, exerting a direct affect on inflammatory gene transcription (Adcock and Caramori, 2001).

Glucocorticoids downregulate pro-inflammatory genes through inhibition of MAPK signalling *via* induction of MAPK phosphatase-1 (MKP1), and inhibition of NFκB (Barnes and Karin, 1997). This switches off transcription of inflammatory genes, and upregulated transcription of anti-inflammatory cytokines such as IL-10 may also occur (Adcock *et al.*, 2004). GC may also prevent desensitization to β_2-agonists by blocking the downregulation of β_2AR caused by repeated exposure to β_2-agonists (Bhatnagar *et al.*, 2006).

1.1.2.3 Leukotriene receptor antagonists

Leukotrienes are pro-inflammatory mediators generated from arachidonic acid and released by immune cells including eosinophils and neutrophils, and by mast cells at degranulation. Leukotriene receptor antagonists (LTRA) act by sequestering leukotriene receptors preventing pro-inflammatory leukotrienes

including LTC4 and LTD4 from binding. The smooth muscle contraction and vascular permeability effected by leukotrienes is thus downregulated. LTRAs are now often prescribed in mild-moderate asthma for their dual bronchodilatory and anti-inflammatory properties.

1.1.2.4 Monoclonal antibody therapy

The reductionist approach to new asthma therapies has increasingly led to the targeting of individual novel components of the asthmatic disease response. A number of monoclonal antibodies have been developed for therapeutic use following this approach.

Omalizumab is a murine anti-human IgE monoclonal antibody that binds circulating IgE at the Fc region, blocking binding to IgE receptors including high-affinity FcεRI. This inhibits binding of IgE to receptors on basophils and dendritic cells, and the binding and subsequent cross-linking of IgE on mast cells (Clark *et al.*, 2006; D'Amato, 2006). Through this sequestration of free circulating IgE the allergic response can be attenuated. This treatment is of use in those individuals with persistent allergic asthma that is poorly controlled by inhaled glucocorticoids, and is not considered suitable for all asthmatics. Other monoclonal antibodies have been trialled including mepolizumab, an IL-5 blocking antibody. Although mepolizumab reduced numbers of circulating eosinophils, it had limited success in reducing tissue eosinophils, and had no effect on asthmatic lung response to inhaled allergen challenge (Büttner *et al.*, 2003).

Many trials have produced disappointing results in reduction of asthma symptoms and/or number of exacerbations. Current research indicates these therapies may show greater efficacy when patients are selected by disease phenotype beforehand, to identify those whose disease is at least partially mediated by the treatment target.

The therapies described were developed either to provide symptomatic relief or resolve inflammation in asthma, and have only limited ability to alter the

remodelling response. Long-term treatment with GC shows a reduction in remodelling markers in some studies (Hoshino *et al.*, 1998c), however there remains an opportunity to identify new targets for manipulation that will alter the AWR response.

Airway wall remodelling may occur prior to the development of diagnosable asthma, and it is not apparent whether this is a purely protective response or contributes to progression and maintenance of the disease state. Alteration of AWR could potentially have unintended downstream effects.

Finally, an understanding of how the asthmatic disease is initiated could aid in identification of targets that are not limited to relief of symptoms, but instead aim to "cure" asthmatic disease.

1.1.3 Asthma initiation

1.1.3.1 Genetics and Environment

Given the heterogeneity of asthma as a disease, there is no consensus on what precludes disease development. It is generally agreed that a combination of genetic and environmental factors act to influence the development of asthma.

In a review of asthma genetics, Weiss *et al.* excluded genetic association studies (where a specific genetic variant is tested for in affected individuals versus controls) with less than 300 total subjects as, in a complex trait such as asthma, smaller sample sizes are statistically underpowered. The studies that remained identified 43 candidate genes, on more than 20 different chromosomes, that could be associated with asthma. A number of genes are also associated with atopy- a known risk factor for asthma (Weiss *et al.*, 2009).

Chromosome	Gene
2q	*CTLA4* *IL1RN*
5q	*IL4* *IL13* *CD14* *IL9*
12q	*IFN-γ* *STAT6*
20p	*ADAM33*

Table 1.1 Chromosomal regions of interest and genes associated with asthma (Blumenthal, 2005; Van Eerdewegh *et al.*, 2002; Wills-Karp and Ewart, 2004).

Around 500 genetic loci in total have been proposed as candidate "asthmatic" genes. As asthma is heterogenous and complex so is the genetic background and heritability of the disease. Phenotypic variation is however currently unlinked to the genome. From the large number of candidate genes identified and the low significance of these gene polymorphisms individually, it is apparent that asthma is the product of accumulations of genetic polymorphisms. It is possible these alone do not induce asthma *per se*, and that gene interactions with environmental stimuli are required to initiate and drive disease development. Studies on the environmental effects on asthma development are often contradictory, however of interest to this project was the finding that house dust mite (HDM) exposure in infancy is a risk factor for subsequent asthma development (Huss *et al.*, 2001).

Understanding of allergic sensitisation in the lung and the factors leading to subsequent development of asthmatic disease is incomplete, and insufficient to identify a definite asthmatic prior to disease manifestation. Characterisation of cell behaviour in the time period between sensitisation and overt disease

could produce information allowing the targeting of asthma initiators in a window of opportunity, thereby precluding further disease development. Until that possibility is realised it is important to extend understanding of established disease mechanisms to subsequently improve treatment regimens for established asthmatic disease.

1.1.3.2 Allergic sensitisation

Although the mechanisms of asthmatic disease initiation are unclear, initiation of an allergic asthmatic response is not. Asthma is a Th2 type disease, typified by the production of cytokines interleukin-4 (IL-4), IL-5 and IL-13, with immunoglobulin E (IgE) a major initiator of pathophysiology (Barnes *et al.*, 2002). Sensitisation to allergen occurs in many asthmatics, with allergen exposure often leading to a symptomatic response (Craig, 2010; Tovey *et al.*, 1981). Briefly, sensitization occurs when dendritic cells in the mucosal lining of the lung take up inhaled antigen and migrate to the lymph nodes, where they function as professional antigen-presenting cells with costimulatory capacity. Antigen presented to naïve T cells as immunogenic peptides, alongside costimulation (*via* CD86) drives CD4+ T cells down the Th2 lineage pathway in asthmatics. When Th2 cells in circulation meet specific antigen, cytokines such as IL-4, IL-5 and IL-13 (encoded on chromosomal region 5q31-32, also known as the IL-4 gene cluster) are secreted. Genetic studies have shown mutations in this region to predispose to the development of asthma. IL-13 induces goblet cell differentiation in the airway epithelium and promotes AHR (Walter *et al.*, 2001; Wills-Karp *et al.*, 1998). IL-4 promotes B cell production of IgE and IL-5 promotes eosinophil production and recruitment from the bone marrow. Antigen specific IgE produced by B cells binds FcεRI receptors on the surface of mast cells. Mast cells are normally resident in the respiratory tract; greater numbers may accumulate in allergic asthma (Brightling *et al.*, 2002). Cross-linking of FcεRI by IgE on mast cells initiates the acute phase asthmatic response (Section 1.1.4.1).

1.1.4 Asthmatic airway inflammation

Asthmatic exacerbation stimuli are broadly definable as allergenic, environmental or mechanical. In the asthmatic airway, the response to these stimuli is abnormal and termed AHR. Approximately 90% of asthmatics may suffer acute asthmatic episodes triggered by allergen, the remainder being induced by environmental or mechanical stimuli. Exercise induced asthma, where symptoms are only present following physical exertion, occurs even in very fit individuals, whilst those with no history of atopy (the predisposition to produce IgE upon low dose protein antigen challenge) can experience wheeze, cough and tightening of the chest in response to environmental triggers such as sudden air temperature change (moving from warm to cold or cold to warm areas) and occupational exposure to chemical agents.

1.1.4.1 The acute phase response

In allergic asthma inhalation of an offending antigen cross-links specific IgE bound to FcεRI receptors on mast cells resident in the respiratory tract. IgE cross-linking signals the release of potent granule contents from the mast cell, initiating the acute phase response. Histamine and prostaglandins released cause smooth muscle contraction and rapidly increase vascular permeability leading to bronchoconstriction.

The immediate smooth muscle contraction and increased mucus production result in the symptoms experienced by sufferers. Increased vascular permeability leads to constriction of the airways by increasing tissue volume. It also contributes to the chronicity of inflammation by allowing influx of a number of inflammatory mediator cells to the area of injury, namely eosinophils, $CD4^+$ T_H2 cells, additional mast cells and neutrophils. These cells are activated in the cytokine milieu of the injury area and release other factors that contribute to the ongoing inflammation. T_H2 cell release of IL-3 and GM-CSF recruits and activate eosinophils, which go on to release further inflammatory mediators. A “chronic wound” scenario may result through these mechanisms, and thus drive further exacerbations and remodelling (Holgate *et al.*, 2004).

The acute phase reaction may be followed 6-8 hours later by the late-phase response.

1.1.4.2 The late phase response

This can be attributed to other mediators released by mast cells, such as LTD4 and TNF-α, and the action of eosinophils and macrophages chemotactically attracted to the lung following mast cell degranulation. Some mast cell-derived molecules and their primary effects are listed in Table 1.2. The late-phase reaction results in a second stage of smooth muscle contraction and sustained oedema; in essence a further asthmatic episode.

Effector molecule	Effect of release
Tryptase, Cathepsin G	Extracellular matrix remodelling
Leukotrienes C4, D4, E4	Smooth muscle contraction Inc. vascular permeability Inc. mucus production
IL-4, IL-13	T_H2 response promotion Mucus production
IL-3, IL-5, GM-CSF	Eosinophil activation Inc. eosinophil production
TNF-α	Endothelium activation Inc. vascular permeability Inc. cytokine production
Histamine	Smooth muscle contraction Inc. vascular permeability

Table 1.2 Effector molecules released by mast cell activation and their major effects.

LABAs are often used as "rescue therapy" during acute asthma attacks, and inhaled GC treatment can dampen underlying inflammation. However these treatments do not resolve the underlying structural changes attributed to AWR processes.

1.1.5 Asthmatic airway wall remodelling

Airway wall remodelling (AWR) is a complex phenomenon with the significant effect of reduction of airway calibre, leading to fixed airflow limitation and

AHR (Aysola *et al.*, 2008; James *et al.*, 1989; Mitchell *et al.*, 1998). Thickening of the airway wall has been shown to greatly increase the degree of airway narrowing that can occur during an acute asthma attack. Airway thickness data collected from post-mortem and surgically resected lungs of normal, COPD and severe asthma patients, was used to model increasing airway resistance caused by calibre reduction using the Poiseuille equation. This equation ($\Delta P = 8l\mu V/\pi r^4$, where ΔP= change in pressure, l= length, μ= gas velocity, V= flow and r= airway radius), equates pressure to laminar flow. Using this model, it was demonstrated that small alterations in the airway radius (narrowing), led to greatly increased pressure (airway resistance) (Bossé *et al.*, 2010; Wiggs *et al.*, 1992).

It is unclear whether remodelling is (partially) driven by the inflammatory response, or whether the non-resolving inflammatory response is due in some part to AWR. With evidence to suggest remodelling occurs prior to or concurrently with the development of airway inflammation, it is possible remodelling begins as a protective mechanism (Bush, 2008). Regardless of the driving factors, AWR has consequences in bronchial architecture and behaviour. AWR should be regarded not only as structural changes, but also as alterations in the relationship between and among structural cells and other airway constituents.

Pathologically, asthma is broadly restricted to the bronchi and bronchioles, which form part of the conducting airway tree. Multiple AWR phenomena have been identified, and this review shall focus on those reported in the epithelium and underlying fibroblasts in the lamina propria.

1.1.5.1 Epithelium

The archetypal respiratory epithelium of the bronchi consists of pseudostratified, ciliated columnar epithelial cells interspersed by non-ciliated mucus-producing goblet cells and small basal cells. The asthmatic epithelium is often described as fragile, with biopsy studies suggesting increased epithelial shedding and damage in asthmatics (Demoly *et al.*, 1995). Basal cell loss of

columnar cell attachments is accompanied by increased EGFR and CD44 expression, suggesting an active process rather than an artefact of biopsy sampling and processing (Lackie *et al.*, 1997; Puddicombe *et al.*, 2000). This is supported by the finding of increased Creola bodies (clumps of epithelial cells) in BAL fluid from asthmatics compared to control subjects (Montefort *et al.*, 1992).

Goblet cell hyperplasia has been identified in asthmatic epithelium (Ordoñez *et al.*, 2001), and their contribution to mucus hyper-secretion is a factor in mucus plugging of the airway seen in fatal asthma (Kuyper *et al.*, 2003). A number of other features have been identified in asthmatic epithelium, including increased PAR-2 expression (Knight *et al.*, 2001), increased STAT6 expression (Mullings *et al.*, 2001) and increased Endothelin (Vittori *et al.*, 1992).

Of relevance to this project are the findings of Chu *et al.* who demonstrated increased TGF-β2 expression in asthmatic biopsy tissue compared to healthy (Chu *et al.*, 2004). Segmental allergen challenge led to increased TGF-β2 in BAL, although in this study basal levels of TGF-β2 were higher in healthy individuals (Batra *et al.*, 2004). This is however indicative of a role for TGF-β2 in maintaining normal lung homeostasis. There is a correlation between TGF-β positive epithelium and reticular basement thickness (Vignola *et al.*, 1997) Furthermore, the identification of increased phospho-Smad-2 signalling and decreased Smad-7 expression in asthmatic epithelium suggests a dysregulation of TGF-β responsiveness (Nakao *et al.*, 2002; Sagara *et al.*, 2002). Decreased Smad-7 expression was inversely correlated to reticular basement thickness and AHR.

1.1.5.2 Reticular basement membrane

Anchoring the epithelium to the underlying connective tissue is the reticular basement membrane (RBM). This layer can become thickened in asthma, due to increased deposition of ECM molecules including the fibrillar collagens I, III and V (Wilson and Li, 1997). Increased RBM thickness has been positively

correlated with AHR (Hoshino *et al.*, 1998a) and with frequency of asthma attacks (Evans *et al.*, 1999). Treatment with inhaled glucocorticoids can reduce RBM thickness (Hoshino *et al.*, 1998c)

Increased deposition of Tenascin-C (Tn-C), collagen III and lumican have been described at baseline in asthmatics, that could be subsequently decreased by anti-IL-5 treatment or inhaled GC (Flood-Page *et al.*, 2003; Laitinen *et al.*, 1997). An allergen challenge study of asthmatics identified a significant increase in Tn-C deposition in the RBM at 24h post-challenge (Phipps *et al.*, 2004b). With regard to this thesis, Thompson *et al.* reported induction of Tn-C expression by bronchial epithelial cells following challenge with exogenous TGF-β2. Expression was also increased following a model "scrape" injury (Thompson *et al.*, 2006).

1.1.5.3 (Myo)fibroblasts

Fibroblasts are the predominant cell type in the lamina propria, responsible for the majority of extracellular matrix production and of the lamina propria itself.

The lamina propria appears ultrastructurally as loose connective tissue composed of collagens and proteoglycans, with longitudinal bundles of elastic fibres of elastin and fibrillin, and nerves. The major components of the extracellular matrix are described in Table 1.3.

Component	Function
Fibrillar collagens (Types I, II, III, V)	Resist tensile forces, mix with elastin to limit stretch and stop tearing of tissue.
Hyaluronan	–ve charge attracts +ve ions which draws water into matrix ∴ can withstand compressive forces
Proteoglycans	Withstands compressive forces. Bind FGF and other factors
Tenascin	Guides cell migration through integrin binding.
Fibronectin	Aids cell attachment to ECM, and cell migration through matrix
Elastin	Allow stretch of tissue.
Laminin	Cell adhesion at basal lamina

Table 1.3 Extracellular matrix components of the bronchi.

Differentiation of fibroblasts to myofibroblasts occurs naturally during the tissue repair response, although persistence is associated with fibrosis (Brewster *et al.*, 1990). Myofibroblasts gain a contractile function due to the accumulation of stress fibres and smooth muscle related proteins; α-smooth muscle actin (α-SMA) is used as a marker of myofibroblast differentiation (Hinz *et al.*, 2007a), and subepithelial collagen deposition in AWR is thought due to greater numbers of myofibroblasts in the lamina propria (Zhang *et al.*, 1994). Increased myofibroblast number correlates with RBM thickness (Gizycki *et al.*, 1997) and differentiation follows TGF-β stimulation (Hu *et al.*, 2003).

Asthmatic fibroblasts challenged with TGF-β2 upregulated smooth muscle related mRNA transcripts to a greater degree than healthy and were maximally primed at 10-fold lower concentration of TGF-β2 than healthy. This suggests that fibroblasts from asthmatic subjects may be more sensitive to TGF-β2 than healthy cells (Wicks *et al.*, 2006).

1.2 Transforming Growth Factor-β

Transforming growth factor- β (TGF-β) is a pro-fibrogenic cytokine implicated in the remodelling and repair processes in the airways. TGF-β is part of the TGF-β superfamily of cytokines, of which there are around 40 mammalian members including bone morphogenetic proteins (BMP), activins and macrophage inhibitory cytokine-1 (MIC-1). TGF-β is considered a vital element in maintaining tissue homeostasis in the healthy lung, with effects on matrix production and turnover key to this process. Complex interlinking pathways of cell activation, proliferation, downregulation and mediator release are affected and regulated by active TGF-β, and disseminating the cytokine's effects on individual cell types has provided data on the possible outcomes of activation *in-vivo*. Three isoforms of TGF-β have been identified in humans: TGF-β1, TGF-β2 and TGF-β3. Of these TGF-β1 is the best characterised. The individual isoforms are encoded on different genes. The majority of published data focuses on TGF-β1, however the role of TGF-β2 in asthmatic remodelling

and repair is gaining prominence and it is the TGF-β2 isoform that shall be evaluated in experimental work presented in this thesis.

1.2.1 TGF-β secretion

TGF-β2 is first transcribed and translated as pre-pro-TGF-β, the pre section being an N-terminal 19 amino acid signal peptide for translocation to the Golgi, where the C-terminal pro-region is cleaved by a furin-like peptidase at a dibasic cleavage site (Figure 1.1). The cleaved pro-peptide forms the 75kDa homodimer of latency associated protein (LAP). The mature TGF-β is a 25kDa homodimer that non-covalently associates with LAP. The molecule is now designated latent TGF-β (LTGF-β) or the small latent complex (SLC). LAP covalently binds a latent TGF-β binding protein (LTBP) forming the large latent complex (LLC) (Figure 1.2) (Todorovic *et al.*, 2005). LTBP is thought to anchor the TGF-β complex in the ECM by co-localising with fibrillins, thereby providing a reservoir of biologically inactive TGF-β (Chaudhry *et al.*, 2007). The majority of this work was carried out on TGF-β1.

The LTBPs are members of the LTBP-fibrillin superfamily as they have structural similarities with the fibrillins. The SLC of TGF-β2 can bind to either LTBP-1 or LTBP-3, both expressed in lung. Each isoform contains 4 cysteine rich (CR) domains. The third CR domain in each binds covalently to LAP. The cysteine rich domain at the N-terminus of the LTBP mediates covalent linking to the extracellular matrix. LTBPs may thus play a role in controlling bioavailability of TGF-β in tissues (Ramirez and Rifkin, 2009).

1.2.2 TGF-β2 activation

The SLC and LLC are biologically inactive complexes containing mature TGF-β. Before receptor binding can occur TGF-β must disassociate from LAP. The activation of TGF-β can occur through several mechanisms. TGF-β2 LAP does not contain an argine-glycine-aspartic acid (RGD) sequence, and so does not appear to bind integrins as a method of activation as TGF-β1 and TGF-β3 do.

TGF-β latency is governed by LAP, as it blocks sites of receptor binding on the mature TGF-β dimer. Immobilisation on the cell surface may be required for presentation to proteases. This can occur via binding of mannose-6-phosphate (M-6-P) moieties of the LAP to M-6-P receptors (M-6-PR) on the cell surface (Yang *et al.*, 2001). In this way plasminogen-derived plasmin has been shown to cleave LAP and activate TGF-β1 (Khalil *et al.*, 1996).

Activation of TGF-β1 and TGF-β2 has been demonstrated in various *in-vitro* systems through the activity of both serine and cysteine proteases. An overview of these studies is given in Table 1.4.

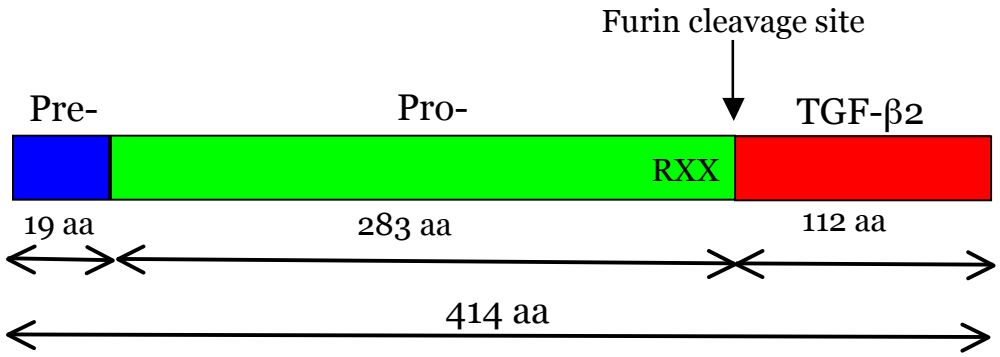


Figure 1.1 Schematic of pre-pro-TGF-β2. The pre-TGF-β signal peptide targets the complex to the Golgi, where the pro-region is cleaved by a furin-like peptidase. TGF-β2 forms a mature, 25kDa homodimer, which non-covalently associates with a homodimer of the pro-region, now termed latency-associated peptide (LAP).

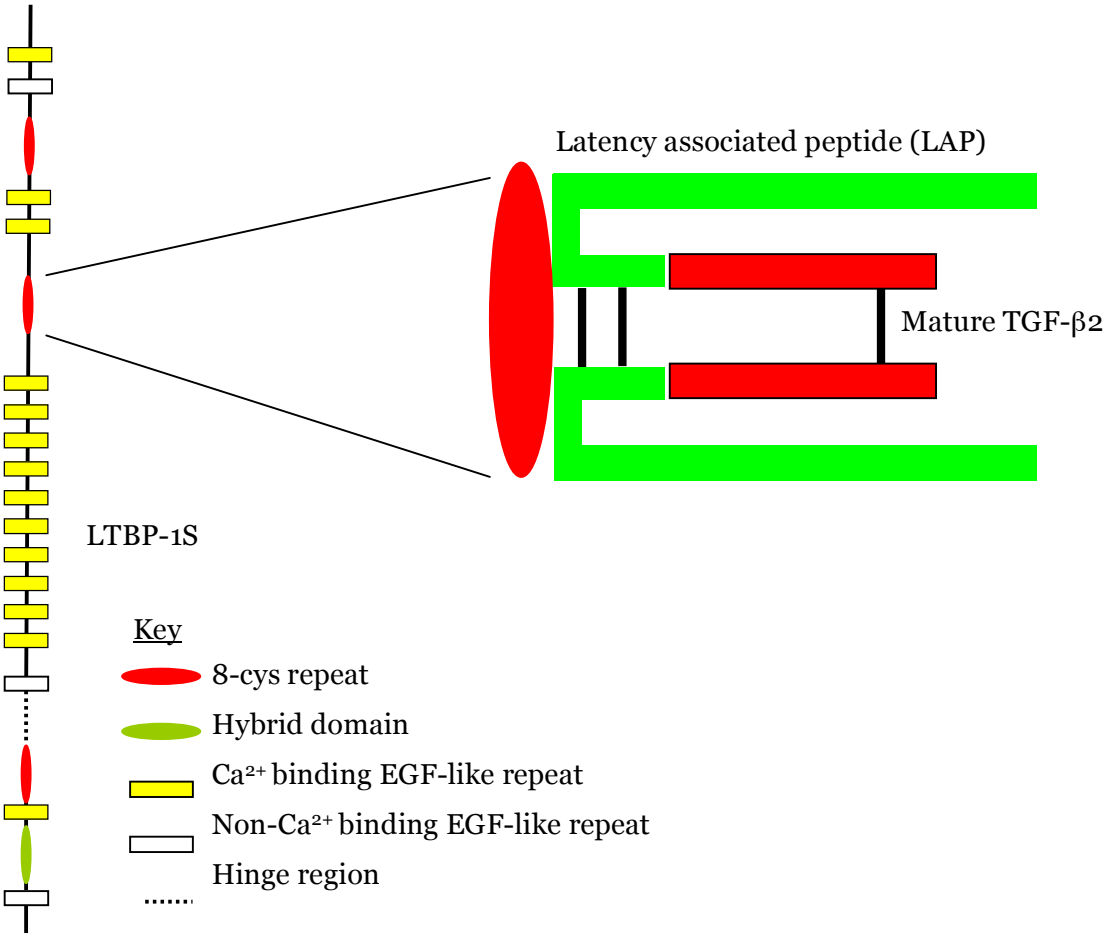


Figure 1.2 Schematic of mature TGF-β2 in the large latent complex (LLC) 25kDa mature TGF-β2 associated with 75kDa LAP forms the small latent complex (SLC). LAP has covalently bound a latent TGF-β binding protein (LTBP-1S), forming the large latent complex (LLC).

Protease	Class	TGF-β isoform	Cell associated?	Reference
Plasmin	Serine	TGF-β1	Cell assoc.	(Khalil *et al.*, 1996; Lyons *et al.*, 1988)
Prostate-specific antigen (PSA)	Serine	TGF-β2	Cell-free & assoc.	(Dallas *et al.*, 2004)
Tryptase	Serine	TGF-β1	Cell-free & assoc.	(Tatler *et al.*, 2008)
Der f 1	Cysteine	TGF-β1	Cell-free	(Nakamura *et al.*, 2009)
Calpain	Cysteine	TGF-β1	Cell-free & assoc.	(Abe *et al.*, 1998)
Cathepsin B	Cysteine	TGF-β1	Cell-assoc.	(Gantt *et al.*, 2003)
Cathepsin B/D	Cysteine	TGF-β1	Cell-free & assoc.	(Oursler *et al.*, 1993)

Table 1.4 Proteinases shown to activate latent TGF-β isoforms

1.2.3 TGF-β Receptors

There are three subsets of transmembrane TGF-β receptors, Type I (TβR-I) and Type II (TβR-II), both of which are required for TGF-β signal transduction and TβRIII. The TβR-I family contains two receptors which bind TGF-β: activin receptor-like kinase 1 (ALK-1) and ALK-5. TβR-I have intrinsic serine/threonine kinase activity in the cytoplasmic portion and a short cysteine-rich extracellular domain, features which are shared by TβR-II. TβR-II differ from TβR-I in a longer cytoplasmic domain and a spacer region lying between transmembrane and kinase sequences. TβR-II lack the characteristic glycine/serine rich domain found next to the kinase domain in TβR-I.

TGF-β2 must bind the 250-350 kDa proteoglycan betaglycan, also known as TGF-β receptor type III (TβR-III), to mediate strong binding to TβR-II, although recent evidence shows a splice variant of TβR-II can bind TGF-β2 without the requirement for betaglycan (del Re *et al.*, 2004a). Once a TβR-II dimer is bound by active TGF-β, the dimeric TβR-I associates forming a tetrameric receptor complex. The cytoplasmic domain of TβR-I is phosphorylated by TβR-II, signalling the start of the signal transduction pathway.

1.2.4 TGF-β-Smad signalling

The primary signalling pathway of TGF-β is Smad-dependent. Smads are a family of eight intracellular transcription factors classified as receptor associated (R-Smad 1, 2, 3, 5 and 8), common (Co-Smad 4) or inhibitory (I-Smad 6 and 7). R-Smads 2 and 3 are activated by TGF-β (and activin) binding. The Smad proteins consist of two globular domains designated Mad-homology 1 (MH1) and MH2 joined by a flexible linker. In the inactive form, the MH1 and MH2 domains are bound together (Massagué *et al.*, 2005).

Upon ALK-5 phosphorylation R-Smad 2 and R-Smad 3 are presented to the receptor complex by the membrane-anchored Smad anchor for receptor activation (SARA) which binds a Smad interaction motif (SIM) in the linker region. R-Smad2 and 3 are phosphorylated by ALK-5 which leads to dissociation from SARA, the MH1 and MH2 domains are forced apart by the conformational change then dimerisation in the cell cytoplasm. The R-Smad2 and 3 heterodimers associate with Co-Smad4 which aids translocation into the nucleus. In the nucleus the complex binds to Smad-binding elements (SBE), specific motifs found in the promoter regions of genes regulated by TGF-β.

R-Smad activation is regulated by kinase activity in the cell cytoplasm. Phosphorylation of residues in the linker regions of R-Smads by MAPKs (mitogen associated protein kinases) such as Erk MAPK in the cytoplasm blocks Smad translocation into the nucleus and therefore abrogates TGF-β signalling.

Nuclear Smad complexes can co-operate with transcription factors and DNA binding proteins to influence gene transcription. Co-Smad4 is important in these interactions stabilises the association of R-Smads with DNA binding factors. Co-factor identity is dependent upon cell type and environment, thus TGF-β isoforms have multiple and wide ranging effects on many cell types. A selection of known Smad2 and Smad3 co-factors are shown in Table 1.5.

Smad Co-factor	Interacting Smad	Results of interaction
Max	Smad3	Inhibition of Smad3-mediated transcription
TFE3	Smad3	Synergistic cooperative effects on PAI-1 and Smad7 genes.
c-Fos	Smad3	Associate at AP-1-dependent target genes
c-Jun, JunB, JunD	Smad3	+ve and –ve regulation of Smad3 activity
FoxH1	Smad2 & 3	Bind Activin-responsive promoters
FoxO	Smad2 & 3	Regulation of p21 expression
Estrogen receptor	Smad3	Repression of Smad3 target genes
Glucocorticoid receptor	Smad3	Inhibition of Smad3 activity
Sp1	Smad2	Activation of numerous target genes eg. P15, PAI-1, Smad7 and collagen.
IRF-7	Smad3	Activation of IRF-7 transactivation effects
NFκB p52	Smad3	Activation of κB site
P53	Smad2	Synergism and antagonism of Smad2 targets

Table 1.5 Smad2 and Smad3 binding partners and their major function. Adapted from (Feng and Derynck, 2005; Van Eerdewegh *et al.*, 2002).

TGF-β1 has been shown to induce expression of cdk inhibitors p15, p21 and p27 in bronchial epithelial cells, and p21 is increased in asthmatic epithelium (Puddicombe *et al.*, 2003). In the case of p21 this occurs when Smad3 interacts with the forkhead protein FoxO and binds to the p21 gene promoter region. In the zinc-finger protein family Sp1 interacts with Smad2, 3 and 4. Association with Smad2 and Smad3 can result in transcription of α2(I) collagen, Smad7 and PAI-1 genes, all of which may have further downstream effects on AWR. Smad3 cooperation with the co-factor TFE3 also leads to transcription of PAI-1 and Smad7, and also the laminin-γ-chain gene (Feng and Derynck, 2005). TGF-β mediated upregulation of these matrix-associated genes could contribute to AWR.

1.2.5 TGF-β in asthma

The predominance of TGF-β2 in the epithelial layer and immediately below in healthy and asthmatic bronchial tissue suggests it may be of interest in AWR. Much of the literature investigating TGF-β in the airway refers to the TGF-β1 isoform.

Bronchial biopsy studies provide a snapshot of bronchial tissue in time. Thirteen atopic asthmatics were biopsied before and 24h following allergen challenge (HDM, cat dander or grass pollen, dependent on most reactive skin prick test reponse), and TGF-β1, TGF-β2 and TGF-β3 stained for. All three isoforms were present in the epithelium in this study, however only TGF-β2 showed a significant increase in expression following allergen challenge. Tenascin-C expression was also increased post-challenge (Torrego *et al.*, 2007). These results are of relevance to this study, however it must be noted that no healthy controls were included.

Batra *et al.* undertook a timecourse study of BAL cytokine levels, with timepoint 0 compared to 24h, 1 week and 2 weeks following allergen challenge (ragweed extract) in both healthy controls and mild asthmatics. TGF-β2 was greater at baseline in healthy controls compared with asthmatics in this study. Following allergen challenge, TGF-β1, TGF-β2, IL-4 and IL-13 were increased in asthmatic samples only, and IL-4 remained elevated the longest. This clearly indicates a role for TGF-β2 in healthy lung, that is dysregulated in asthmatics (Batra *et al.*, 2004).

Following on from Batra and colleagues BAL analysis, Balzar *et al.* reported on a biopsy-based study of severe asthmatics compared with mild asthmatics and healthy controls. All tissue was at baseline, that is no allergen challenge took place. Following assessment of all 3 TGF-β isoforms in biopsy tissue, the authors reported only the TGF-β2 isoform was upregulated in asthmatics. It was also reported that CTGF (a downstream marker of TGF-β activation) was downregulated in all asthmatics. This could be attributable to the decreased TβRI and low levels of TβRIII descibed in the asthmatic tissue (Balzar *et al.*,

2005a). It is possible that this downregulation of receptor expression is due to maintenance of lung homeostasis. If there are consistently high levels of active TGF-β2, then sensitivity to the cytokine may become blunted.

Healthy control and mild asthmatic bronchial biopsy tissue was analysed for expression of TGF-β1 and TGF-β2 at baseline by Chu and colleagues, who demonstrated increased TGF-β2 over TGF-β1 in both sample cohorts. Furthermore, TGF-β2 was significantly increased in asthmatic compared with healthy tissue. The authors also established primary bronchial epithelial cell cultures at air-liquid interface (ALI) from a subset of study participants, and assessed mucin production as a marker of goblet cell hyperplasia following TGF-β exposure. TGF-β2 exposure led to a non-significant increase in mucin protein secretion, however no changes were observed following TGF-β1 exposure (Chu *et al.*, 2004).

The results discussed above strongly indicate a role for epithelial-derived TGF-β2 in AWR. Reactivation of the epithelial-mesenchymal trophic unit (EMTU) of epithelium and underlying mesenchymal cells (fibroblasts) may drive AWR in asthma (Holgate *et al.*, 2004; Knight *et al.*, 2004). Several groups have published *in-vitro* studies purporting to mimic this reactivation through challenge of the epithelium.

Thompson *et al.* determined levels of TGF-β2 from healthy HBECs at baseline and following scrape injury. TGF-β1 was below the level of detection in conditioned medium (CM), however active TGF-β2 rose from 50-70pg/ml to around 150pg/ml following injury; tenascin C was also increased. Exogenous TGF-β2 alone induced Tn-C expression also (Thompson *et al.*, 2006). This is of interest as tenascin C has been suggested as a marker of EMTU reactivation. Exposure of lung fibroblasts either to scrape-injured epithelial CM or in co-culture below the injured cells led to significant increases in α-SMA protein expression, indicative of myofibroblast differentiation. Only healthy cells were used in this study, so again the role of TGF-β2 in maintaining lung homeostasis upon injury is shown.

Tschumperlin *et al.* devised a mechanical stress model for HBEC culture at ALI, to mimic the bronchoconstriction aspect of the asthmatic response. Increased active TGF-β2 secretion was reported, but mRNA levels remained steady, suggesting the epithelial cells contained a pre-formed pool of TGF-β2, and protein was not newly synthesised. CM also resulted in increased collagen synthesis measured by [^{3}H]proline incorporation (Tschumperlin, 2003).

Continuous exposure of HBEC cultures to IL-13 led to a "pro-fibrotic" cell phenotype. HBEC cultures in this study continously secreted higher levels of active TGF-β2 than controls, and exposure of fibroblasts to IL-13 pre-treated cultures led to increases in collagen production. The authors suggest that the continuous exposure to the Th2 cytokine IL-13 models the allergic asthmatic airway (Malavia *et al.*, 2008).

It is apparent from these studies that environmental insult, including physical injury/stress and exogenous TGF-β2 in culture, of epithelia leads to alterations in cell behaviour that drive remodelling in underlying fibroblasts. Allergen challenge of human subjects and subsequent alterations in TGF-β2 levels demostrated a novel mechanism by which AWR responses could be continuously driven.

1.3 The role of house dust mite allergens in asthma

An allergen is an antigen capable of provoking an IgE antibody response. The biological function of an allergen has the potential to affect the physiological response. In allergen nomenclature, the first three letters of the Linnean genus are used, together with a single letter of species name and a number denoting the order in which allergens were purified. Hence, the first purified allergen of house dust mite (HDM) *Dermatophagoides pteronyssinus* becomes Der p 1. *Dermatophagoides pterronysinus* is a common source of allergens and subsequent allergic sensitisation in Western Europe. The pathogenesis of allergic asthma may in part be due to the proteolytic activity of HDM allergens including Der p 1 (Asokananthan *et al.*, 2002; Gough *et al.*, 1999; Wan *et al.*, 1999)

Inflammatory responses to HDM allergens are well documented (Custovic *et al.*, 1996). *In-vitro* challenges of healthy control and asthmatic epithelial cells consistently show release of proinflammatory cytokines such as GM-CSF, IL-6 and IL-8 following challenge that is further upregulated in asthma (Lordan *et al.*, 2002; Pichavant *et al.*, 2005; Rusznak *et al.*, 2001). Activity of a selection of HDM allergens is given in Table 1.6.

Allergen	Biochemical Function	Effect of cell challenge	Reference
Der p 1	Cysteine protease	Inactivated elastase inhibitors Enhanced DC recruitment Cleaved CD25	(Brown *et al.*, 2003; Pichavant *et al.*, 2005) (Gough *et al.*, 1999)
Der p 2	Unknown, no protease activity	GM-CSF, IL-6 & IL-8 release by epithelial cells	(Osterlund *et al.*, 2009)
Der p 3	Serine protease (trypsin)	Cleavage of complement C3 & C5. PAR-2 activation	(Maruo *et al.*, 1997; Sun *et al.*, 2001)
Der p 5	Unknown, no protease activity	IL-6 & IL-8 release by epithelial cells	(Kauffman *et al.*, 2006)
Der p 9	Collagenolytic serine protease	PAR-2 activation	(Sun *et al.*, 2001)

Table 1.6 HDM-derived major allergens and selected effects

As seen in Table 1.6, both proteolytically inert and active allergens activate the respiratory epithelium to varying degrees. Also of importance in asthma pathogenesis is the ability of HDM allergens to disrupt the integrity of the epithelial layer. Wan *et al.* illustrated tight junction breakdown and increase in epithelial permeability in 16HBE14o- cultures challenged with Der p 1 (Wan *et al.*, 2000). The loss of airway epithelial integrity may be key to downstream effects of HDM exposure, however this was not investigated in this study.

The proteolytic activity of HDM allergens is of interest in this study. Exposure of BEAS-2B cells to Der p 1 or Der p 9 revealed that at concentrations required for cytokine (GM-CSF, IL-6, IL-8) release (10μg/ml) IL-6 was proteolytically degraded by both Der p 1 and Der p 9 (King *et al.*, 1998). Cytokine secretion responses are partly mediated by PAR-2 cleavage (Adam *et al.*, 2006). PAR-2

is upregulated in asthmatic epithelium (Knight *et al.*, 2001), and *in-vitro* Der p 1 challenge of primary HBECs showed increased secretion of GM-CSF, IL-6 and IL-8 by allergic asthmatic HBECs over non-atopic controls (Pichavant *et al.*, 2005).

The reports above all used single purified allergens of Der p. Heterogenous mixtures of proteolytically active HDM extracts may exert additonal effects. HDM extracts are variable preparations between companies/lab groups, as they are highly dependent upon the source material and the method of preparation. For example, an aqueous extract prepared from frozen whole mites will contain substantially less Der p 1 than a preparation that contains a variable mix of whole mites, nymphs (the larval stage), fecal pellets, eggs and spent culture media (Jeong *et al.*, 2010; Thomas *et al.*, 2002). Der p 1 and Der p 2 are thought to have a role in the mite gut, and are concentrated in fecal pellets (Park *et al.*, 2000). Variation in cellular responses between groups may be due to different formulations of the extracts used, and this should be borne in mind when assessing results.

Repeated insult to the lung through allergic responses, with the resultant activation of cell types including epithelial cells, fibroblasts, mast cells and eosinophils may promote the airway wall remodelling seen in asthmatic lung, namely increased deposition of collagens and other proteins in the basement membrane and mucosa, myofibroblast differentiation, goblet cell hypertrophy and submucosal gland hyperplasia, and smooth muscle hyperplasia. Holgate *et al.* propose the reactivation of a developmental unit, the epithelial-mesenchymal trophic unit (EMTU), as the source of remodelling phenomena,

1.4. Aims and hypothesis

Decreased lung function in asthma has been partially attributed to airway wall remodelling. Increases in the pro-fibrogenic cytokine TGF-β2 have been reported in asthmatic tissue and BAL fluid. It is therefore hypothesised here that alterations in active TGF-β2 levels contribute to airway wall remodelling in the asthmatic lung.

The aims of this project are:

1. To identify and measure AWR markers in healthy and asthmatic human bronchial biopsy tissue, including reticular basement membrane thickening, myofibroblast differentiation and TGF-β2 expression
2. To identify whether house dust mite extracts can activate latent TGF-β2 secreted by bronchial epithelial cells.
3. To assess whether asthmatic bronchial fibroblasts are intrinsically more sensitive to the pro-remodelling effects of TGF-β2 than healthy fibroblasts.

Chapter 2: Materials and Methods

Materials were obtained from Invitrogen (Paisley, UK), unless otherwise stated.

2.1 Tissue Culture

Human bronchial epithelial and fibroblast cells were required for this project and were obtained from commercial and non-commercial sources. An SV-40 transformed bronchial epithelial cell line was also used: 16HBE14o-. Tissue culture procedures were carried out in a class II microbiological safety cabinet.

2.1.1 Primary bronchial fibroblast isolation and maintenance

Informed consent was obtained from healthy and asthmatic individuals attending Wythenshawe Hospital to undergo a single bronchoscopic procedure performed by a respiratory physician in the bronchoscopy suite of the North West Lung Centre. Information regarding patient smoking history, chest related medication, forced expiratory volume in 1 second (FEV1) and allergy was compiled. Ethical approval was given by South Manchester Ethics Committee.

Bronchial biopsies were taken using 1.8mm alligator cup biopsy forceps (Type 100503, Conmed UK Ltd, Swindon, UK) and dropped into sterile HBSS in the bronchoscopy suite. In the lab, biopsies were rinsed in chilled DMEM containing 100units/ml penicillin, 100ng/ml streptomycin and 25ng/ml amphotericin B before being placed in a 25cm^2 tissue culture flask and allowed to attach for 30mins at 37°C, then 2ml of complete DMEM (containing 10% fetal calf serum (PAA Laboratories, Somerset, UK), 50 units/ml penicillin, 50ng/ml streptomycin and 2mM L-glutamine) with 0.25ng/ml amphotericin B, was added dropwise. Medium was changed after 24 hours, and every 3 days following. Fibroblast-like (spindle-shaped) cell migration from biopsy tissue was seen around 14 days after seeding. Cultures were maintained until areas of 80% confluence were seen, then cells passaged as follows. Medium was

removed and cells washed twice with PBS. 0.05% Trypsin/EDTA was added at 1ml/flask and incubated at 37°C until cells detached. Active trypsin was diluted out by addition of 10ml complete DMEM. The cell suspension was centrifuged at 300*g* for 5 min, the cell pellet resuspended in complete DMEM and subcultured in a 75cm² flask (Corning Life Sciences). Cells were expanded and subcultured at a 1:4 split, before being frozen at a low passage in DMEM freezing mix (80% complete DMEM, 10% FCS, 10% DMSO). Fibroblasts were used at passages 4 through 8. Donor characteristics are given in Table 2.1.

Donor	Age	Sex/Race	FEV1 (% predicted)	Steroid naïve?	Smoking status	Medication
Asthmatic 1	22	M/Caucasian	96%	Yes	Non-smoking	Inhaled β2 agonists
Asthmatic 2	37	M/Caucasian	103%	Yes	Non-smoking	Inhaled β2 agonists
Healthy 2	23	F/Caucasian	108%	Yes	Non-smoking	None

Table 2.1 Donor information for primary fibroblasts

2.1.2 Maintenance of commercially sourced cells

Commercially available primary cells from donors selected according to age, smoking history and health status were purchased from Lonza. One normal human lung fibroblast (NHLF) and three normal human bronchial epithelial cell (NHBEC) donors were used, and information provided by the company is shown in Table 2.2.

Donor	Cell Type	Sex/Race	Age	Smoking status
4F0768	Fibroblast	M/Caucasian	31	Non-smoking
2F1578	Epithelial	M/Caucasian	17	Non-smoking
7F3081	Epithelial	M/Caucasian	49	Non-smoking
7F3000	Epithelial	M/Hispanic	27	Non-smoking

Table 2.2 Donor information obtained from supplier (Lonza).

The SV-40 virally transformed airway epithelial cell line 16HBE140- was obtained from within the University of Manchester (D. Thornton, Faculty of Life Sciences). These cells have epithelial features including tight junction formation and cytokeratin expression, but do not differentiate at ALI.

2.1.2.1 NHBEC maintenance

Cryopreserved NHBEC were initially seeded at 3500 cells/cm^2, as stipulated in Lonza literature, in Bronchial Epithelial Growth Medium (BEGM). BEGM consists of Bronchial Epithelial Basal Medium (BEBM) supplemented with a BEBM SingleQuot™ kit (bovine pituitary extact, epidermal growth factor, epinephrine, gentamicin-amphotericin, hydrocortisone, insulin, retinoic acid, triiodothyronine and transferrin). Medium was changed every 2nd day. When 80% confluent, cells were trypsinised and frozen down. Aliquots of 1 x 10^6 NHBEC were expanded in 75 cm^2 tissue culture flasks before experimental use. NHBEC were not used beyond passage 3 as the ability to differentiate at ALI is decreased in further passages.

2.1.2.2 NHLF maintenance

Initial seeding of cryopreserved cells (a single vial of 565,000 cells) used Fibroblast Basal Medium (FBM), a serum-free formulation supplemented with insulin and recombinant human fibroblast growth factor-B (rhFGF-B), as recommended by Lonza. The supplier's protocol was followed, with cells seeded at 2500 cells/cm^2. Subsequent expansion of NHLF used complete DMEM. Cells were used between passages 4 and 8.

2.1.2.3 Transformed epithelial cell line maintenance

Transformed epithelial cell line 16HBE14o- was grown in 75cm^2 tissue culture flasks (Corning) containing 10ml minimum essential medium with Earle's salts (Sigma-Aldrich), supplemented with 10% fetal calf serum (FCS), 50units/ml penicillin, 50ng/ml streptomycin and 2mM L-glutamine. Medium was changed every 2-3 days. At 70-80% confluence, cells were passaged as for fibroblasts, subcultured at a 1:6 split. Cell stocks were routinely frozen in MEM freezing mix (80% complete MEM, 10% FCS, 10% DMSO).

2.1.3 Air-liquid interface epithelial cell culture

NHBEC are induced to differentiate into ciliated, goblet and basal cells when cultured at an air-liquid interface (ALI) in the presence of retinoic acid. For

ALI culture, hanging inserts (Millipore, UK) were placed in 12-well culture plates, 1ml bronchial epithelial differentiation medium (BEDM: 50% serum-free DMEM + 50% BEGM without triiodothyronine) added basolaterally and 250µl apically to wet the polycarbonate membrane. The prepared inserts were equilibrated at 37°C for 30 min. 82500 BEC were seeded into each insert in 250µl BEDM, to total 500µl BEDM apically. On day 1 media was changed apically and basolaterally, and every 2-3 days thereafter until cells reached around 80% confluence at day 7 after seeding. Cultures were brought to ALI by removing all medium, and replacing basolateral medium only with 1 ml BEDM Differentiation markers (mucus-production, cilia formation) were observed from day 7 onward. ALI cultures were maintained for up to 21 days.

2.1.4 Bronchial epithelial cell challenge assays

Primary HBEC challenges were carried out on cultures maintained at ALI for a minimum of 7 days to allow cell differentiation. Scratch assays were based on the protocol of Thompson *et al.* (Thompson *et al.*, 2006). Briefly, a 200µl pipette tip was used to make a single score down the centre of the insert membrane, disrupting the epithelial cell layer. Cells were monitored microscopically, and conditioned medium taken from the basal compartment at specified timepoints. For mRNA analysis, culture membranes were cut from inserts and immersed in 1ml TRIzol reagent, cells lysed by repeated gentle pipetting, and frozen at -80°C until further processing (Section 2.5.1).

To mimic environmental stresses, house dust mite preparations were applied to the apical surface, dissolved in serum-free DMEM. Control wells were exposed to SF-DMEM only. Cells were monitored and samples taken as for scratch assay cultures. LPS (Sigma-Aldrich) was added to basal media.

Epithelial cell line (16HBE140-) experiments were carried out under submerged culture conditions in 6 well culture plates at 80% confluence. Scratch assays were carried out as for primary BEC, using a 200µl pipette tip to make two scratches in a cross and immediately changing the medium, to

remove apoptotic scratched cells that may produce a confounding effect on results.

2.1.5 Bronchial fibroblast challenge studies

Fibroblasts were seeded in 6 well tissue culture plates (Corning Life Sciences) and grown to 80% confluence in complete DMEM. Cells were quiesced for 24 hours in serum-free DMEM prior to experimental procedures.

For primary BEC conditioned media challenge studies, conditioned media was collected and pooled from individual donors from days 7-21 ALI, when apical-basal polarity and differentiation were established. Scratch-injured BEC culture medium and HDM-exposed BEC culture medium were applied to fibroblast cultures and cells harvested at specified timepoints. TGF-β2 stimulated fibroblast cultures were harvested in the same manner.

Q-proteome mammalian protein kit was used (Qiagen, Crawley, UK) for whole cell lysate preparation, and the manufacturer's protocol followed to produce samples stored at -20°C until further use.

Collagen protein production was assessed in both culture supernatant and the cell layer using the Sircol collagen assay. For cell-associated collagen, 500µl 0.5M acetic acid containing 1mM EDTA was pipetted onto cells, scraped into a 1.5ml eppendorf and frozen at -20°C until use. Conditioned media for the assessment of soluble secreted collagen was collected and stored at -20°C until assayed.

For mRNA extraction, cultures were washed briefly with PBS prior to the addition of TRIzol reagent at 1ml/well (see Section 2.5.1).

2.1.6 Rat tail tendon collagen extraction

Type I collagen was isolated from rat tail tendons as previously described (Piez, 1967). In a laminar flow hood ten frozen rat tails were thawed in 70%

IMS and the skin and tendon sheath removed to expose tendon fibres. Fibres were removed and submerged in 500ml sterile 0.5M acetic acid. The mixture was stirred for 48 hours at 4°C, then centrifuged at 1600*g* for 3 hours. The precipitate was discarded, and supernatant added to an equal volume of sterile 1.5M NaCl. This was precipitated at 4°C for 48 hours, then centrifuged at 1700*g* O/N. Supernatant was discarded and the pellets (now composed primarily of Type I collagen) were redissolved in 500ml sterile 0.5M acetic acid. The acidic collagen solution was dialysed against sterile water 3 times at 4°C using 10000 MWCO dialysis tubing (Perbio Science, Crawlington, UK) for a total of 48 hours. The resultant Type I collagen solution was decanted into a sterile Duran bottle and stored at 4°C. Soluble collagen concentration was obtained using the Sircol assay kit (Biocolor Ltd., Ireland), following the manufacturer's protocol. Rat tail derived collagen concentration was read from the standard curve prepared.

2.2 Histological methods

2.2.1 Bronchial biopsy tissue donor characteristics

The characteristics of biopsy tissue donors used in Chapter 3 are shown in Table 2.3. All donors were caucasian, steroid naïve and reported no respiratory illness in 3 months prior to sampling.

Group	Sex Ratio M:F	Age range (years)	FEV1 % predicted range	Current medication
Asthmatic	9:3	19 - 58	85% - 115%	β2-agonists only
Healthy	5:5	22 - 47	94% - 117%	None

Table 2.3 Bronchial biopsy tissue donor demographics

2.2.2 Tissue processing for histological analysis

Biopsy samples were fixed O/N at 4°C in formal buffered saline (10%, pH 7.4), and stored in 50% industrial methylated spirits (IMS) prior to tissue processing. Samples were processed in a Tissue-Tek Vacuum Infiltration Processor (Bayer Diagnostics, Newbury, UK) running a dehydration and wax infiltration program as detailed in Table 2.2. Samples were embedded in

paraffin wax blocks and allowed to set O/N. Wax blocks were trimmed and sectioned at 4-5μm on a Reichert-Jung 2030 microtome (C. Reichert AG, Wien, Austria) fitted with disposable Accu-edge blades (Sakura Finetek, Zoeterwoude, The Netherlands). Sections were floated on cold deionised water then transferred to a 40°C water bath to smooth "wrinkling" of the tissue samples. Sections were collected onto Poly-L-Lysine coated microscope slides (Menzel GmbH, Braunschweig, Germany) and dried on a 40°C heated block before storage at room temperature, protected from light.

Step	Solution	Time (min)	Temperature (°C)
1	50% IMS	45	Ambient
2	70% IMS	45	Ambient
3	90% IMS	60	35
4	100% IMS	30	35
5	100% IMS	30	35
6	100% IMS	60	35
7	Toluene	30	35
8	Toluene	30	35
9	Toluene	60	50
10	1st Wax	30	60
11	2nd Wax	30	60
12	3rd Wax	30	60
13	4th Wax	60	60

Table 2.2 Tissue-Tek Vacuum infiltration processor program for wax embedding of biopsy tissue. In each step an ambient pressure/vacuum cycle ensured complete infiltration of the tissue.

2.2.3 Histological staining techniques

Tissue sections were dewaxed by immersion in two consecutive xylene baths, followed by rehydration through a series of graded alcohols (IMS) (100%, 100%, 90%, 70% and 50%), and immersion in tap water. Haemotoxylin and eosin staining was used to demonstrate general tissue architecture, whilst Masson's trichrome identified the collagen and Miller's elastin stain the presence of elastic fibres throughout the tissue. Standard histological stains as listed in "Theory and Practice of Histological Techniques" (Bancroft and Stevens, 1990) were prepared and followed with the exception of periodic acid solution and Schiff's reagent, which were purchased from Sigma-Aldrich (Poole, UK). For further details of staining techniques, see Appendix A.

2.3 Immunohistochemistry

Tissue sections were dewaxed and rehydrated as for histological staining. Briefly, endogenous peroxidase activity was quenched by incubation with 1% hydrogen peroxide solution in methanol. Serum from the animal in which the

secondary antibody was raised was used to block sites of non specific binding, before samples were incubated with primary antibody in BSA-containing antibody diluent. After washing, incubation with biotin-conjugated secondary antibody followed, or antibody diluent without antibody as negative control, for 1 hour. Primary antibodies used are shown in Table 2.4. After further washing, 30 minutes of avidin-biotin conjugation using a Vectastain Elite Standard ABC kit (Vector Labs Ltd., Peterborough, UK) was carried out at RT. Slides were washed, rinsed in distilled water and diaminobenzidine (DAB) (Vector Labs Ltd.) substrate incubated on sections for between 1 and 5 min. Slides were rinsed in distilled water and counterstained with Harris's haematoxylin for 30 seconds before dehydration through a series of alcohols into xylene, and mounted using Depex mounting medium (BDH Lab Supplies, Poole, UK).

Antigen	Raised in	Dilution used	Protein concentration	Supplier
TGF-β1	Rabbit	1/400	200μg/ml	Santa Cruz
TGF-β2	Rabbit	1/400	200μg/ml	Santa Cruz
TβRIII	Goat	1/200	200μg/ml	Santa Cruz
α-SMA	Mouse	1/400	4.5mg/ml	Sigma-Aldrich
Tenascin C	Rabbit	1/500	100μg/ml	Monosan
Occludin	Goat	1/400	200μg/ml	Santa Cruz
β-tubulin IV	Mouse	1/400	500μg/ml	Sigma-Aldrich
Anti-rabbit IgG, biotinylated	Donkey	1/1000	500μg/ml	Amersham
Anti-goat IgG, biotinylated	Rabbit	1/2000	500μg/ml	Dako
Anti-mouse IgG, biotinylated	Goat	1/2000	0.5 mg/ml	Dako

Table 2.4 Primary and secondary antibodies used in biopsy assessments

2.4 Microscopy and Image analysis

Standard histological stains (H&E, Masson's trichrome, PAS-Alcian blue) were used to highlight tissue architecture. Stained sections were viewed using an Axiostar plus light microscope (Zeiss, Hertfordshire, UK), with 4x, 10x, 20x and 40x objective lenses. Images were captured using a Spot-32 digital camera (Diagnostic Instruments Inc, USA) coupled with Spot-RT version 2.1.2 software running on a Dell Optiplex GX620 computer. Images of a 100μm graticule (Psyer, SKI Limited, UK) were taken at the same magnification for calibration purposes.

All image analysis was performed using Image Pro-Plus software (Media Cybernetics, California, USA), using a graticule image to calibrate. Scoring was performed blinded, and repeat scoring/measurements were made at least 2 weeks apart.

2.4.1 Determination of basement membrane thickness

For RBM measurements, either Masson's trichrome or PAS-Alcian blue stained sections were analysed as both allow clear differentiation between the reticular basement membrane and underlying mucosa.

Three sections with sufficient intact epithelium to confirm correct orientation of the reticular basement membrane were selected from throughout each biopsy. Images were taken at x200 magnification, and an outline was traced around the horizontal sections of reticular basement membrane on each section. The selections were filled and converted to objects (Figure 2.1). Objects were converted to white against a black background, and merged with an image of horizontal lines set at 2μm intervals. All bright object pixel heights created were calculated, and converted to microns. This produced a measurement at 2μm intervals along the selected RBM. A graticule image at x200 magnification was used to calibrate measurements. Each section was viewed and analysed on three separate occassions to give an average, and ensure intra-observer variation was within acceptable limits (below 10%).

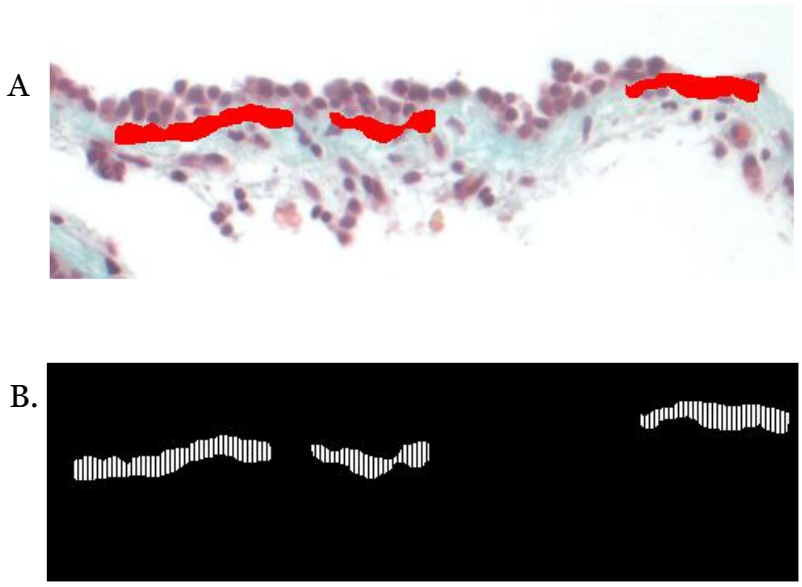


Figure 2.1 Measurement of reticular basement membrane thickness.
Images taken at an original magnification of 200x were orientated using the Image Pro-plus software package so maximal length of basement membrane was horizontal. A. Basement membrane sections were selected. B. A grid mask was applied to convert selected areas to a set of objects. Object heights were calculated, and the average taken.

2.4.2 α-Smooth muscle actin immunoreactive cell counts

To quantify levels of myofibroblast differentiation in healthy and asthmatic bronchial biopsy tissue, three 5μm sections of tissue from throughout each biopsy were immunostained for the presence of α-smooth muscle actin (α-SMA) protein, a commonly used marker of muscle-like cells, with a monoclonal antibody raised in mouse. Smooth muscle bands within the tissue were used as a positive control, and normal mouse IgG as a negative control. Images of α-SMA immunostained tissue were taken at x20 magnification for assessment. Positive cells were counted to a depth of 100μm below a 1mm length of RBM, giving a total area analysed of 100μm^2. Blood vessels and smooth muscle bundles were not included. The area examined was limited to this depth to assess cells directly involved in the EMTU. Results were expressed as a percentage of total cells identified.

2.4.3 Immuno-quantitation of TGF-β2, Tenascin C and TβRIII

A single section was selected from each biopsy. The standard immunohistochemistry protocol was followed, with the addition of a sodium citrate antigen retrieval step prior to endogenous peroxidase reduction. Slides were fully submerged in 10mM citrate buffer pH 6.0, heated on full power in a 900watt microwave for 4min then allowed to cool for 20min before proceeding. For TGF-β2 stained sections, the epithelium, lamina propria and smooth muscle were scored seperately on a scale of 0-3: 0= no/background immunoreactivity, 1= low, 2= moderate and 3= intense immunoreactivity. In tenascin C stained sections the area to a depth of 100μm below the RBM, and including the epithelial layer, was assessed on scale of 0-3 as for TGF-β2. TβRIII stained sections were assessed for total staining as for tenascin C.

2.5 Molecular Biology

Analysis of messenger RNA levels of cells was carried out to determine changes in gene expression following exposure to TGF-β2, conditioned medium or other challenge. Although gene expression levels do not necessarily correlate to increased protein levels, analysis of mRNA identifies increases or decreases in gene transcription levels in response to stimulation. All materials were molecular biology grade.

2.5.1 RNA isolation

Cultured cells in monolayer were washed twice in pre-warmed PBS, then TRIzol reagent added at 20μl/cm² or 1ml/sample, whichever was the greater. Cells were scraped from the culture surface using a disposable cell scraper and transferred to a 1.5ml microtube. For membrane grown BEC, after washing the membrane was cut from the insert and placed into a 1.5ml eppendorf before adding TRIzol reagent. BEC were lysed by repeated gentle pipetting through a 200μl tip. Samples were incubated at room temperature for 5 min to allow dissociation of nucleoprotein complexes. Following centrifugation at 12000*g* for 10 min at 4°C, the supernatant was transferred to a fresh microtube. 200μl chloroform per ml of TRIzol reagent was added and shaken vigorously for 15

sec before incubating at room temperature for 2-3min. Samples were then centrifuged at 12000*g* for 15 min at 4°C. The upper aqueous phase containing RNA was pipetted into a clean microtube, and the same volume of isopropyl alcohol added. Following incubation at -20°C for 1 hour, samples were centrifuged at 12000*g* for 10 min at 4°C. The resulting pellet was washed with 1ml of 75% ethanol, mixed by brief vortexing and re-centrifuged at 7500*g* for 5 min at 4°C. The pelleted sample was then air-dried before 30µl water was added and the sample incubated at 60°C in a water bath for 10 min. The purified nucleic acids were stored at -40°C, or -80°C for longer term storage.

2.5.2 Determination of RNA Quantity and Quality

A ND-1000 Nanodrop spectrophotometer (Lab-tech International, East Sussex, UK) coupled with ND-1000 version 3.5.2 software was used to determine RNA quantity and quality. A 1µl sample was used in this analysis. Measurement of absorbance at 260nm (A260) and at 280nm (A280) wavelength was taken, and the A260/A280 ratio determined. An A260/A280 ratio of between 1.7 and 2.1 was accepted as being of sufficient quality for subsequent use.

2.5.3 DNase removal of of contaminating genomic DNA

A commercial DNase kit (Sigma Aldrich, UK) was used to eradicate DNA contamination. Thin-walled PCR tubes containing 1µg sample RNA in 8µl molecular grade water, 1µl 10x reaction buffer and 1µl DNase I (1Unit/µl) were incubated at RT for 15 min, then 1µl stop solution added, and tubes heated to 70°C for 10 minutes to destroy residual DNase activity before chilling on ice.

2.5.4 Reverse transcription of mRNA to cDNA

1µl dNTP mix and 1µl random nonamers were added to the 1µg sample of DNase-treated mRNA, mixed and incubated at 65°C for 5 min. Microtubes were then placed on ice before adding: 4µl 5x First Strand buffer, 2µl DTT and 1µl RNase OUT. Microtubes were incubated at 42°C for 2 min, 1µl Superscript

II Reverse Transcriptase added, and samples incubated at 25°C for 10 min, 42°C for 50 min then the reaction stopped by heating to 70°C for 15 min. cDNA samples were stored at -20°C short-term, or at -80°C for longer term.

2.5.5 Quantitative Real Time PCR

cDNA samples were serially diluted 10-fold with molecular grade water. One sample was used to produce a standard curve by assigning arbitrary values of concentration to each dilution. SensiMixPlus SYBR 2x mastermix (Quantace, Finchley, UK) containing heat-activated DNA polymerase**,** dNTPs, 6mM $MgCl_2$ and SYBR Green I dye, was used for all reactions. Primers were reconstituted in molecular grade water, and used in reactions at 15µM. For each 25µl reaction, 12.5µl mastermix, 0.225µl each of forward and reverse primers, 2.05µl water and 10µl cDNA was prepared. Reactions were pipetted into a 96 well plate (MJ Research, Hertfordshire, UK) in triplicate, alongside negative controls (water and non-transcribed RNA). Plates were sealed with Microseal 'B' sealer film (Bio-Rad, UK) and PCR performed on a MJ Research thermocycler that monitored sample fluorescence. PCR cycling was as follows:

1. Denaturation 10 min 95°C

Followed by 40 cycles of:

2. Denaturation 15 sec 95°C
3. Annealing 1 min 60°c
4. Extension 30 sec 72°C (sample fluorescence read)

Following cycle completion, a melt curve was performed to assess product specificity. The mixture is gradually heated and fluorescence measured. SYBR Green fluoresces when bound to double stranded DNA, so at the temperature the double stranded DNA is denatured, there is a sudden decrease in fluorescence. When this decrease is plotted as an inverse function of time, a single peak will show one specific product is formed.

All primer sets were designed using BLAST searches and Primer3 web-based software. Primer pairs resulted in one specific product as assessed by melt curve analyses.

A threshold crossing the amplification curve in the linear phase was set to begin analysis of sample concentration. From the intercept of the threshold and the amplification curve the cycle threshold value was taken. This value was used to determine the arbitrary concentration of each standard sample. The concentrations of the standard were plotted against the cycle threshold to produce a standard curve. An R^2 value of greater than 0.995 was required for the standard curve to be acceptable for determining concentrations of other samples in the reaction. When the arbitrary concentrations of interest were determined, all results from genes of interest were normalised against housekeeping genes, selected for stable expression as described in Section 2.5.6. For fibroblast experiments the housekeeping genes used were *GAPDH* and *ATP5B*, and epithelial experiments used *GAPDH*. Results were analysed for statistical significance with Prism software package, using one-way ANOVA followed by Dunnett's post-hoc with confidence intervals of 95%.

2.5.6 Housekeeping gene selection for gene expression studies.

Primer sets for ten candidate housekeeping genes were purchased from PrimerDesign Ltd, UK. and gene expression evaluated in control and experimentally modulated (TGF-β2 stimulated) NHLF cultures, following the protocol supplied. Results were analysed using the GeNorm software program (PrimerDesign Ltd) as previously described (Vandesompele *et al.*, 2002). This identified the most stably expressed genes. Phospholipase A2 (*YWH*) and ubiquitin C (*UBC*) were least stable and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Adenosine tri-phosphate synthase (*ATP5B*) were most stably expressed following TGF-β2 stimulation (Figure 2.2). Fibroblast q-PCR experimental results were therefore normalised to the geometric mean of *GAPDH* and *ATP5B* in all qPCR analyses.

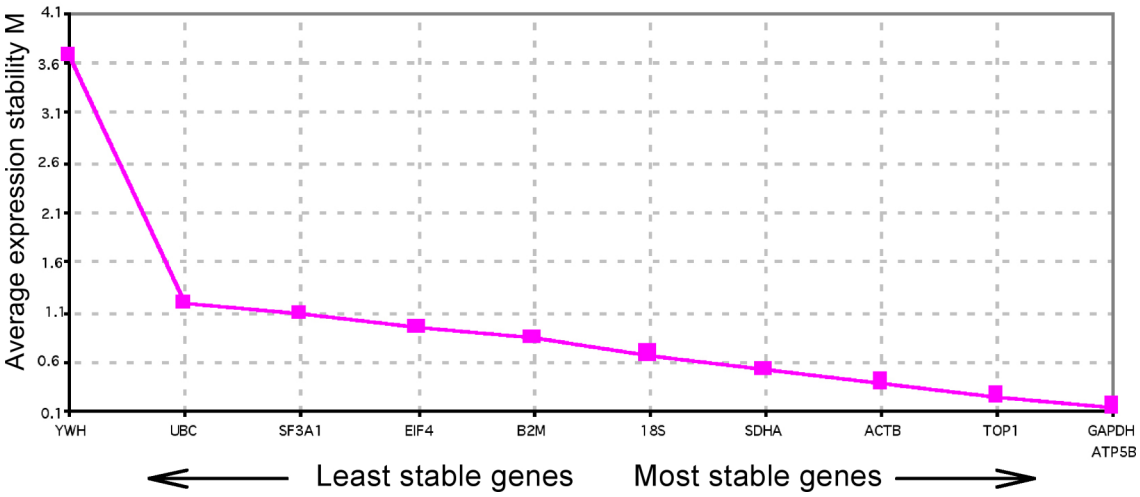


Figure 2.2 Expression stability values of control genes.
3 control and 3 TGF-β2 (1ng/ml) stimulated NHLF cultures were harvested at 24h, mRNA extracted and RT-PCR performed with 10 genes. GeNorm analysis of the results identified *GAPDH* and *ATP5B* as most stably expressed following TGF-β2 exposure.

2.5.7 Formaldehyde gel electrophoresis

A 1.2% Formaldehyde agarose gel was prepared (Table 2.5) and cast. Once cool, the gel was equilibrated in 1x FA buffer for 30 min, before 1µg RNA samples in a total volume of 8µl were prepared in 2µl of 5x Loading buffer (Table 2.6), heated to 65°C for 5 min and chilled on ice before loading into gel. The gel was run at 100V for between 60-90 min and visualised with UV light.

1.2% FA Gel	10x FA Gel Buffer (pH 7.0)
1.2g Agarose	200mM MOPS
10ml 10x FA buffer	50mM Sodium acetate
90ml RNase free water	10mM EDTA
1.8ml 37% Formaldehyde	For 1x buffer, 100ml 10x + 20ml 37% formaldehyde + 880ml RNase free water
1ul 10mg/ml Ethidium bromide	

Table 2.5 Gel and gel buffer for nucleic acid quality assessment

16µl saturated aqueous bromophenol blue
80µl 500mM EDTA pH8
720µl 37% Formaldehyde
2ml 100% Glycerol
3084µl Formamide
4ml 10x FA gel buffer
RNase free water to 10ml

Table 2.6 5x RNA loading buffer

2.6 Intra-cellular protein detection by immunoblot

Fibroblasts were grown in 6 well plates in complete DMEM until 80% confluent. Wells were rinsed with serum-free DMEM and cells quiesced in SF-DMEM for 24 hours prior to challenge. Cells were stimulated with a range of TGF-β2 concentrations (1ng-20ng/well), for 15 minutes at 37°C. Cells were washed with ice-cold PBS, 500µl ice-cold lysis buffer added, and cells scraped into a 1.5ml eppendorf. Cell lysates were mixed on a rotory mixer for 30 min at 4°C, then centrifuged at 13000*g* for 5 min. The supernatant was retained and stored at -80°C until use in western blotting analyses.

2.6.1 SDS-PAGE

This technique allows the seperation of proteins according to molecular weight. 10% bis-tris acrylamide gel solutions were prepared and cast in 1mm BioRad glass plates. 20µg total protein samples in 2x Laemmli SDS-PAGE

sample buffer were loaded into wells. Electrophoresis was carried out at 200V for approximately 1hour.

2.6.2 Western Blotting

SDS-PAGE gels were transferred to a nitrocellulose membrane at 30V for 90 min. After transfer, gels were discarded and the membrane probed with Ponceau stain (Sigma Aldrich) to confirm transfer of proteins onto the membrane. Non-specific protein binding was reduced by blocking the membrane in 5% non-fat milk powder in blotting buffer (10mM Tris-base, 100mM NaCl, 0.1% Tween) for 1 hour at RT. Primary antibody diluted in blocking buffer was incubated on the membrane for 1 hour at RT or O/N at 4°C, with constant agitation. The membrane was washed 6 times in blotting buffer before incubation with HRP-conjugated secondary antibody for 1 hour at RT. The membrane was washed as before then exposed to ECL reagent (Pierce) for 1 min. The membrane was drained, wrapped in clingfilm for protection and stored in a lightproof cassette until film exposure (within 30 min of ECL reaction). In a darkroom, light sensitive film (Kodak) was placed against the membrane in the cassette and exposed for 1 min. The film was then removed and placed in developing solution until bands appeared, transferred to a fixative solution then rinsed in water before allowing to dry.

2.8 Proteolytic activity assay

The synthetic amino acid substrate, N-benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride (Sigma-Aldrich), was dissolved in methanol to a stock concentration of 25mM. All other components are listed in Table 2.7.

Component	Diluent	Final concentration	Supplier
Trypsin	PBS	3U/ml	Sigma-Aldrich
Papain	PBS	200mU/ml	Sigma-Aldrich
HDM Skin Prick Test Extract	SF-DMEM	100000U/ml	ALK-Abello
HDM Fecal pellet prep.	SF-DMEM	1mg/ml (saturated)	Indoor Biotechnologies
E64	PBS	10μM	Sigma-Aldrich
PMSF	Methanol	1mM	Sigma-Aldrich

Table 2.7 Proteolytic activity assay components

50μl protease samples were added to a 96 well plate in triplicate. 10μl reducing agent (1mM DTT) was added to cysteine protease samples and allowed to equilibrate for 5min at RT. 20μl protease inhibitor, cysteine protease inhibitor E-64, serine inhibitor PMSF or Complete Mini protease inhibitor cocktail (Roche, UK) stock prepared as per the manufacturers instructions, was added to negative controls. All samples were made up to 150μl with PBS and allowed to stand for 15min at RT. Amino acid substrate was diluted to a final concentration of 1mM, and 50μl added to each well. The plate was protected from light and held at 37°C. Absorbance readings at 405nm were taken at 20min intervals over 2h and reaction progression curves prepared.

2.9 TGF-β detection by ELISA

TGF-β1 and TGF-β2 E_{max} Immunoassay Systems were used according to manufacturer's instructions (Promega UK Ltd, Southhampton, UK). These kits allow detection of biologically active TGF-β1 and TGF-β2 isoforms. 96-well Nunc Maxisorp ELISA plates (Thermo Fisher Scientific, UK) were coated with either a monoclonal TGF-β1 or -β2 specific capture antibody (supplied), O/N at 4°C in carbonate coating buffer. The plate was warmed to RT for 15 min and emptied, then 270μl of blocking buffer (supplied) pipetted into each well. The plate was sealed and incubated at 37°C for 35 min. Samples and standards were prepared. Active TGF-β standards are supplied at 1μg/ml, and were used between 15.6pg/ml and 1000pg/ml. The standard curve is linear between

32pg/ml and 1000pg/ml. Samples were either untreated to detect the active portion only or heated at 80°C for 6min (which activates any latent TGF-β present) to detect total (latent + active) TGF-β. Following activation samples were chilled on ice until use. Samples and standards (100µl/well) were incubated for 90 min on a plate shaker at RT. The plate was washed 5 times using TBS-Tween wash buffer then incubated with 100µl of rabbit anti-human TGF-β polyclonal antibody diluted 1:2000 in sample buffer (supplied), for 2 hours at RT, on a plate shaker. Plates were washed 5 times as before, and an HRP-conjugated anti-rabbit antibody diluted 1:100 in sample buffer added at 100µl well. A 2 hour incubation at RT with shaking followed. After plate washing, 100µl of room temperature TMB One solution was added to wells, and incubated at RT for 15 min. The chromogenic reaction was stopped with the addition of 100µl 1N HCl to each well. Absorbance was read at 450nm using an Asys UVM340 microplate reader (Biochrom Ltd., UK). A standard curve was created and sample concentration calculated.

2.9 Data analysis and statistics

All statistical analyses were carried out using Prism version 5.0b (GraphPad Software, USA). Values for immunohistochemistry measurement and scoring were analysed by Student's unpaired t-test. Subsequent correlations were computed by Spearman's rank correlation coefficient. Values for mRNA expression and protein production were analysed by one way ANOVA followed by Dunnett's test where stimulated samples were compared to control only, or followed by Tukey's test when all samples were compaired pair-wise. In all analyses a p value of <0.05 was considered significant.

Chapter 3: Evidence of airway wall remodelling in mild asthmatic bronchial biopsy tissue

3.1 Overview

Airway wall remodelling (AWR) contributes to the decline in respiratory function and symptom persistence observed in many asthmatics (Bai, 2009). AWR is independent of inflammation and largely unaffected by current medications (Bourke *et al.*, 2010; Ward and Walters, 2005). Thickening and/or stiffening of the conducting airway wall may reduce airflow by reduction of airway diameter (Aysola *et al.*, 2008; James *et al.*, 1989; Mitchell *et al.*, 1998).

Alterations in the composition of the asthmatic airway wall have been described, and include changes in both matrix and cellular components (Johnson and Burgess, 2004; Li and Wilson, 1997; Roche *et al.*, 1989). Increased deposition of ECM molecules including collagens I, III and V in the lamina reticularis have been shown to lead to reticular basement membrane thickening (Wilson and Li, 1997), and have been linked to measures of airway hyper-responsiveness and asthma severity (Kariyawasam *et al.*, 2007; Westergren-Thorsson *et al.*, 2002). Differentiation of fibroblasts to a myofibroblastic phenotype can occur. Myofibroblasts are associated with fibrotic conditions and identified by accumulation of stress fibres and smooth muscle related proteins (Hinz *et al.*, 2007b). Alterations in matrix production may follow this phenotypic change due to increased activation and matrix secretory properties of myofibroblasts. Additional structural changes observed in asthma include goblet cell hypertrophy, submucosal gland hyperplasia, smooth muscle hypertrophy and increased angiogenesis (Johnson and Burgess, 2004; Ordoñez *et al.*, 2001).

Reactivation of the epithelial-mesenchymal trophic unit has been suggested as an initiator of AWR in asthma (Holgate *et al.*, 2004). Comprising the epithelium, lamina reticularis and cells immediately adjacent in the lamina

propria, the EMTU is identified during embryonic development by tenascin C deposition. Tenascin C is increased in asthmatic bronchial biopsy tissue following segmental allergen challenge and increased expression has been identified in asthmatic biopsy tissue (Flood-Page *et al.*, 2003; Phipps *et al.*, 2004b). The EMTU represents the primary site of environment-host interaction in the lung, and continuous phenotypic or behavioural changes in cells residing in this area may be indicative of the breakdown of normal homeostasis.

If the purported effects of AWR in the asthmatic individual arise from matrix component alterations and/or increases, it leads to the question: what drives the remodelling response? As a pro-fibrotic cytokine, expressed in healthy lung and identified as an inducer of pathology in a number of fibrotic diseases, transforming growth factor-β has been investigated as a determinant of AWR in several studies. Correlations between TGF-β1 positive cell number and TGF-β intra-cellular signalling with RBM thickness have been found (Sagara *et al.*, 2002; Vignola *et al.*, 1997). It has been demonstrated that the TGF-β2 isoform predominates in bronchial tissue (Balzar *et al.*, 2005a; Minshall *et al.*, 1997; Torrego *et al.*, 2007), and expression is increased upon epithelial cell injury (Holgate *et al.*, 2000).For this reason, the TGF-β2 isoform was chosen for further investigation in this project.

3.2 Aims and Hypothesis

This chapter aims to address the hypothesis: there is no significant difference in AWR between healthy and asthmatic bronchial biopsy tissue.
The hypothesis shall be tested through the following aims:

1. Quantify remodelling phenomena: RBM thickness, myofibroblast differentiation and tenascin C deposition.
2. Determine relative expression of TGF-β2 in healthy compared with asthmatic tissue.
3. Assess correlation of TGF-β2 expression with remodelling phenomena.

3.3 Results

Bronchial biopsy tissue collected from 10 healthy and 12 mild asthmatic individuals was analysed. Paraffin wax embedded sections (5μm) were cut and collected onto poly-L-lysine coated slides. Standard histological stains were used to highlight tissue architecture and selected features.

3.3.1 Reticular basement membrane thickness

The RBM, or lamina reticularis, is composed primarily of collagens I, III and V and lies immediately below the true basement membrane- a thin meshwork of type IV collagen. RBM thickness has been has been used in several studies as a measure of AWR. In this study, periodic acid-Schiff's /alcian blue or Masson's trichrome stained sections were utilised, as both allowed a clear differentiation between the RBM and the underlying lamina propria.

Three representative sections from each biopsy were analysed to produce an average measurement per sample. All 12 asthmatic and 10 healthy biopsy samples were examined and data normally distributed according to the Shapiro-Wilk test. Healthy tissue mean measurement range was 3.13-9.21μm (Figure 3.1), and 3.03-10.18μm in asthmatic tissue (Figure 3.2). Mean RBM thickness was significantly different between groups as calculated by unpaired t-test (Figure 3.3), with asthmatic tissue showing a significantly thickened RBM (healthy mean ± SEM = 6.038 ± 0.562; asthmatic mean ± SEM = 7.824 ± 0.537; $p<0.05$).

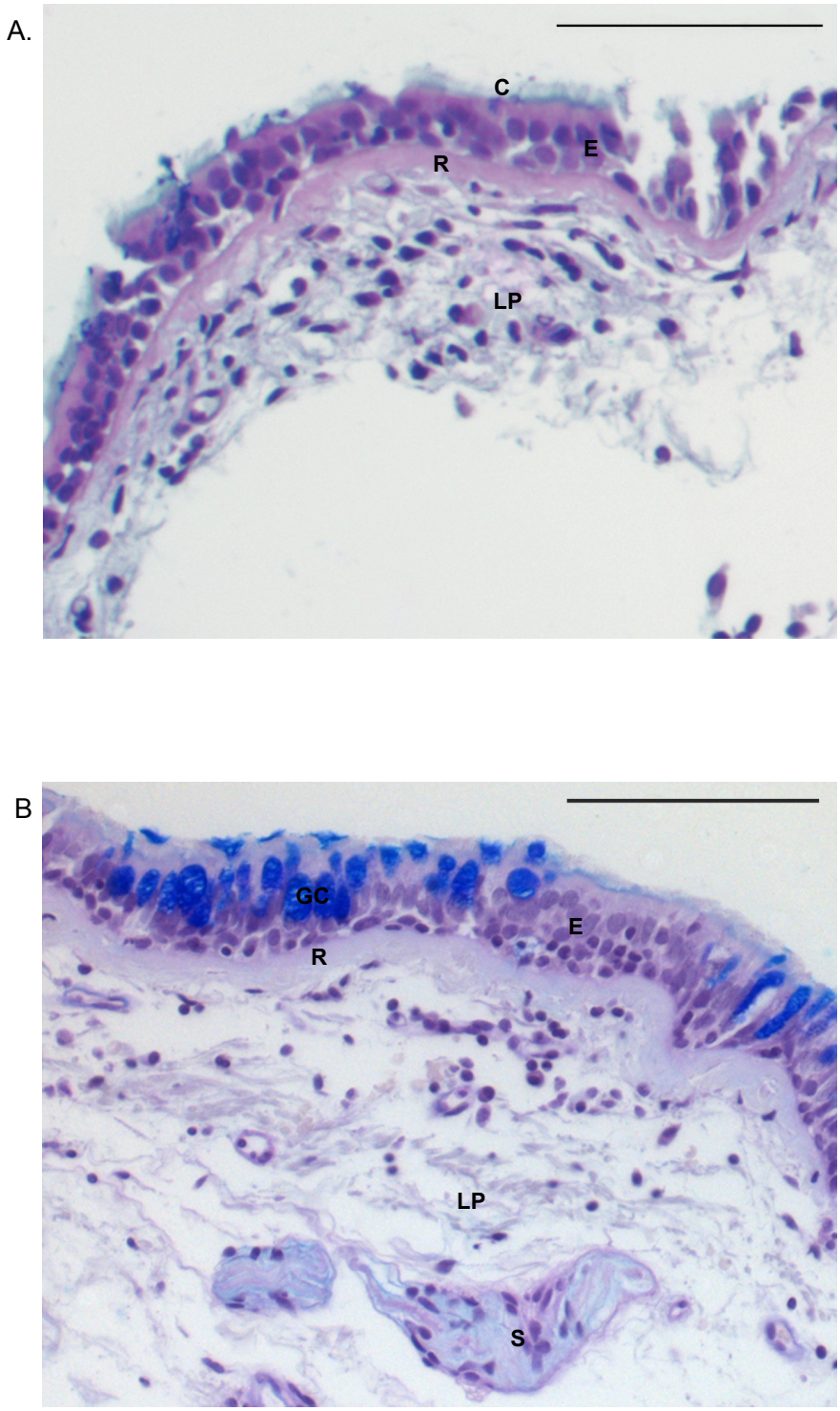


Figure 3.1 Appearance of RBM in healthy bronchial biopsy tissue.
Mean RBM thickness A=3.13µm and B= 9.21µm. PAS/Alcian blue stained 5µm sections of biopsy tissue were imaged using a Spot camera and analysed using Image Pro-plus. C- cilia; E- Epithelium; GC- Goblet cell; LP- Lamina Propria; R-RBM; S- Smooth muscle. Scalebar - 100µm.

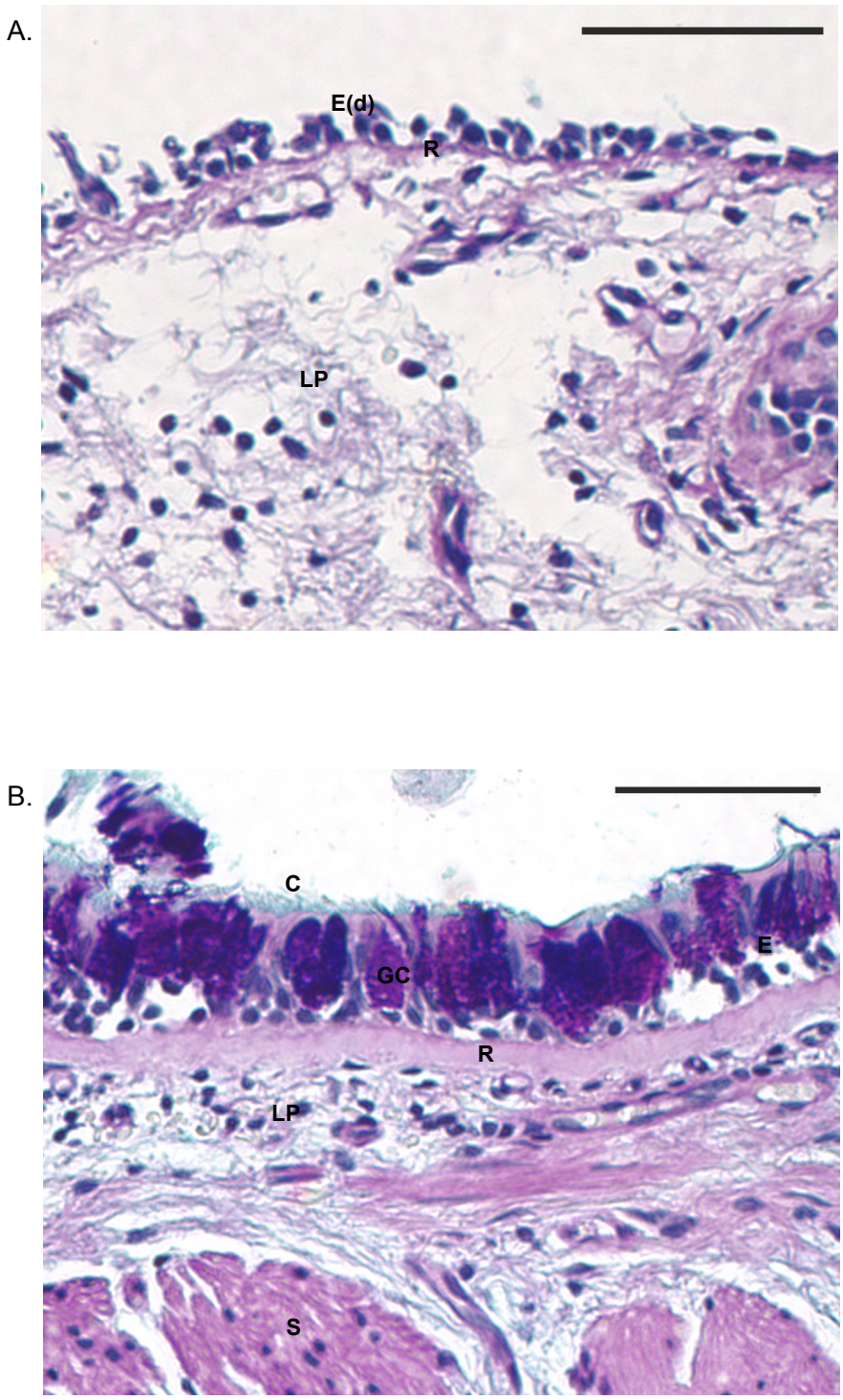


Figure 3.2 Appearance of RBM thickness in asthmatic bronchial biopsy tissue. Mean RBM thickness in A=5.28μm and B= 10.18μm. PAS/Alcian blue stained 5μm sections of biopsy tissue were imaged using a Spot camera and analysed using Image Pro-plus. C- cilia; E- Epithelium; E(d)- Epithelium (denuded); GC- Goblet cell; LP- Lamina Propria; R-RBM; S- Smooth muscle. Scalebar - 100μm.

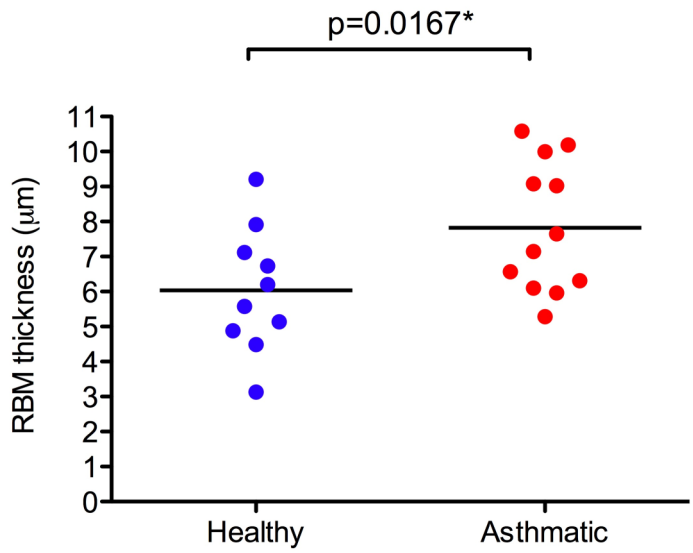


Figure 3.3 Mean RBM thickness in healthy vs. asthmatic biopsy tissue. Asthmatic RBM was significantly thicker than healthy sample RBM. Each data point represents the average measurement in μm from 3 sections of one biopsy, one biopsy per subject. Healthy mean ± SEM = 6.038 ± 0.562, range 3.13-9.21; Asthmatic mean ± SEM= 7.824 ± 0.537, range 5.28-10.57; $p<0.05$ (n=10 healthy & n=12 asthmatic).

3.3.2 Myofibroblast frequency in lamina propria

To assess myofibroblast differentiation, three sections from each biopsy were probed with a monoclonal anti-α-smooth muscle actin antibody. Bands of smooth muscle within the tissue were used as an internal positive control. α-SMA positive cells in each section were counted, limited to a depth of 100μm beneath the true basement membrane as the interest here was on cells directly involved in epithelial-mesenchymal interactions. Sections where dehydration of the tissue had resulted in diffusely spread collagen fibres were excluded to ensure a similar area of complete lamina propria was assessed in each case. Smooth muscle, sub-mucosal gland and blood vessel-associated positive cells were not counted. Where cell type was in doubt, such as around small blood vessels, cell shape was accounted for, and cells of the characteristic spindle-shape of fibroblasts were included. Data are expressed as the mean percentage of total cells stained positively (Figure 3.4).

Myofibroblast frequency was significantly increased in asthmatic compared with healthy tissue (Figure 3.5), as assessed by unpaired t-test.

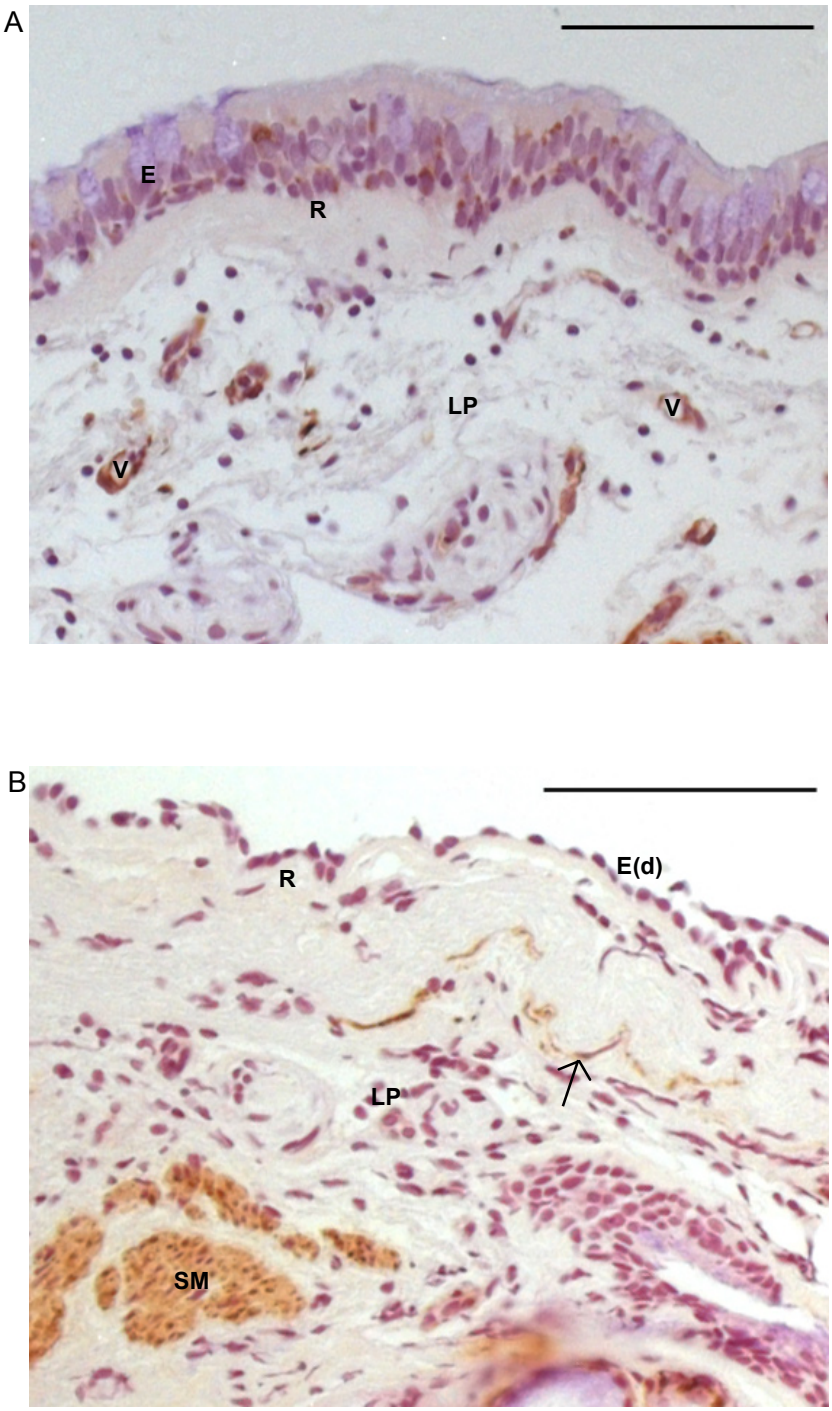


Figure 3.4 α-SMA immunoreactivity in healthy and asthmatic bronchial biopsy tissue. 5μm sections were probed with a monoclonal anti-α-SMA antibody, developed with DAB and counterstained with haematoxylin. A. healthy biopsy tissue, and B. asthmatic biopsy tissue with smooth muscle "block" staining used an internal positive control. Arrow identifies a line of myofibroblast-like cells immediately below the RBM. E- Epithelium; E(d)- Epithelium (denuded); LP- Lamina Propria; R-RBM; S- Smooth muscle; V- vessel. Scalebar - 100μm.

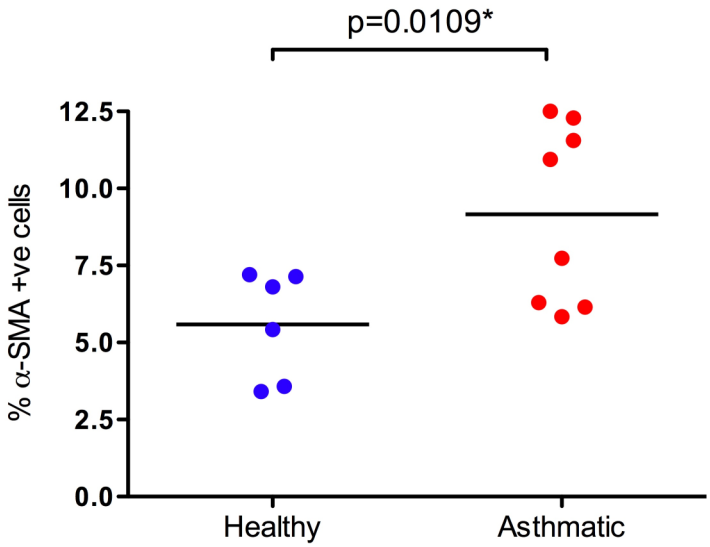


Figure 3.5 Frequency of α-SMA immunopositive fibroblast-like cells in healthy and asthmatic bronchial biopsy tissue. Data points represent percentage of positive cells in lamina propria area to depth of 100μm from basement membrane, in 3 sections from each sample. Healthy mean ± SEM = 5.593 ± 0.714; Asthmatic mean ± SEM = 9.164 ± 1.037; $p<0.05$ (n=6 healthy & n=8 asthmatic).

3.3.3 Tenascin C immunoreactivity

Tenascin C expression has been reported in the RBM of asthmatics during "active" remodelling as assessed following segmental allergen challenge. *De novo* deposition has been suggested as a marker of EMTU re-activation and subsequent remodelling in asthmatic lung.

A single section from each biopsy was immunostained with an anti-tenascin C antibody and total immunoreactivity scored on a scale from 0-3. Tenascin C immunoreactivity was seen in the RBM against the true basement membrane as expected. Expression was not significantly different between groups as assessed by unpaired t-test (Figure 3.6). Both healthy and asthmatic tissue showed a range of staining intensity, however no asthmatic samples scored 0 (no staining) compared with 2 healthy samples, indicating possible increased basal levels of repair and regeneration in asthmatic tissue.

3.3.4 Transforming Growth Factor β-2 immunoreactivity

It has been reported that asthmatic lung tissue shows increased TGF-β2 levels. Increased TGF-β2 in the asthmatic lung may be an indicator of fibrotic and/or anti-inflammatory activity. Several studies report an increase in TGF-β2 levels following allergen challenge, while TGF-β1 levels remain unchanged.

A single section from each biopsy was probed with a TGF-β2 polyclonal antibody and scored from 0-3. In this study, TGF-β2 was localised primarily to the epithelial layer and smooth muscle bands, with limited scattered cell positivity in the lamina propria in both healthy and asthmatic subjects (Figures 3.7 & 3.8). There was no significant difference in TGF-β2 score in either the epithelium or smooth muscle compartments (Figure 3.9), or the lamina propria (data not shown). Figure 3.10 shows the frequency distribution of total (epithelium + smooth muscle + lamina propria) TGF-β2 scoring of 10 healthy and 12 asthmatic biopsy samples. There is a trend towards increased TGF-β2 scoring in the asthmatic group that is not statistically significant.

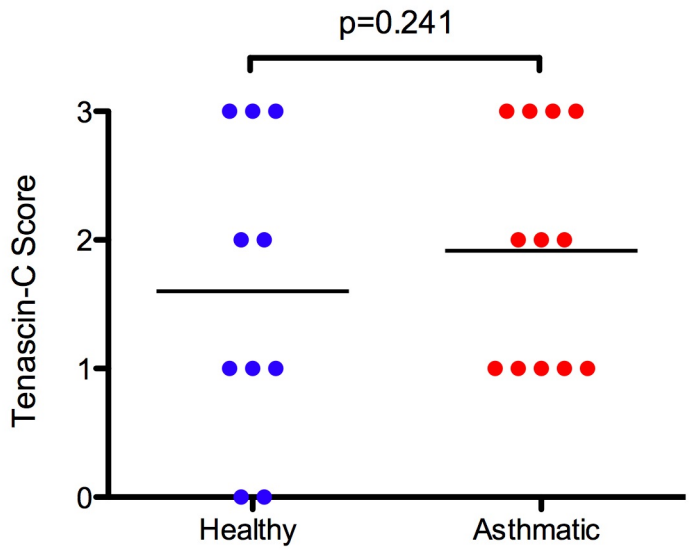


Figure 3.6 Tenascin-C immunoreactivity score of healthy and asthmatic biopsy tissue. 5μm tissue sections were immunostained with a monoclonal tenascin C antibody, developed with DAB substrate and counterstained with haematoxylin. Images were taken with a Spot-32 camera and scored blindly in 3 independent screenings. Healthy mean ± SEM= 1.600 ± 0.371; Asthmatic mean ± SEM= 1.917 ± 0.259; $p>0.05$. 0= no staining, 1=mild, 2= moderate, 3= intense immunostaining.

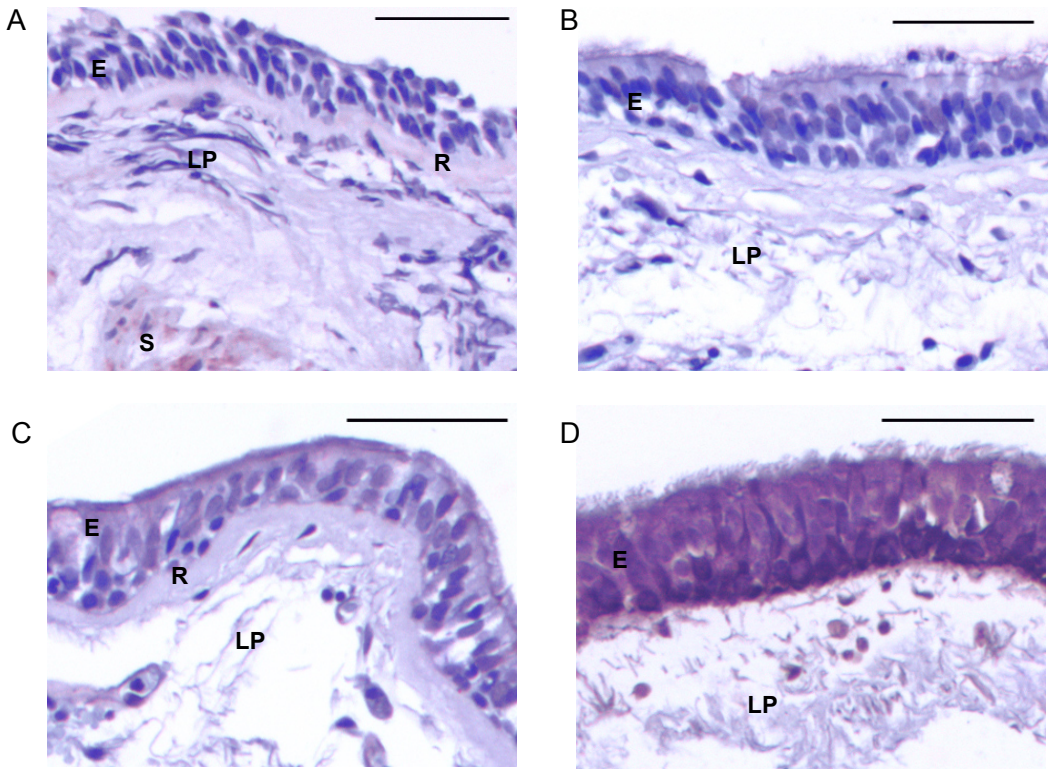


Figure 3.7 TGF-β2 immunoreactivity in healthy bronchial biopsy tissue. 5μm sections were probed with a polyclonal TGF-β2 antibody, developed with DAB substrate and counterstained with haematoxylin. Images were taken with a Spot-32 camera and analysed. Epithelial staining intensity scored as A=0, B=1 mild, C=2 moderate and D=3 intense. E- Epithelium; LP- Lamina propria; R- RBM; S- Smooth muscle. Scalebar - 50μm.

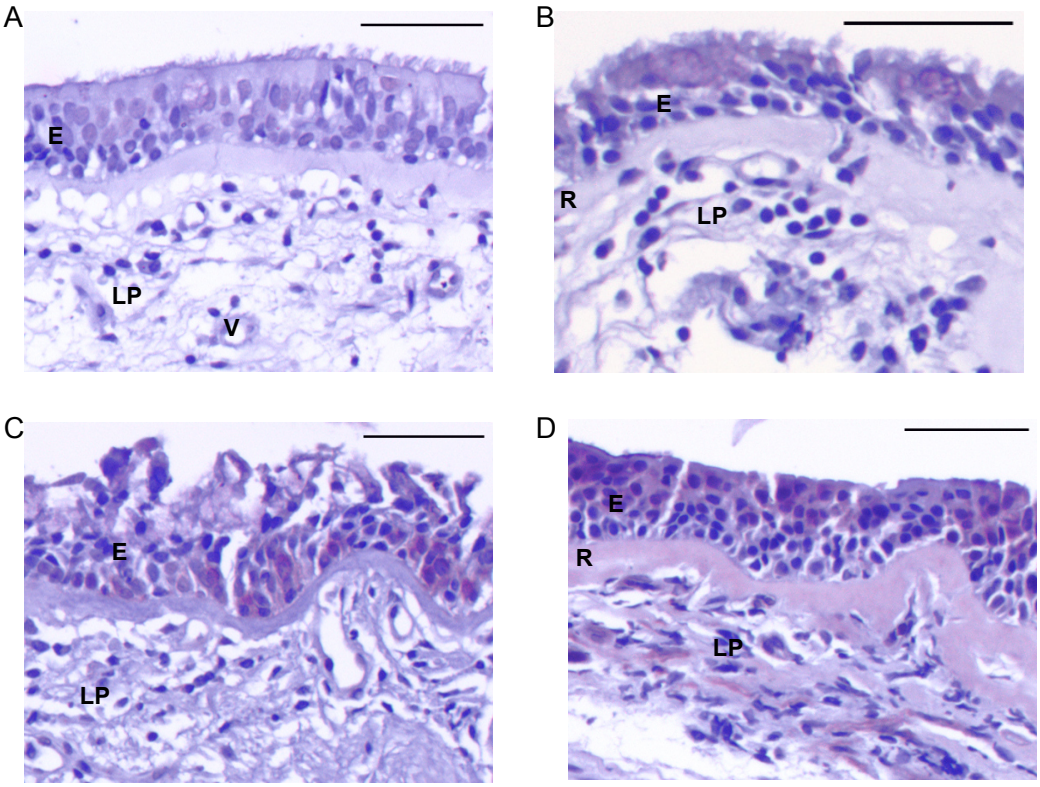


Figure 3.8 TGF-β2 immunoreactivity in asthmatic bronchial biopsy tissue. 5μm tissue sections were incubated with a polyclonal TGF-β2 antibody, developed with DAB substrate and counterstained with haematoxylin. Images were taken with a Spot-32 camera and analysed. Epithelial staining intensity scored as A=0, B=1, C=2 and D=3. E- Epithelium; LP- Lamina propria; R- RBM; V- Vessel. Scalebar - 50μm.

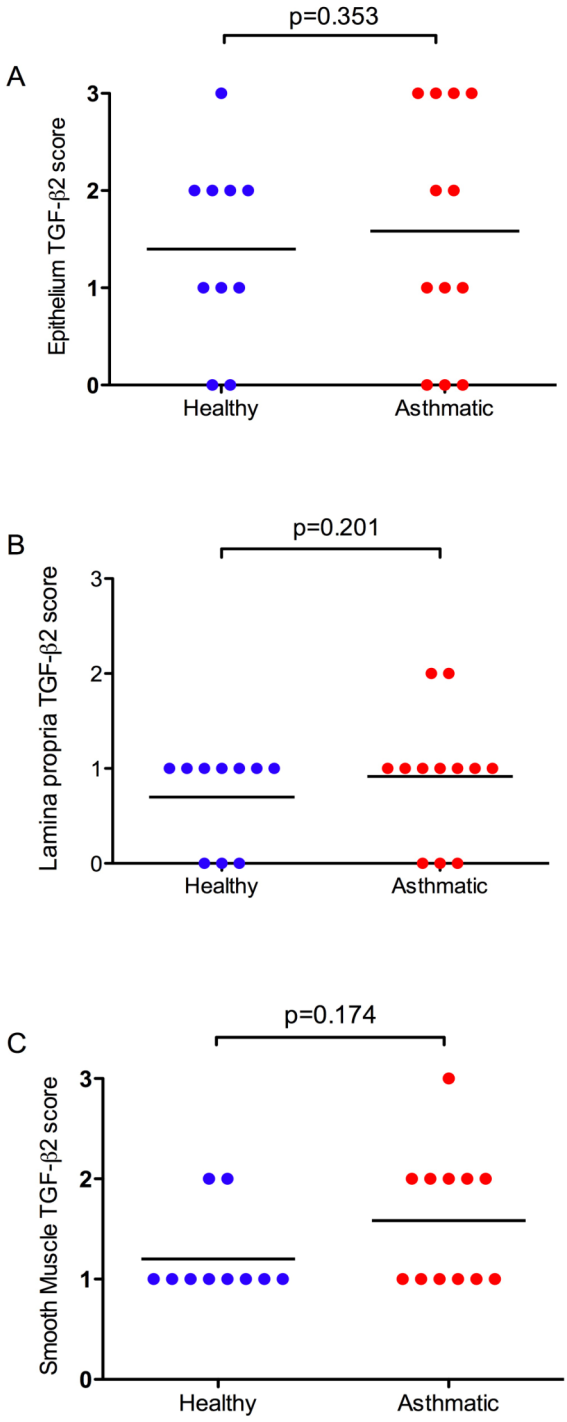


Figure 3.9 TGF-β2 immunoreactivity scores of epithelial and smooth muscle bronchial tissue compartments. Tissue sections were scored blindly on a scale from 0-3 as for Tn-C. Healthy mean± SEM: epithelium=1.40±0.305, lamina propria=0.700±0.152, smooth muscle= 1.20±0.133. Asthmatic mean ± SEM: epithelium= 1.58±0.358, lamina propria= 0.9167± 0.1930, smooth muscle = 1.46± 0.215. Unpaired t-test showed no significant difference.

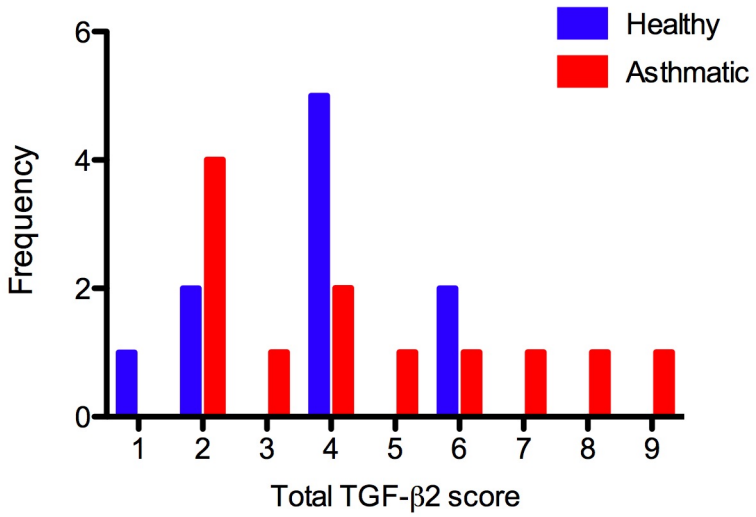


Figure 3.10 Frequency of total TGF-β2 score distribution. Total of epithelium, lamina propria and smooth muscle scores for each sample, shown as the number of samples (frequency) to attain each total score. A trend towards increased asthmatic total score, and therefore biopsy immunoreactivity, is apparent.

3.3.5 TGF-βReceptor III (TβRIII)

TGF-β2 has low binding affinity for the TβRI - TβRII hetero-dimer receptor complex that facilitates signal transduction. In contrast to other isoforms, TGF-β2 requires the TβRIII receptor (betaglycan) to present the TGF-β2 dimer to the signalling complex.

A single section from each biopsy was probed with a polyclonal TβRIII antibody and examined (Figure 3.11). Positive immunoreactivity was observed primarily in the epithelium, with scattered weak staining in the lamina propria and smooth muscle, similar to that reported by Balzar *et al.* (Balzar *et al.*, 2005a). No difference was observed between healthy and asthmatic samples.

3.3.6 Correlation of TGF-β2 and remodelling phenomena

TGF-β2 is a pro-fibrogenic cytokine, and expression has been linked to increased AWR in asthmatics (Chu *et al.*, 2004). To investigate whether this finding was repeated in this sample cohort, Spearman's rank correlation coefficient (R_S) was calculated for all samples, assessing correlation between epithelial, lamina propria, smooth muscle and total TGF-β2 scores with RBM thickness, myofibroblast frequency and TnC expression. The R_S values for each calculation are shown in Table 3.1. There were no statistically significant associations between TGF-β2 and remodelling, with one exception: healthy epithelial TGF-β2 scores associated with myofibroblast frequency, $p=0.024$, $R_S=-0.771$. Asthmatic lamina propria TGF-β2 and myofibroblast frequency approached significance at $p=0.057$ and $R_S=-0.617$. Following on from the work of Brewster *et al.* (Brewster *et al.*, 1990), the inter-relation betweeen RBM thickness and myofbroblast frequency was assessed, shown in a scatter plot in Figure 3.12. Significant correlation was not found in this sample cohort, however clustering of samples by disease status is apparent.

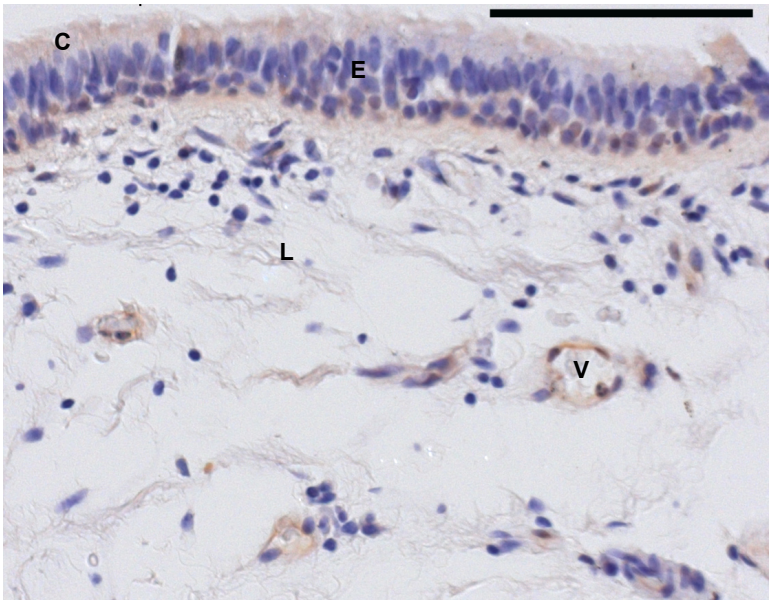


Figure 3.11 TGF-β Receptor III expression in bronchial biopsy tissue. 5μm sections of healthy and asthmatic biopsy tissue were immunostained with anti-TβRIII, counterstained with haematoxylin. A representative image taken using a Spot camera is shown. Scalebar - 100μm.

Remodelling / TGF-β2 Score	RBM thickness	Myofibroblast freq.	Tenascin-C score
Epithelium	0.050 -0.222	-0.252 -0.771*	-0.272 -0.396
Smooth muscle	-0.377 -0.037	-0.026 -0.612	-0.178 -0.284
Lamina propria	0.011 0.174	-0.617 0.474	-0.302 0.215
Total tissue score	0.242 -0.039	0.024 -0.298	0.028 -0.383

Table 3.1 Spearman Rank (R_S) correlation statistics. Remodelling markers and TGF-β2 score were analysed together in asthmatic (red) and healthy (blue) tissue.

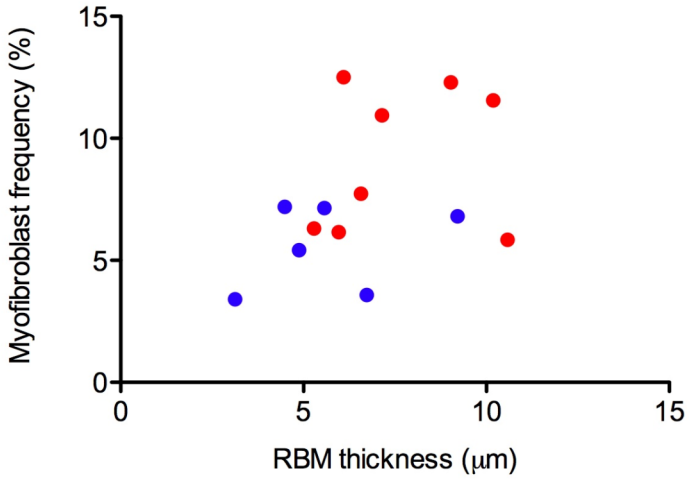


Figure 3.12 RBM thickness and myofibroblast frequency correlation. Asthmatic (red) and healthy (blue) scores were plotted. Spearman rank correlation analysis indicated no significant association between these variables, although clustering of each phenotype is apparent. Healthy p=0.401, R_S=0.142; Asthmatic p=0.303, R_S= -0.187.

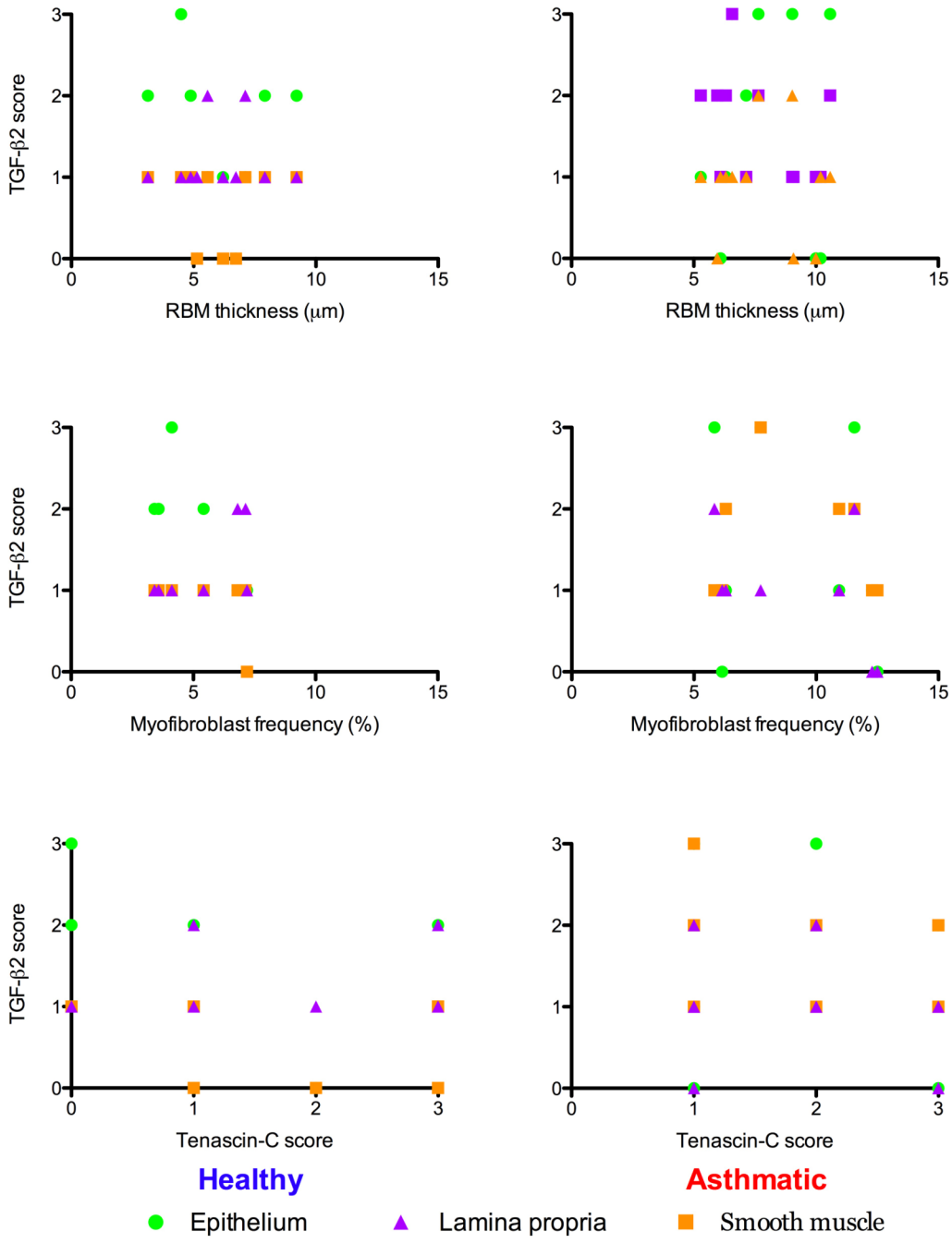


Figure 3.13 TGF-β2 expression scores of epithelium, lamina propria and smooth muscle plotted against RBM thickness, myofibroblast frequency and tenascin-C expression. No statistically significant differences between healthy and asthmatic samples were observed, but a minor trend of healthy samples grouping on the lower end of the scale, and asthmatic towards the upper is apparent.

Scatter plots of TGF-β2 scores (for individual tissue compartments) vs. remodelling scores are shown (Figure 3.13). Although not significant, a trend towards clustering of healthy samples to lower scale, and asthmatic to the upper is apparent, suggestive of transient increases in TGF-β2 expression in asthmatic tissue.

3.4 Summary

This study assessed bronchial biopsy tissue from 12 mildly asthmatic and 10 healthy individuals, for remodelling phenomena and TGF-β2 expression. Additionally, association of TGF-β2 expression with remodelling features was investigated. Evaluation of biopsy tissue was undertaken to prime future *in-vitro* work using primary bronchial cells, to inform whether mildly asthmatic EMTU cells have an altered phenotype and could thus produce different responses in culture.

For RBM thickness measurements results were calculated from 3 independent evaluations of each image. Intra-observer variation was below 8%, similar to that reported by Chu *et al.* Asthmatic samples displayed a significantly thicker RBM compared with healthy control tissue, similar to and in agreement with other published studies (Chu *et al.*, 1998; James *et al.*, 2002; O'Shaughnessy *et al.*, 1997)

Frequency of myofibroblast differentiation was determined in lamina propria cells. Asthmatic tissue had a statistically significant increase in myofibroblast frequency compared to healthy tissue.

Tenascin C expression levels were not significantly different between healthy and asthmatic tissue. However, none of the asthmatic samples scored 0, suggesting a greater incidence of stress/injury and therefore subsequent TnC response in that group.

TGF-β2 expression was evaluated in a single section of each biopsy using a polyclonal antibody following sodium citrate antigen retrieval. Expression in

separate anatomical "compartments" was scored, comprised of the epithelium, lamina propria and smooth muscle. There were no statistically significant differences in these samples, however a trend towards increased total TGF-β2 expression levels in asthmatic tissue was observed.

In correlation analyses, only epithelial TGF-β2 and myofibroblast frequency expression in healthy samples reached statistical significance. Asthmatic lamina propria TGF-β2 expression and myofibroblast frequency approached significance at p=0.057.

In summary, measurable statistically significant evidence of remodelling was shown in mildly asthmatic tissue used in this study, therefore the null hypothesis that there is no significant difference in measures of AWR between healthy and asthmatic bronchial biopsy tissue can be rejected.

3.5 Discussion

Airway wall remodelling (AWR) encompasses alterations both in the extracellular matrix and cells resident in the airway, and contributes to the symptom persistence and decrease in respiratory function observed in many asthmatics (Bai, 2009). AWR is largely untreated by current medications and as such, understanding of the processes that drive AWR may lead to improved treatment options. Reactivation of the epithelial-mesenchymal trophic unit has been posited by some researchers as an initiator of AWR (Holgate *et al.*, 2004). This study aimed to quantify commonly held markers of the remodelling process: reticular basement membrane (RBM) thickness, myofibroblast differentiation and tenascin C (TnC) deposition, in bronchial biopsy tissue. The pro-fibrotic, anti-inflammatory cytokine TGF-β2 was investigated as it is transiently upregulated in allergic asthma, and may also be a contributory factor in AWR processes.

RBM thickening is the typical measure of AWR in asthma. Investigating the hypothesis that AWR led to thickened airway walls and thus decreased respiratory function, a number of studies showed RBM thickness correlated with increased overall thickness of the bronchial wall measured by high-

resolution computerised tomographic scanning (James *et al.*, 2002; Kasahara *et al.*, 2002). Other studies have shown a positive correlation between increasing RBM thickness and inhaled glucocorticoid use (indicating increased severity of asthma), with greater increases demonstrated in atopic compared with non-atopic asthmatics (Amin *et al.*, 2000). Whether RBM thickness may be used as a marker of asthma severity is a point for debate. Several groups have shown a positive correlation between increased RBM thickness and decreased FEV1% predicted(Bourdin *et al.*, 2007; Hoshino *et al.*, 1998b; Shiba *et al.*, 2002) whilst others show no association(Chu *et al.*, 1998; Hoshino *et al.*, 1998a; Liesker *et al.*, 2009; Tillie-Leblond *et al.*, 2008). The disparity between groups may be explained by the use of single biopsies from different levels of the bronchi, the grade of asthmatic severity used (mild vs. moderate vs. severe), and the criteria used to to determine these classifications. Different methods used in measuring RBM thickness may also play a role.

Two methods of calculating RBM thickness are predominant in the literature. Sullivan *et al.* devised a multiple point-to-point method of measurement, whereby the top and bottom of the RBM are marked then multiple top-to-bottom measurements made along the length of the selection (Sullivan *et al.*, 1998). Wilson & Li selected whole areas of RBM and performed area/length calculations (Wilson and Li, 1997). To ensure their method was robust, Wilson & Li used transmission electron microscopy (TEM) measurements as a "gold standard" for comparative purposes. Use of TEM was not practical for this study due to the requirement for multiple biopsies for specialised sample preparation; with a single biopsy available per patient, wax-embedding tissue allowed both histological and immunohistochemical analyses. A comparative study found both methods produced similar results, as long as sufficient length of RBM was included in the analysis (Bourdin *et al.*, 2007). The point to point method of Sullivan *et al.* was chosen for this study as it was marginally less reliant on biopsy tissue with continuous straight lengths of RBM to generate reliable data.

All asthmatic biopsy samples used in this study were obtained from mild, steroid-naïve asthmatic subjects. Asthmatic RBM was significantly thicker

compared with healthy samples, even within the small sample size. Studies using similarly designated tissue, reported results within comparable limits. Chu *et al.* report RBM thickness increasing with severity of disease, from control bronchial biopsy tissue at 5.2μm, mild asthmatic 5.5μm, to moderate and severe at 6.8μm and 6.5μm respectively, although as in this study each subset contained a wide range of measurements (Chu *et al.*, 1998). It is of note that the severe asthmatic mean was marginally less than the moderate. This may be attributable to the use of oral steroids in this subset, which can exert a small reducing effect on RBM thickness during treatment, as demonstrated in other studies (Shiba *et al.*, 2002). RBM thickness appears fairly fixed by 6 years of age in asthmatics, and so variability in age of study participants should not affect results (Bush, 2008). RBM thickening is not confined to asthmatic lung and has been quantified in eosinophilic bronchitis, allergic rhinitis and cystic fibrosis (Brightling *et al.*, 2002; Hilliard *et al.*, 2007). It is unknown whether the mechanism of thickening is the same. That it is identifiable in other disease states points to it being a response to bronchial stress. It has been suggested that RBM changes may protect from bronchospasm (Milanese *et al.*, 2001).

Myofibroblasts are fibroblast-like cells with increased matrix synthesis capacity and augmented contractile features. They are associated with tissue repair and fibrosis and are the main matrix-producing cell in tissue repair events throughout the body (Brewster *et al.*, 1990). The physical strain of asthma attacks and increased TGF-β isoform production provide an ideal environment for fibroblast differentiation, which requires both active TGF-β exposure and mechanical strain (Desmoulière *et al.*, 1993). Myofibroblasts were quantified in a region of tissue limited to 100μm depth from the basement membrane. This method was selected as a consistent way to differentiate collagen deposition capacity in bronchial biopsy tissue (O'Shaughnessy *et al.*, 1997). The statistically significant increased myofibroblast representation amongst the cell population in the asthmatic samples found here is significant due to the increased matrix secretory capacity of these cells. The origin of myfibroblasts in subepithelial fibrosis is not defined. The majority differentiate from resident fibroblasts, but these

may be supplemented by migrating smooth muscle cells and circulating fibrocytes that differentiate into myofibroblast-like cells (Hinz *et al.*, 2007a). Increased secretion of cytokines by myofibroblasts has been identified, and may contribute to the continual activation state of cells resident in the asthmatic EMTU and beyond (Ward *et al.*, 2008). Previously published work used BAL fluid to assay MMP and TIMP levels to evaluate possible collagen turnover, however BAL fluid was not available for this study.

The dynamic deposition pattern of glycoprotein tenascin C at points of tissue injury/stress make it a useful marker of remodelling activity in the asthmatic bronchi. In a murine model of lung epithelial cell injury, Tn-C was increased at day 1 after injury, returning to baseline by day 3, confirming the limited temporal increase after epithelial injury of this protein (Snyder *et al.*, 2009). In human tissue, Tn-C was increased in asthmatics at baseline (Flood-Page *et al.*, 2003), and also significantly increased 24h after segmental allergen challenge in asthmatics (Phipps *et al.*, 2004a). In this study there was no significant difference between healthy and mild asthmatic biopsy tissue. All asthmatic samples were scored above background, unlike healthy tissue samples. It may be hypothesised that positive immunoreactivity in healthy and asthmatic samples is indicative of remodelling in some form being part of healthy lung homeostasis. However, as only a single section was scored from each biopsy, this cannot be considered indicative of the lung tissue.

Remodelling may be driven by increased TGF-β activity in the asthmatic lung. An investigation into TGF-β isoform expression in healthy and asthmatic biopsy tissue showed TGF-β2 to be the predominant isoform, localised primarily to the epithelium with limited expression in smooth muscle. TGF-β2 was also the only isoform to be elevated in asthmatic tissue (Balzar *et al.*, 2005b). In BAL fluid, Batra *et al.* found TGF-β2 expressed at slightly higher levels at baseline in healthy controls compared to atopic asthmatics. Allergen challenge led to a large spike in TGF-β2 levels at 24h in asthmatic subjects that was not seen in healthy controls. TGF-β1 levels also spiked in asthmatics, but in all cases TGF-β1 levels were consistently lower than TGF-β2, at all timepoints (Batra *et al.*, 2004). The results in that study did not reach

significance, however there was a trend towards increasing TGF-β2 expression in the asthmatic tissue samples. These results were echoed here, whereby a general trend towards increased TGF-β2 expression was observed but did not reach statistical significance. All the asthmatic tissue was phenotypically mild and steroid naïve; Balzar *et al.* showed TGF-β2 increasing in line with asthma severity, whilst other groups have identified increasing TGF-β1 levels proportional to asthma severity in biopsy and lavage respectively (Minshall *et al.*, 1997; Tillie-Leblond *et al.*, 1999) . Results shown here may be limited by the mild tissue phenotype used; the inclusion of moderate/severe asthmatic tissue samples may produce statistically significant increases in TGF-β2 expression.

Increased phosphorylated Smad2 protein has been described in the asthmatic epithelium at baseline (Sagara *et al.*, 2002) and after allergen challenge (Phipps *et al.*, 2004b). Upregulation of TGF-β receptor signalling could be partly explained by the finding of Nakao *et al.*, who illustrated decreased basal Smad7 (a negative regulator of the TGF-β-Smad signalling pathway) expression in the epithelial layer in asthmatic biopsy tissue (Nakao *et al.*, 2002). The reduction in downregulatory/control capacity this could effect exemplifies the multi-layered mechanisms by which TGF-β activity may be modulated in the asthmatic airway. Results such as those cited above indicate there may be increased TGF-β activity in the asthmatic lung that exclude the need for concomitant increases in active TGF-β2 levels.

Active TGF-β2 must bind to specific receptors and signal intracellularly to produce downstream effector functions. TGF-β2 presentation to the TβRI-TβRII heterodimer TβRIII expression was assessed due to the role of this molecule in TGF-β2 receptor binding. No differences were found between healthy and asthmatic tissue expression levels in this study. Animal studies have identified alterations in TβRIII expression induced by periods of hypoxia, however there is no published data on TβRIII expression in asthma (Vicencio *et al.*, 2002). It has been reported that a splice variant of TβRII (TβRII-B) is sufficient for TGF-β2 signalling so bypassing the requirement for TβRIII,

however commercial antibodies were unavailable (del Re *et al.*, 2004b; Rotzer *et al.*, 2001).

The causal relationship between the AWR features discussed earlier and asthmatic disease manifestations is broadly unknown. Delineation of these relationships relies on correlation between remodelling phenomena and other features of asthmatic disease. Increased RBM thickness has been positively correlated with AHR (Hoshino *et al.*, 1998a) and with frequency of asthma attacks (Evans *et al.*, 1999). However, other groups have found asthma exacerbations predict decline in lung function, and not the extent of remodelling (O'Byrne *et al.*, 2009). AHR measures were not taken from all subjects whose tissue was acquired for inclusion in this study, and asthma exacerbation frequency was very low in the mild steroid naïve asthmatic subjects used, with no exacerbations in the previous month as a standard minimum. Regarding TGF-β, the majority of published data concerns the -β1 isoform, and increased TGF-β1 expression in the submucosa has been identified. The same study also highlighted increased sub-epithelial fibrosis (RBM thickness) correlated to increased asthma severity (Minshall *et al.*, 1997), again indicating remodelling may directly affect AHR. Sagara *et al.* established that TGF-β specific signalling *via* p-Smad2 was increased in asthmatic bronchial tissue over healthy controls, correlated with increases in RBM thickness (Sagara *et al.*, 2002). Although correlation analyses in this study did not lead to any significant associations, there was a clear separation of the samples into their respective groups when remodelling scores were plotted against TGF-β2 expression. Again, statistical significance may only be reached with increased sample number, possibly including asthmatic biopsy tissue from moderate/severe phenotypes.

The studies discussed above add weight to the hypothesis that TGF-β2 can promote remodelling responses in asthma. It is clear from results presented here that mild asthmatic tissue undergoes remodelling processes. Cells derived from such subjects may show altered responses compared with healthy controls *in-vitro*, to be investigated in further chapters.

Regarding the mild asthmatic phenotype studied, it would be possible to differentiate between "inactive" and "active" allergic asthma through the enumeration of mast cells in the tissue. Tissue and BAL fluid from "active" asthmatics shows increased mast cell infiltration, however with the mild phenotype accessed a significant difference may not be identifiable.

The majority of TGF-β2 immunoreactivity in this study was found in the epithelial layer, but other studies have focused on TGF-β levels in specific cell populations to identify drivers or promotors of remodelling. Such work could be repeated in this sample cohort, perhaps through identification of co-localization of TGF-β2 expression with activated eosinophils, identified by EG2 immunoreactivity. Additionally, in-situ hybridisation to identify which cells are actively producing TGF-β2, or laser capture microdissection of cell populations followed by RT-PCR may provide additional information on TGF-β2 and it's downstream effects.

Chapter 4: Expression and activation of TGF-β2 following experimental injury of human bronchial epithelial cells

4.1 Overview

The upper respiratory tract functions to warm, moisten and filter inhaled environmental factors from air before it reaches the lower respiratory tract. Despite this, the bronchial epithelium is challenged by a proportion of environmental matter, and acts to maintain tissue homeostasis and appropriate barrier function through the mucociliary escalator, epithelial repair and immunomodulation. TGF-β2 is transiently upregulated at some sites of epithelial injury, promoting wound repair. Dysfunctional repair and incomplete resolution of inflammation are thought to occur in the asthmatic epithelium leading to airway wall remodelling (AWR). Increased TGF-β2 expression levels have been identified in the asthmatic epithelium following segmental allergen challenge in human bronchial biopsy studies (Batra *et al.*, 2004), and *in-vitro* studies have demonstrated the increased sensitivity of asthmatic fibroblasts to TGF-β2 mediated myofibroblast transition effect (Wicks, 2006). Myofibroblasts go on to increase extracellular matrix molecule production, contributing to remodelling. Increased TGF-β2 production by the bronchial epithelium, possibly arising from a non-resolving wound response, could therefore drive some aspects of AWR. Expression and activation of TGF-β isoforms are tightly regulated processes both temporally and spatially however,

House dust mite is a prominent source of environmental allergens in the western hemisphere, present at levels sufficient for sensitization (Platts-Mills *et al.*, 1992). Approximately 21 allergens have been identified from house dust mite species thus far and at least 12 have been isolated from the European house dust mite, *Dermatophagoides pterronysinus* (Thomas *et al.*, 2002). Many of these allergens have proteolytic activity. Group 1 allergen, Der p 1, was identified in 1980 as a cysteine protease (Chapman and Platts-Mills,

1980), with sequence homology to papain (Chua *et al.*, 1988). Subsequent purification identified allergens including Der p 3 and 6, with serine protease activity (Stewart *et al.*, 1989; Yasueda *et al.*, 1993). The proteolytic activity of these allergens can affect epithelial permeability through degradation of the tight junction protein occludin (Wan *et al.*, 2001; Wan *et al.*, 1999), and can activate epithelial cells by cleavage of protease activated receptor-2 (PAR-2) (Kauffman *et al.*, 2006).

Latent TGF-β1 can be activated by several proteases *in-vivo*, including the serine proteases plasmin (Nunes *et al.*, 1997), tryptase (Tatler *et al.*, 2008), chymase, neutrophil elastase and thrombin (Taipale *et al.*, 1992; Taipale *et al.*, 1995), and the cysteine protease cathepsin B (Gantt *et al.*, 2003). Abe *et al.* presented evidence of latent TGF-β1 activation *in-vitro* in the presence of cells by the cysteine protease calpain (Abe *et al.*, 1998). An investigation into increased TGF-β activation following glucocorticoid treatment identified increased cathepsin-B protein secretion (a cysteine protease) by osteoblast cells as a possible mechanism; osteoblast conditioned medium alone was sufficient to activate latent TGF-β1 (Oursler *et al.*, 1993). Less is known in terms of TGF-β2 activation by proteases. *In–vitro*, Dallas *et al.* established the preferential activation of recombinant latent TGF-β2 by the serine protease prostate specific antigen, from primary human cells (Dallas *et al.*, 2004). These findings are of interest in asthma as the major dust mite allergen Der p 1 is a cysteine protease, and Der p 3, 6 and 9 are serine proteases. Furthermore, it was recently shown that purified Der f 1 (from the North American dust mite *Dermatophagoides farinae*), can proteolytically cleave and activate latent TGF-β1 (Nakamura *et al.*, 2009). However, the effect of European house dust mite allergens with protease activity on latent TGF-β activation has not been investigated.

4.2 Aims and Hypothesis

The aims of this chapter are:

1. To characterise the proteolytic activity of commercial HDM preparations

2. To investigate whether these HDM preparations activate native latent TGF-β2
3. To assess whether HDM exposure leads to increased expression of TGF-β2 by transformed and primary bronchial epithelial cells

It is hypothesised that the proteolytic activity of HDM can activate latent TGF-β2 produced by human bronchial epithelial cells.

4.3 Results

4.3.1 Commercial amino acid enzyme substrate is cleaved by both serine and cysteine proteases

Prior to performing experimental work with HDM preparations, the proteolytic cleavage susceptibility of the selected enzyme substrate was confirmed. The amino acid substrate N-benzoyl-Phe-Val-Arg-*p*-nitroanilide-hydrochloride (NBPVANA) has been reported to be sensitive to both serine (trypsin) and cysteine (papain, Der p 1) protease activity (Sehgal, 2005). NBPVANA cleavage produces a colorimetric product measurable by absorbance at 405nm.

The NBPVANA substrate was incubated with trypsin (3U/ml) or papain (200mU/ml) at 37°C for 2 hours, and 405nm absorbance readings taken at 20min intervals, following the protocol of Sehgal. Absorbance readings were plotted against time, and the reaction progression curves show both trypsin (serine) and reduced papain (cysteine) proteolytically cleaved the NBPVANA substrate. Trypsin reached a reaction plateau by 20 min, with an activity rate of 3.86μM/min in this time period. Papain showed a rate of substrate breakdown of 1.53μM/min over the same time. Class-specific protease inhibitors of trypsin (PMSF) and papain (E64) were included to confirm their efficacy in excess, while unreduced papain (no DTT reduction) showed no activity (Figure 4.1).

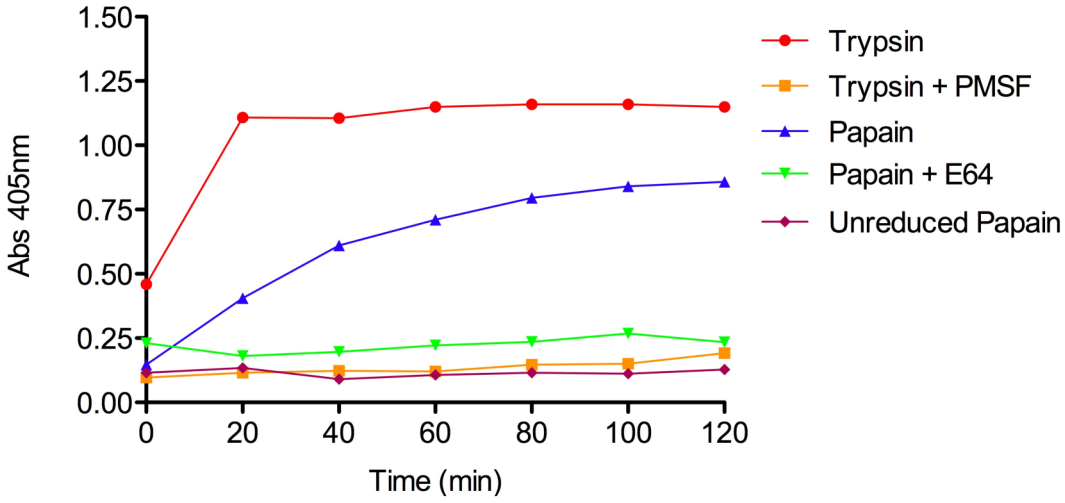


Figure 4.1 Reaction progression curves of substrate cleavage by a serine or cysteine protease. NBPVANA substrate (125µM **)** was incubated with serine protease trypsin (3U/ml) or cysteine protease papain (200mU/ml), with and without class-specific inhibitors PMSF (1mM) and E64 (10µM). Unreduced papain showed no activity, confirming reduction of the thiol group is essential for cysteine protease activity.

4.3.2 Serine and cysteine proteolytic profiles of commercial house dust preparations

Following confirmation of substrate suitability, a commercially available HDM extract (HDM SPT) (ALK-Abello, Netherlands) was assessed in the assay. The extract is used in skin prick testing and is therefore expected to contain the major allergens of *Dermatophagoides pterronysinus*. An initial experiment using 100,000 and 10,000U/ml showed that the lower concentration had very limited proteolytic activity. The extract was therefore used in the assay undiluted, at 100,000U/ml as this produced progression curves comparable to the concentrations of other enzymes selected. Units given are not indicative of protease activity units, instead they are a measure of skin prick test responsiveness as determined by the manufacturer. Protease activity and allergen level data is not supplied or available for this product.

HDM SPT continous rate assays show that activity was not affected by reduction (Figure 4.2). Incubation with class-specific protease inhibitors showed that HDM SPT has serine protease activity. Combined protease inhibitor cocktail (PIC) inhibited all activity, as did the serine protease inhibitor PMSF alone, with no appreciable protease activity remaining. Cysteine protease inhibitor E64 showed no effect. Substrate breakdown rate for reduced HDM SPT was 1.88μM/min, and for unreduced HDM SPT was 1.81μM/min in the linear phase of the reaction.

Comparison of HDM SPT extract activity with cysteine protease papain confirmed that reduction was not necessary for HDM SPT proteolytic activity (Figure 4.3).

The lack of measurable cysteine protease activity was unexpected as Der p 1, reportedly the most abundant allergen in house dust mite, is a cysteine proteae with structural homology to papain. It is possible that serine protease activity outweighed cysteine protease activity simply because there was a greater amount of serine protease activity present in the preparation. The manufacturers do not divulge data on the mixture/proportion of allergens present. Allergen levels can vary widely in preparations from different suppliers (Ford *et al.*, 1985).

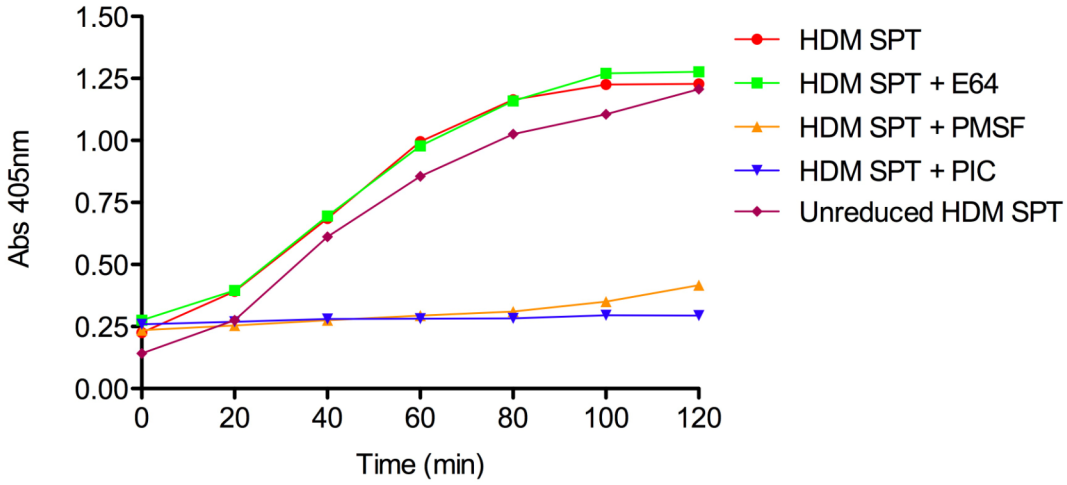


Figure 4.2 Reaction progression curves of substrate cleavage by a commercial HDM extract. HDM extract (100000U/ml) was incubated with and without serine (PMSF- 1mM), cysteine (E64- 10μM) and a complete protease inhibitor cocktail (PIC-20μl stock) prior to the addition of 125μM NBPVANA substrate. Unreduced HDM extract maintained over 95% activity, revealing minimal contribution from cysteine proteases.

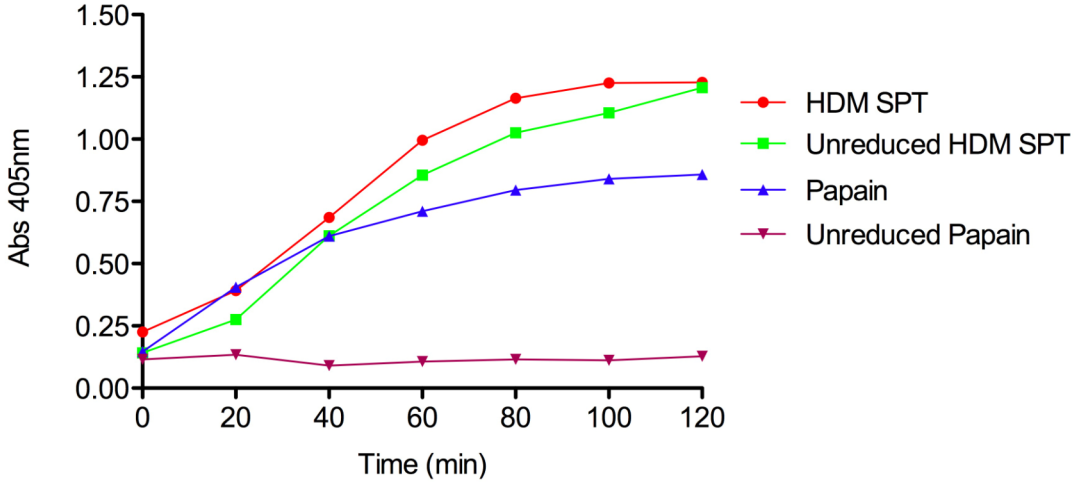


Figure 4.3 Comparison of papain and HDM extract proteolytic activity. Reaction progression curves of substrate cleavage over time by HDM extract (100,000U/ml) and cysteine protease papain (200mU/ml). Unreduced papain shows no activity, but unreduced HDM extract maintains a similar activity profile to the reduced sample.

The HDM SPT extract used in this study is a standardised mixture of allergens, however it does not bear much resemblance to environmental presentation of HDM. Alternatively, mite stocks can be cultured in the lab, where they secrete major allergens into the culture media. Lyophilised spent culture media, consisting of a mixture of HDM fecal pellets, eggs, nymphs and other variables (HDM FP) was acquired for use in this study (Indoor Biotechnologies Ltd, Warminster, UK). This product is for research use only and is not used in any clinical setting. HDM FP was reconstituted at 1mg/ml in solution, although a small proportion of the matter proved insoluble.

Unreduced and reduced HDM FP showed similar proteolytic activity of 3.16μM/min and 2.97μM/min respectively, in the linear phase of the reaction between 20-40min (Figure 4.4). The reaction plateaued at 60min. HDM FP incubated with cysteine protease inhibitor (E64) did not differ from HDM FP alone. Serine protease inhibitor PMSF at 1mM slowed the rate of reaction to 1.84μM/min, but by 2h the reaction reached the plateau. Complete protease inhibitor cocktail (PIC) completely abrogated all protease activity.

HDM SPT and HDM FP were then assayed together. The HDM preparations showed almost identical proteolytic profiles (Figure 4.5). Breakdown of NBPVANA substrate in the linear phase (20-40min) was 3.37μM/min by HDM SPT and 3.6μM/min by HDM FP. The reaction plateaued at 60min, and protease inhibitor cocktail blocked activity.

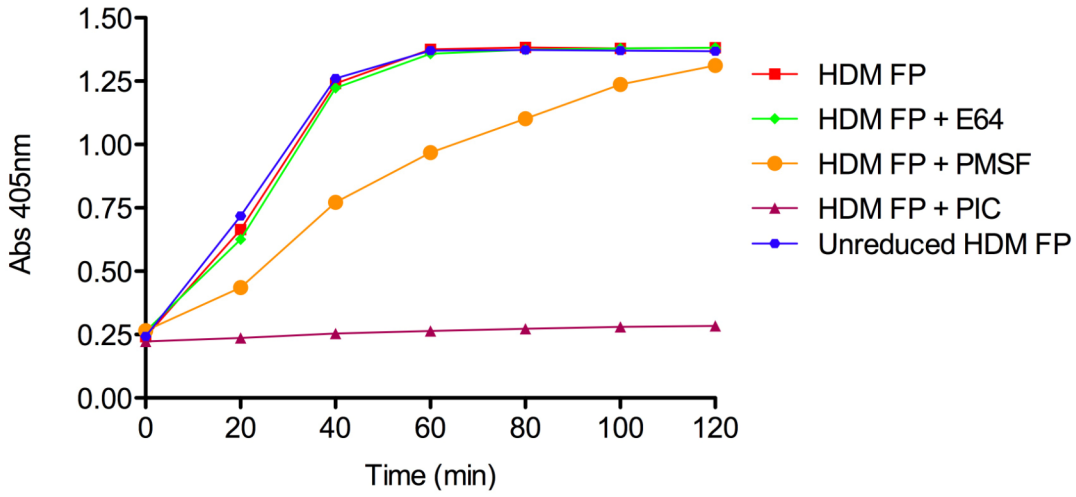


Figure 4.4 Serine and cysteine proteolytic activity of HDM fecal pellet solution. Reaction progression curves of NBPVANA substrate breakdown over time by HDM FP (1mg/ml saturated). Inhibitors of serine (PMSF- 1mM) and cysteine (E64- 10μM) activity were included, and protease inhibitor cocktail (PIC).

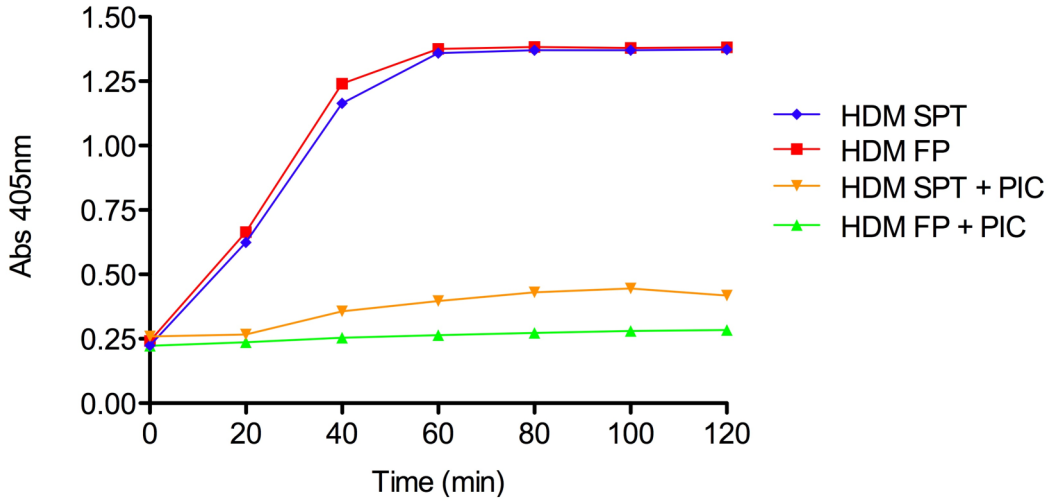


Figure 4.5 HDM SPT extract and HDM FP proteolytic activity.
HDM SPT (100000U/ml) and HDM FP (1mg/ml) were reduced prior to addition of 125μM NBPVANA substrate. Protease inhibitor cocktail was effective in both HDM SPT and HDM FP samples.

A single substrate concentration of 125μM was used in all reactions, and HDM preparations contain a mixture of enzymes, so the results were not suitable for Michaelis-Menten analysis to determine reaction kinetics.

This section presented evidence for proteolytic activity in 2 different HDM preparations. Both preparations showed similar serine protease activity levels on the substrate. The quantity of PMSF used (1mM) was insufficient to entirely block serine protease activity of HDM FP though, and this may be due to greater serine activity, or possibly due to a different class of protease. There was no measurable cysteine protease activity in either extract. This may be due to low levels of Der p 1 in the preparations used.

4.3.3 Activation of LTGF-β2 by proteolysis in an acellular environment

It was hypothesised that proteolytic activity of HDM extract could activate latent TGF-β2. Proteolytic activation *in-vivo* is often dependent on cell- or matrix-association of the latent cytokine, as for the plasmin activation of LTGF-β1. However, initial assessments of proteolytic activation were performed in cell-free conditioned medium culture supernatant containing native latent TGF-β2. LTGF-β2 was not available from a commercial source. Omitting cells ensured no cell-mediated effects would interfere, for example through upregulation of TGF-β2 production or secretion of cellular proteases in response to HDM stimulation. The experiments in this section utilised cell-free conditioned medium (CM) from the SV-40 transformed bronchial epithelial cell line 16HBE14o- as a source of TGF-β2 protein. These cells constitutively secrete latent and active TGF-β2. TGF-β2 protein levels were assessed by ELISA.

Serum-free conditioned medium from 16HBE14o- cells was collected at 24h, and exposed to different protease preparations for 24h at 37°C, in order to analyse the ability of HDM proteases to activate latent TGF-β2 (Figure 4.6). HDM SPT extract was used at 2000U/ml (Salib *et al.*, 2005), HDM FP at 20μg/ml, papain at 100mU/ml and plasmin at 2U/ml (Lyons *et al.*, 1990).

Levels of active TGF-β2 were not affected by protease incubation with the exception of papain-exposed samples that showed complete loss (degradation) of the active cytokine.

Levels of total TGF-β2 (active + latent) were analysed in CM by heating to 80°C for 6mins prior to assaying, to investigate degradation of LTGF-β2 due to protease activity (Figure 4.7). HDM FP degraded a small but significant proportion (17%) of the native LTGF-β2, and papain degraded the majority of both active and latent TGF-β2.

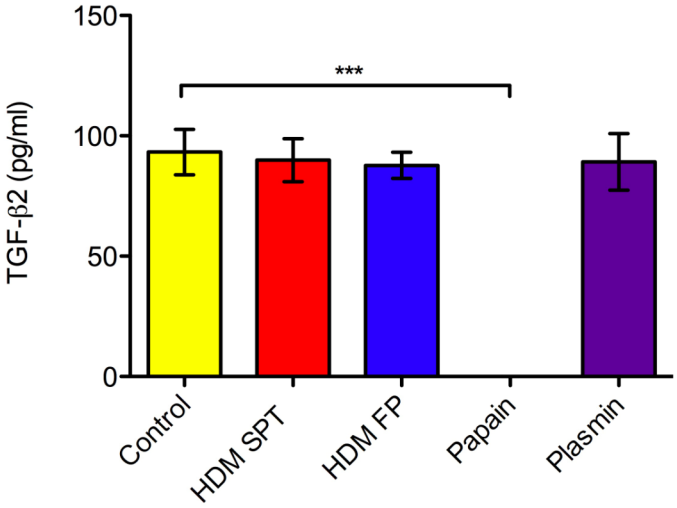


Figure 4.6 Active TGF-β2 in 16HBE14o- conditioned media following 24h incubation with protease preparations. CM was assayed by ELISA following incubation with HDM SPT (2000U/ml), HDM FP (20μg/ml), papain (100mU/ml) or plasmin (2U/ml). Data were analysed by one way ANOVA followed by Dunnett's test (n=3, ***=p<0.005).

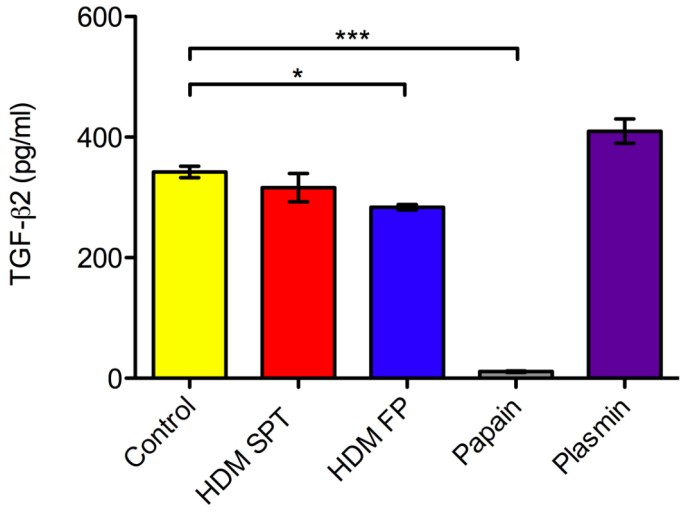


Figure 4.7 Total TGF-β2 in 16HBE14o- conditioned media following 24h incubation with protease preparations. CM was heat treated and assayed by ELISA following incubation with HDM SPT (2000U/ml), HDM FP (20μg/ml), papain (100mU/ml) or plasmin (2U/ml). Data were analysed by one way ANOVA followed by Dunnett's test (n=3, *=p<0.05 ***p<0.005).

Although there was no significant increase in TGF-β2 activation, a decrease in total TGF-β2 was identified in HDM FP exposed samples. A comparison of active *vs*. total (latent + active) TGF-β2 levels showed no significant difference in between HDM preparation exposed and control levels with the exception of papain-exposed samples that were almost entirely degraded (Figure 4.8).

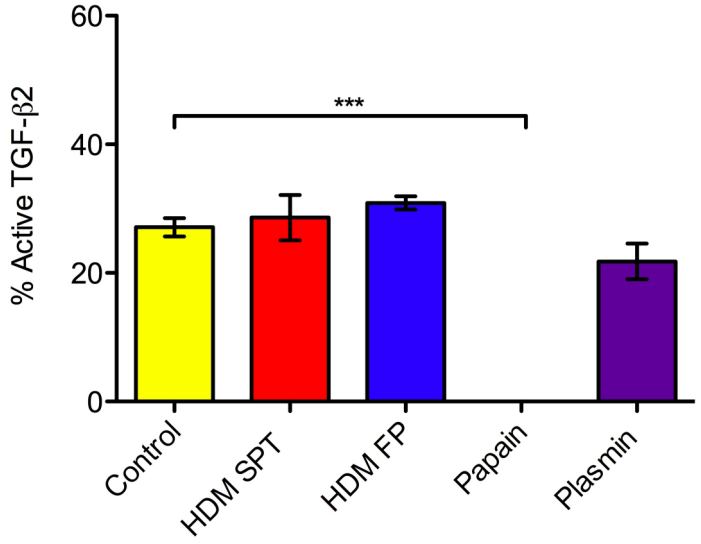


Figure 4.8 Percentage active TGF-β2 in 16HBE14o- conditioned media following 24h incubation with protease preparations. Assayed by ELISA following incubation with HDM SPT (2000U/ml), HDM FP (20µg/ml), papain (100mU/ml) or plasmin (2U/ml). Data were analysed by one way ANOVA followed by Dunnett's test (n=3, *** $p<0.005$).

This section showed that HDM preparations and plasmin did not activate native LTGF-β2 in a cell-free environment. The experimental procedure was then altered to investigate the effect of protease preparations on submerged 16HBE14o- cultures TGF-β2 secretion and activation.

4.3.4 Secretion and activation of TGF-β2 by confluent 16HBE14o- cell cultures following protease challenge

Confluent 16HBE14o- cells were exposed to protease preparations for 24h at 37°C, as in the CM study. The supernatant was harvested and assayed by ELISA for both active and total TGF-β2. To ensure detectable levels of TGF-β2 were present, cell challenge began 24h after the final media change, so media assayed was collected at 48h, and not 24h as the CM samples used in section 4.2.2. Tryptase (15mU/ml) was also included in these experiments as it has been shown to activate cell-associated latent TGF-β1 at this concentration

(Tatler *et al.*, 2008), and is secreted by mast cells in the asthmatic airway (Woodman *et al.*, 2008) (Figure 4.9).

Challenge of cultured cells rather than conditioned media with HDM preparations produced marked differences in levels of active TGF-β2. Papain again degraded TGF-β2 secreted, and is not shown here. A significant decrease ($p<0.005$) was observed in HDM FP exposed cultures. Challenge with tryptase led to a small but significant ($p<0.05$) increase in active TGF-β2. Similar levels of active TGF-β2 were measured in control cultures at 48h as seen in the 24h cultures.

A significant decrease in total TGF-β2 was observed in HDM SPT exposed cultures, and a greater decrease in HDM FP exposed cultures. Both tryptase and papain exposure resulted in significant increases in total TGF-β2 (Figure 4.10).

To identify increases in LTGF-β2 production and effects on active TGF-β2 levels following protease exposure, the percentage of active TGF-β2 was calculated. Figure 4.11 shows percentage active TGF-β2 in HDM protease-challenged 16HBE14o- cell cultures. HDM SPT exposure did not differ from control proportions (around 40%). HDM FP, tryptase and plasmin all showed a decreased proportion compared to control cultures, of 28%, 33% and 17% respectively. These results indicate that HDM FP may be degrading both active and latent forms of TGF-β2, although may also be affecting TGF-β2 secretion by cells. Tryptase and plasmin exposure increased TGF-β2 production by cells.

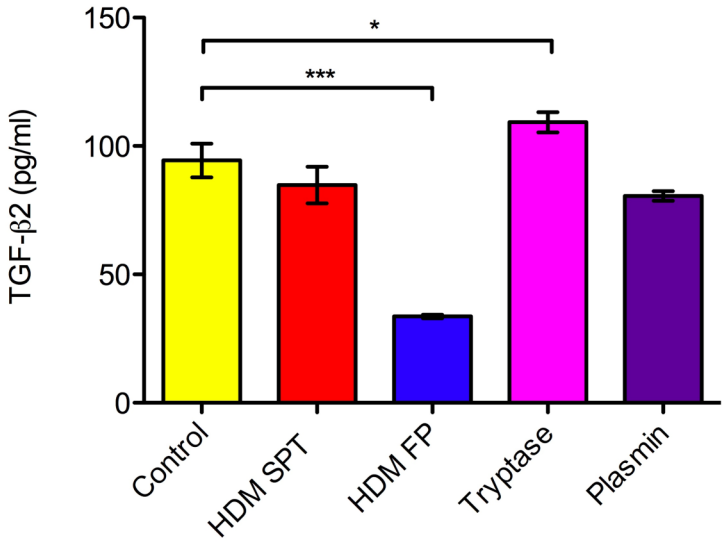


Figure 4.9 Active TGF-β2 secreted by confluent 16HBE140- cultures following 24h protease exposure. Supernatant was harvested at 24h and assayed by ELISA following exposure to HDM SPT (2000U/ml), HDM FP (20μg/ml), tryptase (15mU/ml) or plasmin (2U/ml). Data were analysed by ANOVA followed by Dunnett's test (n=3, *p<0.05, ***p<0.005).

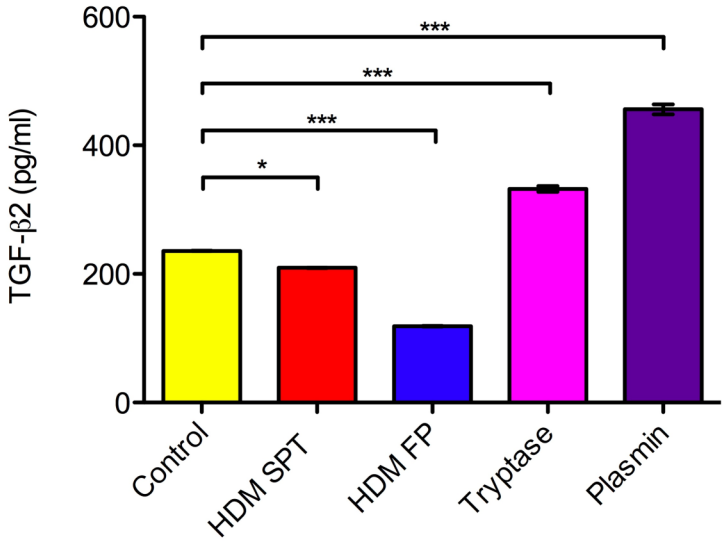


Figure 4.10 Total TGF-β2 secreted by confluent 16HBE140- cultures following 24h protease exposure. Supernatant was harvested at 24h, heat treated and assayed by ELISA following exposure to HDM SPT (2000U/ml), HDM FP (20μg/ml), tryptase (15mU/ml) or plasmin (2U/ml). Data were analysed by ANOVA followed by Dunnett's test (n=3, *p<0.05, ***p<0.005).

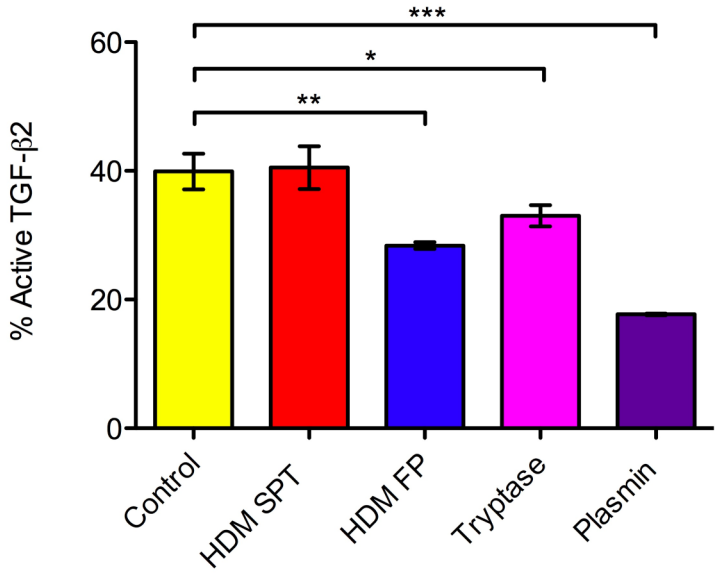


Figure 4.11 Percentage active TGF-β2 in confluent 16HBE14o- cell supernatant following 24h protease exposure. Supernatant was harvested at 24h and assayed by ELISA following exposure to HDM SPT (2000U/ml), HDM FP (20μg/ml), tryptase (15mU/ml) or plasmin (2U/ml). Data were analysed by ANOVA followed by Dunnett's test (n=3,*p<0.05, **p<0.01, ***p<0.005).

In summary, the proteolytic activity present in HDM preparations did not appear to activate native LTGF-β2 in the cell-associated environment. HDM SPT had no effect either on the activation of or the expression of LTGF-β2, while HDM FP exposure appeared to degrade TGF-β2 protein and may also downregulate TGF-β2 expression.

4.3.5 Comparison of TGF-β2 secretion and activation by 16HBE14o- cell cultures following injury or allergen challenge

Injury to the epithelial layer in asthma may drive increased TGF-β2 expression. The use of "scratch" assays has become the standard method of injury in bronchial epithelial culture (Howat *et al.*, 2002; Thompson *et al.*, 2006). Confluent cultures are subjected to a scratch across the surface using a sterile pipette tip. This physical injury is intended to mimic the epithelial sheet shearing thought to occur during the acute asthmatic response. HDM extracts may contain variable levels of lipopolysaccharide (LPS), a Toll-like receptor 4

(TLR4) ligand, but the proteolytically inactive allergen Der p 2 (concentrated in fecal pellets) can activate TLR4 in the absence of LPS (Trompette *et al.*, 2009). To confirm whether LPS exposure was sufficient to alter TGF-β2 levels in a protease-independent manner, LPS was added to cell cultures at a concentration of 100ng/ml as a control.

Cell challenge began 24h after the final media change, so supernatant assayed was from a total 48h. A significant decrease ($p<0.05$) in active TGF-β2 was observed in HDM FP–exposed cultures, whereas HDM SPT did not differ from control. Scratch injury and LPS exposure did not significantly alter active TGF-β2 levels (Figure 4.12A).

When total TGF-β2 levels were assayed, there was a significant increase in TGF-β2 in HDM SPT exposed and scratch injured cultures. No difference compared with control cultures was seen following HDM FP or LPS exposure (Figure 4.12B). The increase in total TGF-β2 in scratch injured cultures suggests this method of injury provokes a wound healing response, and correlates with previously published work (Howat *et al.*, 2002).

The percentage of active TGF-β2 was calculated, to identify alterations in the proprotion of active TGF-β2 present following HDM preparation exposure or injury (Figure 4.12C). HDM SPT and HDM FP exposure significantly decreased the proportion of active TGF-β2 present. HDM FP decreases are likely due to proteolytic degradation of the active and latent protein. A significant decrease was also observed in LPS exposed cultures, but there was no significant difference between scratch injured and control cultures.

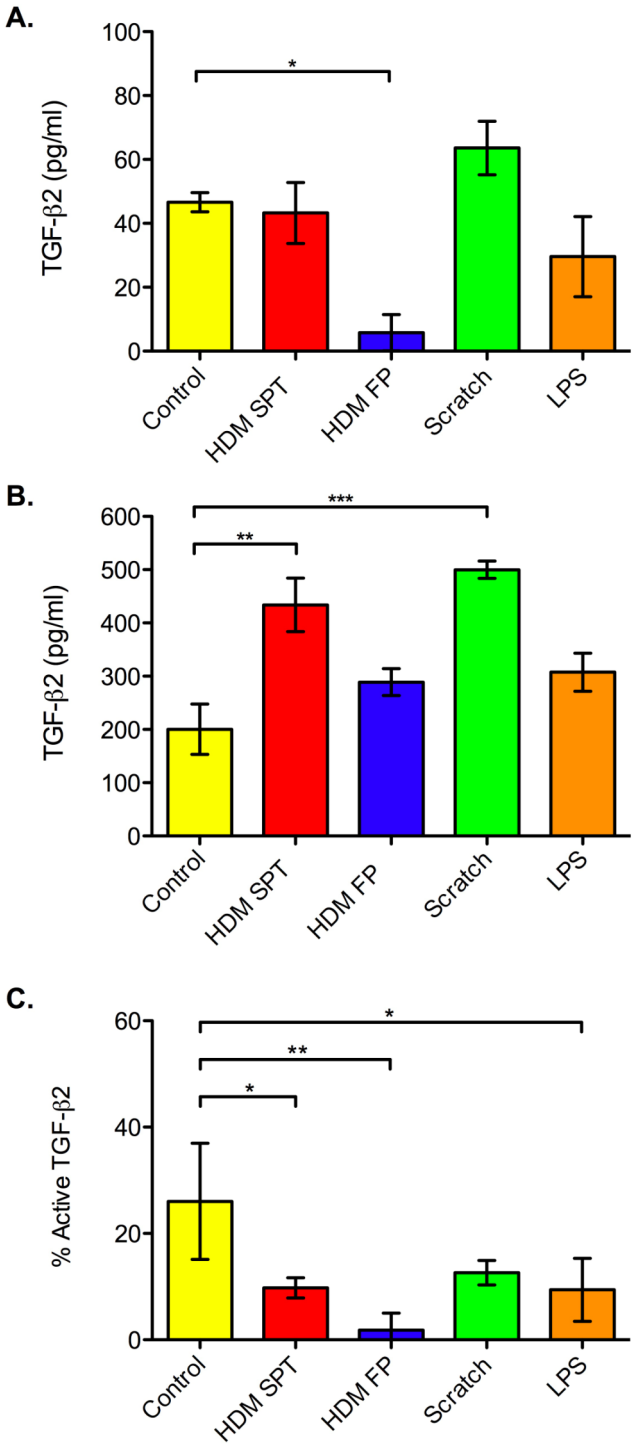


Figure 4.12 TGF-β2 production by 16HBE14o- cells 24h following HDM exposure or experimental injury. Cells were challenged with HDM SPT (2000U/ml), HDM FP (20μg/ml), LPS (100ng/ml) or scratch injury. Media was collected at 24h for assay by ELISA. A. Active TGF-β2, B. Total TGF-β2 in heat-treated media and C. Percentage active TGF-β2 produced. Data were analysed by ANOVA followed by Tukey's test (n=3, *p<0.05, **p<0.01, ***P<0.005).

To summarise, HDM challenge did not lead to increased TGF-β2 activation in the transformed bronchial epithelial cell line 16HBE140-. Therefore, the next set of experiments investigated TGF-β2 activation following allergen exposure using a more physiological model, consisting of healthy primary human bronchial epithelial cells grown at air-liquid interface (ALI).

4.3.6 Secretion and activation of TGF-β2 by primary bronchial epithelial cells following injury or allergen challenge

Primary human bronchial epithelial cells (HBEC), from 3 different donors, were seeded into polycarbonate membrane hanging inserts in 12 well plates and grown submerged until confluence. When cells had formed a confluent layer, the apical media was removed, allowing cell differentiation at the air-liquid interface. All challenges took place after a minimum of 7 days culture at ALI, to allow apical-basal polarity to be established, mature tight junctions to form and for cells to differentiate to ciliated or goblet type. Culture at ALI provides a more physiological epithelial layer for *in-vitro* experiments than submerged culture (Kikuchi *et al.*, 2004; Vanwetering *et al.*, 2007).

Cell challenge began 24h after the final media change to ensure detectable levels of TGF-β2 were present. The culture media assayed was therefore collected at a total of 48h, following 24h challenge. As HDM FP appeared to degrade TGF-β2, only HDM SPT was used in these experiments as an allergen challenge. Following 24h exposure to HDM SPT (2000U apically), scratch or LPS (100ng/ml basally), culture media was harvested and assayed by ELISA for both active and total levels of TGF-β2.

Figure 4.13A presents data on active TGF-β2 production from the first donor (2F1578). HDM SPT exposure led to a significant increase in active TGF-β2, and scratch injury a slight increase (not significant). There was a decrease in LPS exposed cultures that was not significant. The apparent increase in active TGF-β2 following HDM SPT exposure could be explained by a concomitant increase in total TGF-β2. HDM SPT, scratch and LPS all resulted in increased TGF-β2 levels over control (Figure 4.13B). The relative levels of active TGF-β2 are shown in Figure 4.13C. The percentage active TGF-β2 did not differ

significantly from control in any experimental challenge. This may be due to the wide variation seen in control unstimulated samples.

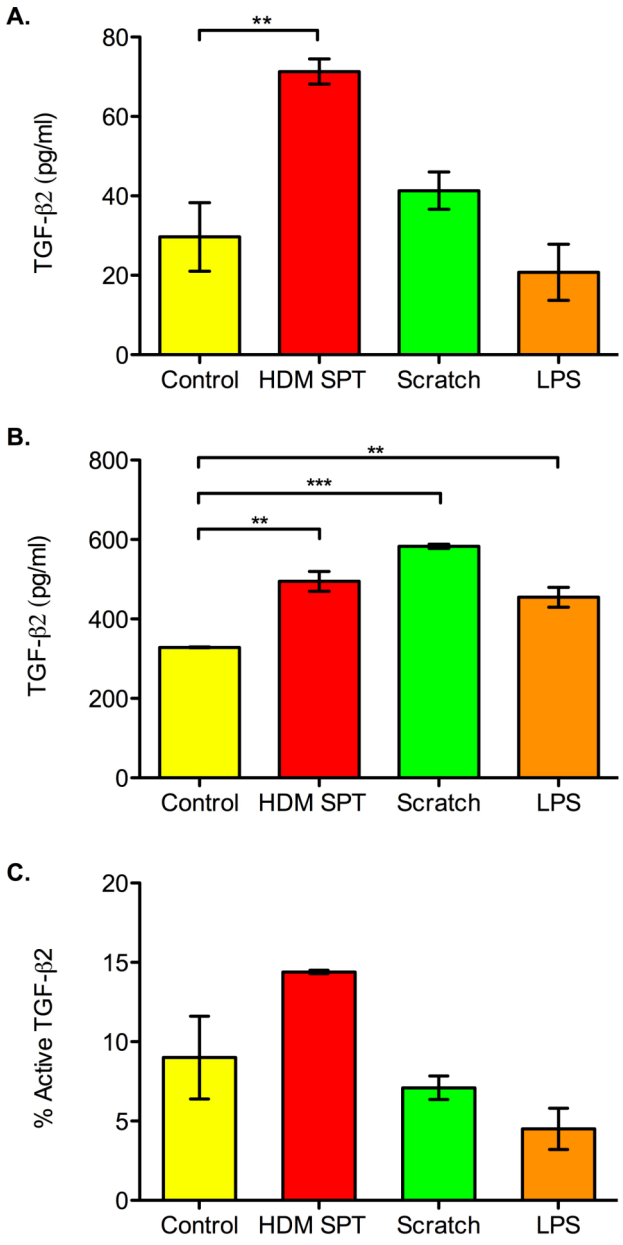


Figure 4.13 TGF-β2 production by NHBEC donor 2F1578 24h following HDM challenge or injury. ALI cultures were challenged by HDM SPT (2000U), LPS (100ng/ml) or scratch injury. Media was collected at 24h for assay by ELISA shown as A. Active TGF-β2, B. Total TGF-β2 after heat-treatment of media and C. Percentage active TGF-β2. Data were analysed by ANOVA followed by Tukey's test (n=3, **p<0.01, ***p<0.005).

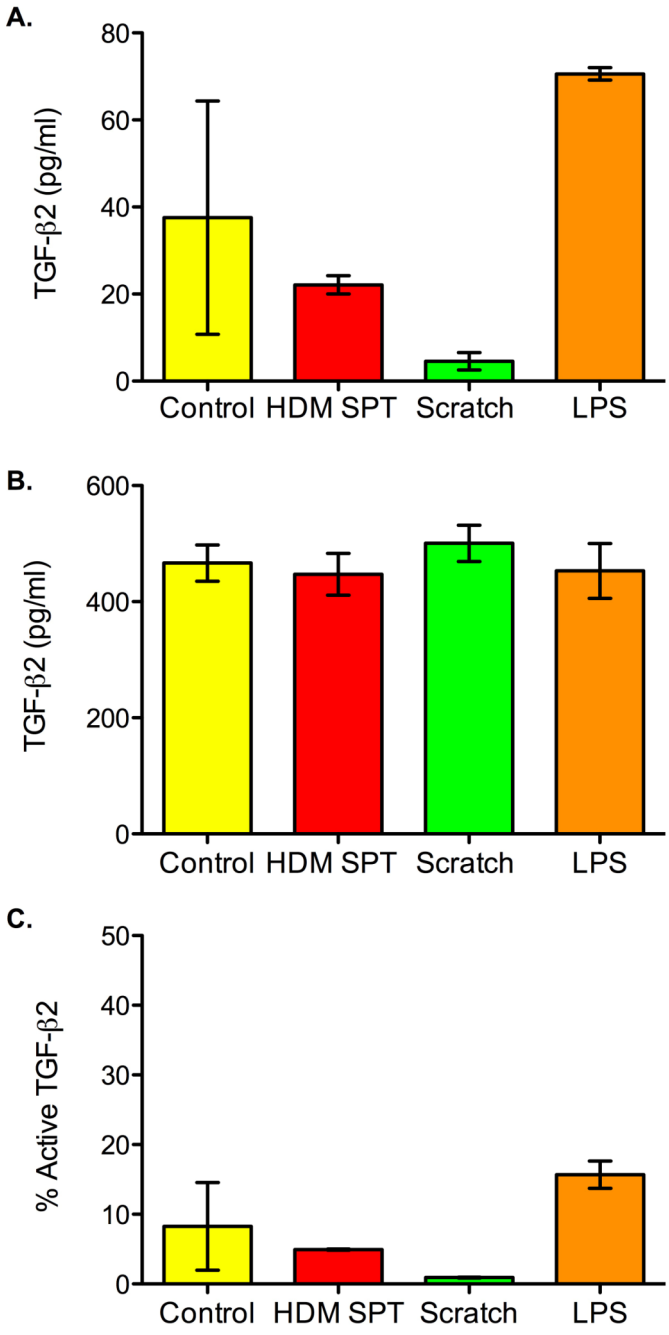


Figure 4.14 TGF-β2 production by NHBEC donor 7F3081 following HDM exposure or injury. ALI cultures were challenged by HDM SPT (2000U), LPS (100ng/ml) or scratch injury. Media was collected at 24h for assay by ELISA shown as A. Active TGF-β2, B. Total TGF-β2 after heat-treatment of media and C. Percentage active TGF-β2. Data were analysed by ANOVA followed by Tukey's test (n=3, **p<0.01).

Primary cells from the final donor, 7F3000, were limited and only 3 conditions were possible, taken at the 24h timepoint (Figure 4.15). There was wide variation in active TGF-β2 levels following HDM SPT, and also in control unstimulated samples. Scratch injury produced very little active TGF-β2. Results were not however statistically significant (Figure 4.15A).

Differences in total TGF-β2 levels were not statistically significant due to wide variation and overlap between repeats and conditons. There was however an observable decrease in HDM SPT compared to control samples (Figure 4.15B). The proportion of active TGF-β2 present following challenge did not differ significantly across experimental conditions (Figure 4.15C).

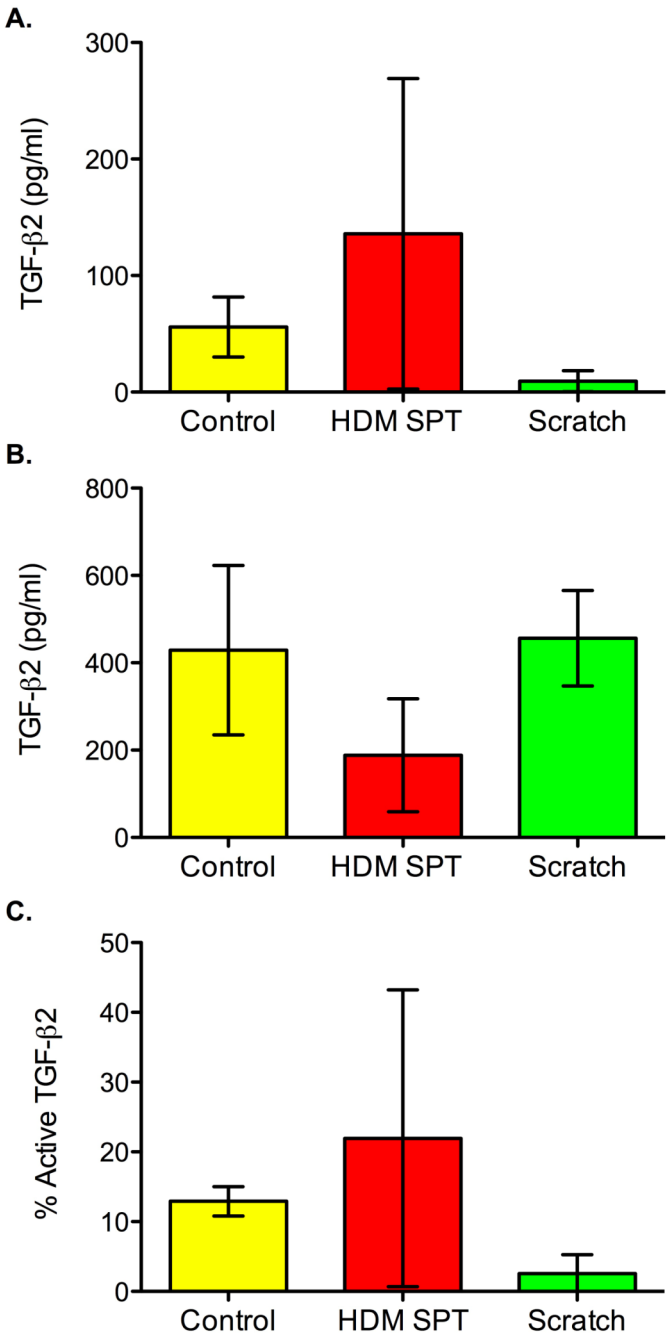


Figure 4.15 TGF-β2 production by NHBEC donor 7F3000 following HDM exposure or injury. ALI cultures were challenged by HDM SPT (2000U) or scratch injury. Media was collected at 24h for assay by ELISA shown as A. Active TGF-β2, B. Total TGF-β2 after heat-treatment of media and C. Percentage active TGF-β2. Data were analysed by ANOVA followed by Tukey's test (n=3, **p<0.01).

To determine the temporal pattern of TGF-β2 production and activation following HDM exposure or injury, ALI cultures were challenged and media collected for assay at earlier timepoints alongside the 24h timepoint. Two donors were available for this study: 2F1578 and 7F3081.

The levels of active and total TGF-β2 produced at 1h, 4h and 24h post-challenge by donor 2F1578 are shown in Figure 4.16A and 4.16B respectively. For active TGF-β2, control samples peaked at 4h, whereas HDM SPT exposed cultures displayed a continual increase. Low levels of active TGF-β2 were measured throughout the timecourse following LPS exposure, while scratch led to a 1h peak then drop at 4h before increasing to a level similar to control. All samples showed an increase in total TGF-β2 levels over time, regardless of experimental condition.

Production of TGF-β2 by donor 7F3081 at 2h, 8h and 24h post-challenge is shown in Figure 4.17. Active TGF-β2 levels in control samples were variable whilst HDM SPT exposure resulted in a peak 2h following challenge, and did not exceed control levels at 24h. Scratch injured levels fell between 8 and 24 hours (Figure 4.17A). Active TGF-β2 production by NHBEC donor 7F3081 was disimilar to donor 2F1578. Total TGF-β2 production did not differ significantly between experimental groups in donor 7F3081 (Figure 4.17B), with the exception of HDM SPT exposure at 2h ($p<0.05$).

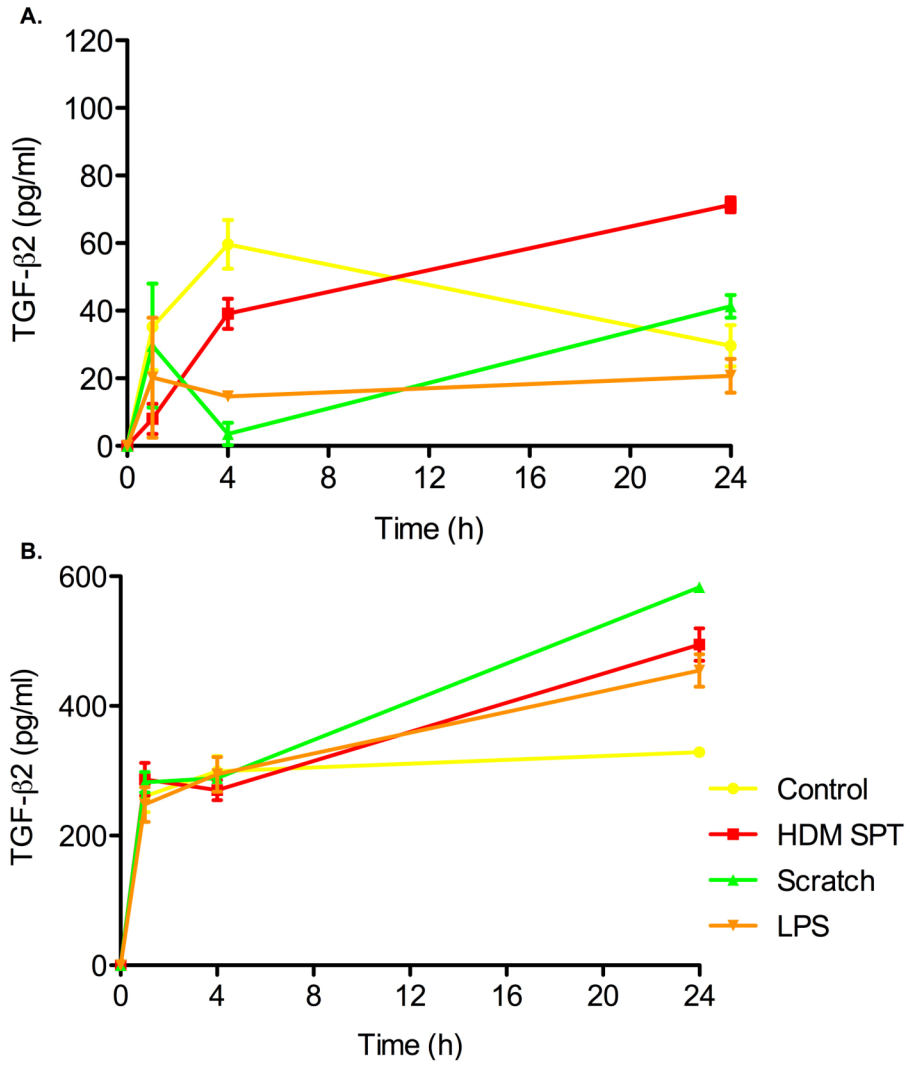


Figure 4. 16 Timecourse of TGF-β2 production by NHBEC donor 2F1578.
ALI cultures were challenged by HDM SPT (2000U), LPS (100ng/ml) or scratch injury. Media was collected at 1, 4 and 24h for assay by ELISA shown as A. Active TGF-β2 and B. Total TGF-β2 after heat-treatment of media (n=3 at each timepoint).

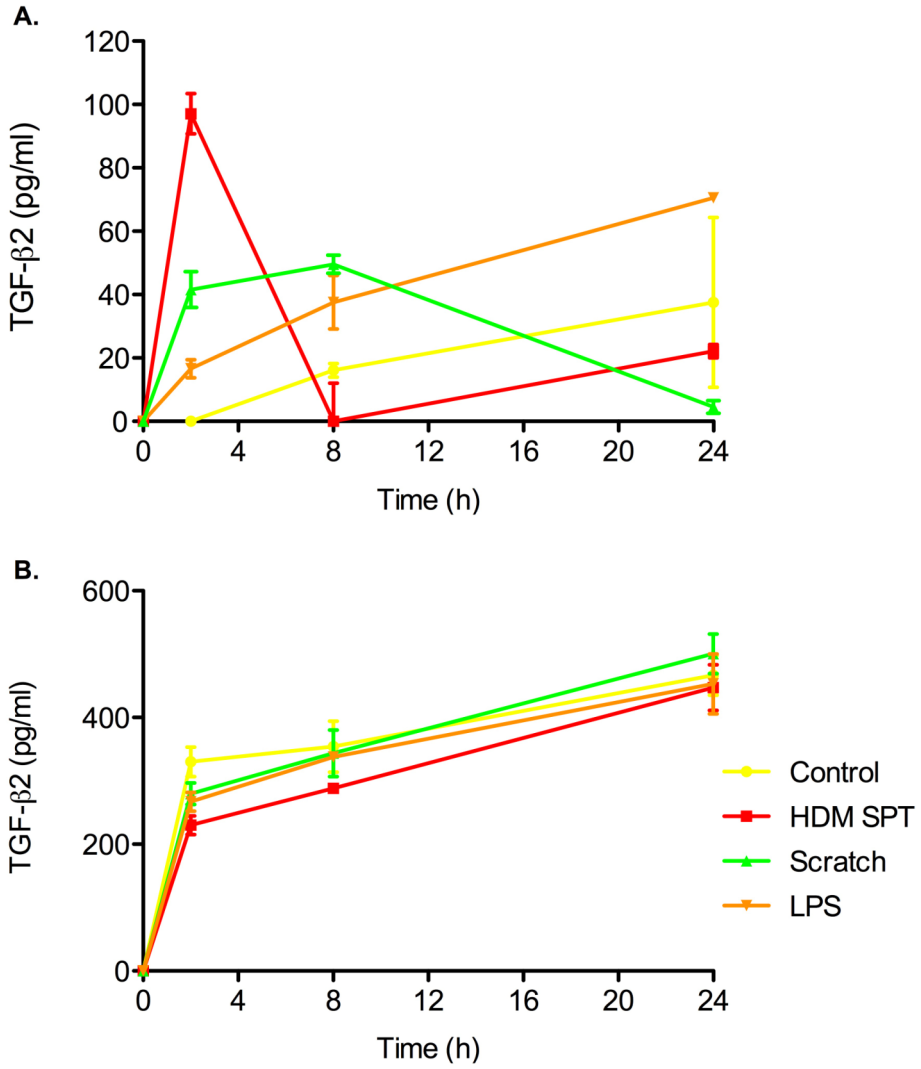


Figure 4. 17 Timecourse of TGF-β2 production by NHBEC donor 7F3081.
ALI cultures were challenged by HDM SPT (2000U), LPS (100ng/ml) or scratch injury. Media was collected at 1, 4 and 24h for assay by ELISA shown as A. Active TGF-β2 and B. Total TGF-β2 after heat-treatment of media (n=3 at each timepoint).

In all samples the majority of TGF-β2 was secreted by 1-2h, followed by a gradual accumulation. This may be due to the media change prior to challenge that removed any previously secreted soluble factors, inciting cells to replenish cytokine levels. Active TGF-β2 levels did not correlate well with total TGF-β2; the fluctuations in active TGF-β2 levels imply a complex and dynamic turnover in the presence of TGF-β responsive primary cells.

Wide donor variability was apparent in this set of experiments. This was not unexpected, as donors are randomly acquired independent samples from the human population, and not a transformed cell line from a single source. They are a good model for respiratory research in this respect, as the irregularity in responses is indicative of responses amongst the wider population, but means that increased sample numbers are required to formulate an over-arching concept of normal cellular responses to challenge.

The scope of donor variability was examined. Statistical analyses (one way ANOVA followed by Tukey test) were carried out between different donors for each challenge, with differences in active and total TGF-β2 levels shown in Figure 4.18A and 4.18B respectively. Scratch injured and LPS challenged samples showed statistically significant differences in active TGF-β2 levels between donors. Variation in control samples between donors is marked, and response to HDM extract also shows pronounced differences within and between donor samples. Active TGF-β levels were consistently variable across challenge types however, with total TGF-β2 appearing more regular. Total TGF-β2 levels (Figure 4.18B) were broadly consistent across the NHBEC donors. Only HDM extract challenge resulted in a statistically significant difference. The similarity in challenge response is especially surprising given the variation in control unstimulated cultures. This suggests that the injury/damage response is tightly regulated even in monotype culture with regard to LTGF-β2. Much greater variation was apparent in active TGF-β2 levels.

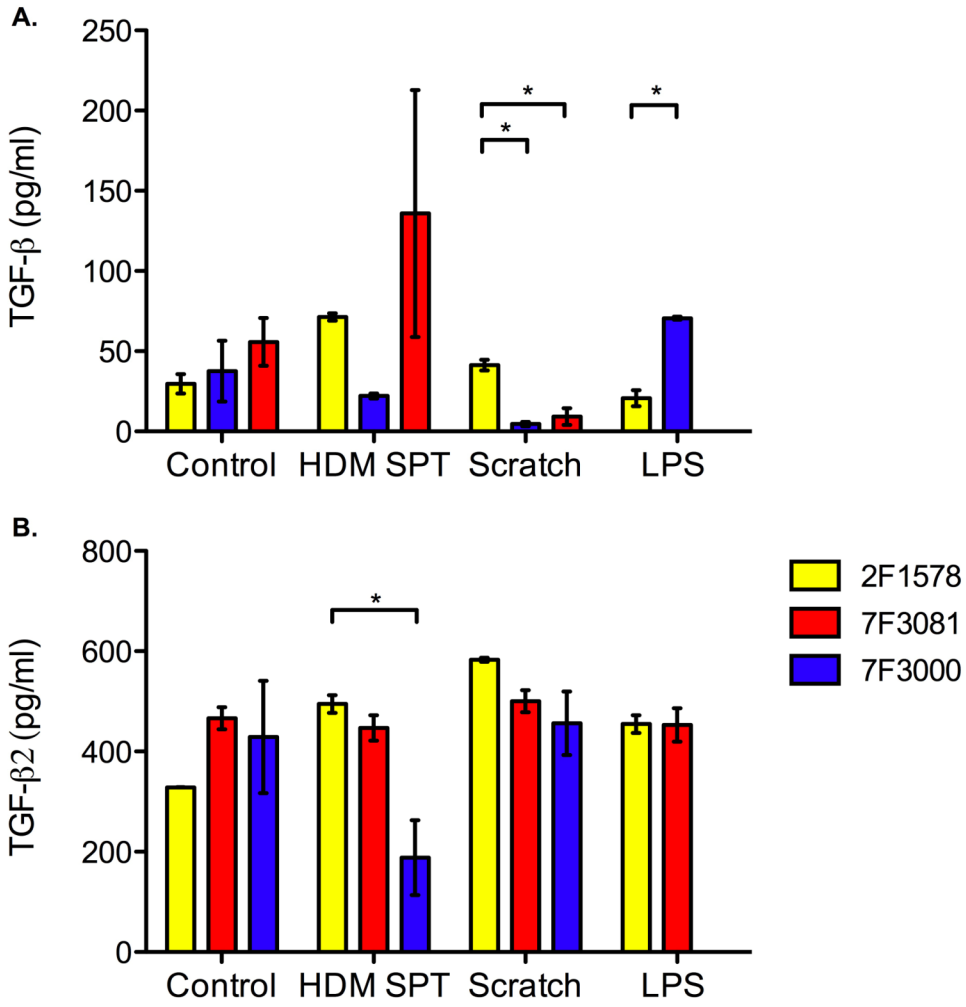


Figure 4.18 Comparison of TGF-β2 production 24h post-challenge in 3 independent NHBEC donors. ALI cultures were challenged by HDM SPT (2000U) LPS (100ng/ml) or scratch injury. Media was collected at 24h for assay by ELISA shown as A. Active TGF-β2 and B. Total TGF-β2 after heat-treatment of media. Data were analysed by ANOVA followed by Tukey's test (n=3, *p<0.05).

4.4 Summary

In this chapter, a house dust mite extract containing major allergens, used in skin prick testing (HDM SPT), and a preparation of lyophilised HDM spent culture matter (HDM FP) were shown to have detectable serine but not cysteine protease activity.

HDM preparations did not directly activate native LTGF-β2 in a cell-free environment, although HDM FP partially degraded LTGF-β2 and TGF-β2 to a lesser extent than the archetypal cysteine protease papain.

In the 16HBE14o- transformed cell line, HDM FP decreased both active and latent TGF-β2 levels, through proteolytic degradation of the cytokine and possibly other cell-mediated effects. HDM SPT decreased total TGF-β2 but had no significant effect on active cytokine levels. Plasmin and tryptase exposure increased total TGF-β2 levels, but the proportion of active cytokine was lowered by plasmin exposure. Scratch injury increased active and total TGF-β2 levels, in agreement with previously published work.

Bronchial epithelial cells from healthy donors grown at ALI responded variably to HDM exposure and injury. Of 3 donors, one (2F1578) increased both active and total TGF-β2 levels following HDM SPT exposure.

It was hypothesised that the proteolytic activity of HDM could activate latent TGF-β2 produced by human bronchial epithelial cells. From the results shown here, this was not apparent and so the hypothesis cannot be accepted.

4.4 Discussion

Allergen sensitisation is a contributing factor in up to 90% of asthmatic disease (Craig, 2010; Tovey *et al.*, 1981). House dust mite (HDM) exposure is a known risk factor for the subsequent development of asthma (Platts-Mills *et al.*, 1997; Sporik *et al.*, 1992). Purified allergens from HDM have been used in some *in-vitro* studies to assess cellular responses to allergen challenge

(Kauffman *et al.*, 2006; King *et al.*, 1998), however an *in-vivo* study by Van der Veen et al. demonstrated that the asthmatic response was greater following exposure to a HDM extract than to purified Der p 1 or Der p 2 (Van Der Veen *et al.*, 2001). This chapter aimed to identify whether HDM preparations could activate native latent TGF-β2 and whether HDM exposure led to increased expression of TGF-β2 by transformed and primary bronchial epithelial cells. TGF-β activation may be a multi-step proteolytic process, therefore this study utilised two HDM extracts containing a heterogenous mix of proteases. The HDM SPT extract is intended for clinical use in skin prick testing, and contains unspecified levels of major *Dermatophagoides pterronysinus* allergens. The HDM FP extract was derived from spent culture media of the Der p mite.

The NBPVANA substrate had previously established protease activity of affinity-purified Der p 1 (Sehgal, 2005; Wan *et al.*, 2000), and was cleaved here by the cysteine protease papain. However, cysteine protease activity was not detected in either HDM SPT or HDM FP using NBPVANA substrate. HDM SPT at 100,000U/ml contains around 9000ng/ml of total Der p protein (Rusznak *et al.*, 2001), however specific levels of Der p 1 are unknown. The lack of cysteine protease activity could possibly be due to sensitivity of the assay in this case. HDM FP appeared to cleave both latent and active TGF-β2, and it is possible (although unlikely) that following solubilisation the protease mixture cleaved self proteins including Der p 1. Both preparations showed serine protease activity. HDM extracts can also contain collagenolytic (Der p 9) and amylase-like (Der p 4) proteins, and may contain many more active substances (Thomas *et al.*, 2002). The HDM preparations were not assayed for other protease classes.

Latent TGF-β can be activated directly by proteolysis (Lyons *et al.*, 1990; Oursler *et al.*, 1993), although cell and/or matrix interactions may also be required (Abe *et al.*, 1998; Sato *et al.*, 1993). Nakamura *et al.* demonstrated activation of the small latent complex (SLC) of TGF-β1 in a cell-free system by the proteolytic action of purified Der f 1, an allergen from the North American *Dermatophagoides farinae* dust mite. Der p 1 and Der f 1 share 82% sequence

homology (Takai *et al.*, 2005), so it may be supposed that Der p 1 has the potential to activate the SLC of TGF-β1. There are no similar studies of latent TGF-β2 activation.

Results from TGF-β1 activation studies should not be directly extrapolated to TGF-β2. TGF-β1, -β2 & -β3 have 75% amino acid homology and share common receptors and signalling pathways. In contrast, their respective LAPs have 45-50% amino acid homology, and can be further altered by glycosylation (Saharinen *et al.*, 1999). LAP of TGF-β2 does not contain an RGD amino acid sequence and is therefore not thought to undergo integrin-mediated conformational or proteolytic activation. These differences may lead to differences in activation susceptibility, although the physicochemical activation profiles (*via* heat or acidification) of all three SLC isoforms are very similar (Brown *et al.*, 1990). Activation by proteolysis is however more sensitive to sequence and structural differences that arise as these may affect enzyme recognition sites. Indeed, previous reports suggest that protease activation experimentally activates only 15-20% of the latent pool of TGF-β, but also degrades a proportion of the cytokine (Brown *et al.*, 1990). Degradation of TGF-β2 by papain and HDM preparations was observed in cell-free and cell-associated experiments in this study. It should also be noted that no cell viability assays were carried out following protease exposure. It is therefore possible that decreases in TGF-β2 levels observed were due to decreased numbers of viable cells in the population. This could be ascertained through Annexin V staining of cultures, or trypan blue exclusion analysis of cell layers.

The configuration of LTGF-β2 produced by bronchial structural cells is unknown. In the kidney, 5 different arrangements of latent TGF-β1 have been identified, and 3 in the prostate, due to secretion as either the SLC or large latent complex (LLC), and alternative splicing of LTBP proteins (Hyytiäinen *et al.*, 2004). It would be of interest to determine the composition of latent TGF-β2 complexes secreted by cells used in this study as these differences may be

important physiologically in determining the susceptibility of the latent cytokine to activation.

The majority of cell types secrete TGF-β as a LLC, therefore removal of both LTBP and LAP to expose the receptor binding site of mature TGF-β2 is a requirement of activation. Cleavage of LTBP-1 in bone matrix secreted by osteoclasts has been identified through the proteolytic action of plasmin, elastase, MMP-2 and MMP-9 (Dallas, 2002). This freed the SLC for secondary steps of activation. Additionally, plasmin activation of TGF-β1 in the SLC was shown in a separate study to occur by proteolytic "nicking" of the latency-associated peptide (LAP) (Lyons *et al.*, 1988). Proteolytic processing of TGF-β from a latent to an active state can therefore occur *via* a multi-step process, and may involve multiple proteases.

The serine protease tryptase is released by a a subset of mast cells upon degranulation. Tryptase-mediated activation of LTGF-β1 was previously shown by Tatler *et al.* in the presence of human airway smooth muscle cells (ASM), however total levels of TGF-β2 remained unchanged (Tatler *et al.*, 2008). In this study tryptase activated TGF-β2 in the presence of cells, and increased the total level of LTGF-β2 (Section 4.2.5). This discrepancy between results may be attributable to the use of a different cell type (ASM vs. transformed bronchial epithelial cells). Tryptase-mediated activation of cells provides a possible mechanism by which allergic asthmatics show increased epithelial TGF-β2 levels.

Salib *et al.* investigated TGF-β production following HDM challenge (with a skin prick test formulation of HDM) of the nasal epithelial cell line RPMI 2650, and found increased total TGF-β2. This is in contrast to the results from the transformed cell line 16HBE140- reported here (Section 4.2.5). However, this may be due to differences arising from the use of transformed cells.Challenge with IL-4 or IL-13 did not alter TGF-β2 expression (Salib *et al.*, 2005). This is in contrast to data from primary HBEC challenge reported by Richter *et al.*, whereby IL-4 and IL-13 exposure stimulated HBEC TGF-β2

release (Richter *et al.*, 2001). This highlights the potential differences between transformed and primary cells, and upper (nasal) and lower (bronchial) airway epithelial responses.

The increase in active and total levels of TGF-β2, above that of physical injury, following HDM SPT challenge of a primary HBEC donor reported here is a novel finding. However, variability of primary cell responses means increased donor numbers are required for further investigations.

Chapter 5: TGF-β2 mediated remodelling responses of healthy and asthmatic bronchial fibroblasts

5.1 Overview

Chapter 3 demonstrated that mild asthmatic bronchial biopsy tissue had significant reticular basement membrane thickening and increased myofibroblast-like cells in the lamina propria. Chapter 4 established possible bronchial epithelial cell modulation of TGF-β2 secretion and activation linked to environmental challenge. Other authors have shown increased TGF-β2 in asthmatic tissue, both at baseline and following allergen challenge (Balzar *et al.*, 2005a; Batra *et al.*, 2004; Chu *et al.*, 2004).

It has been suggested that fibroblastic cells derived from asthmatic donors may show alterations at the level of morphology and baseline proliferation (Westergren-Thorsson *et al.*, 2010). Increased collagen production has been identified in asthmatic lung, and linked with TGF-β1 expression and eosinophil infiltration (known secretors of TGF-β2 in the lung) (Nomura *et al.*, 2002). Previous publications by other groups using asthmatic fibroblasts demonstrated significant phenotypic changes using 500pg/ml TGF-β2 (Thompson *et al.*, 2006; Wicks *et al.*, 2006).

5.2 Aims & hypothesis

This chapter addresses the hypothesis that bronchial fibroblasts derived from mildly asthmatic donors are more sensitive to the pro-remodelling effects of TGF-β2 in comparison to healthy donor cells.

The hypothesis will be tested through the following aims:

1. Investigate alterations in ECM production following TGF-β2 exposure through pro-collagen Iα1 and pro-collagen III mRNA expression changes, and acid-soluble collagen protein measurements.

2. Evaluate myofibroblast differentiation by measurement of α-SMA expression levels.
3. Evaluate non-contact communication from bronchial epithelial cells to underlying fibroblasts, by challenge of NHLF with HBEC conditioned media. ECM production and α-SMA changes will be monitored following challenge.

5.3 Results

5.3.1 Morphology and proliferation of primary bronchial fibroblasts from healthy and asthmatic donors

The cells used in this study between passages 4-8 were grossly examined for alterations in appearance between healthy and asthmatic cells (Figure 5.1).

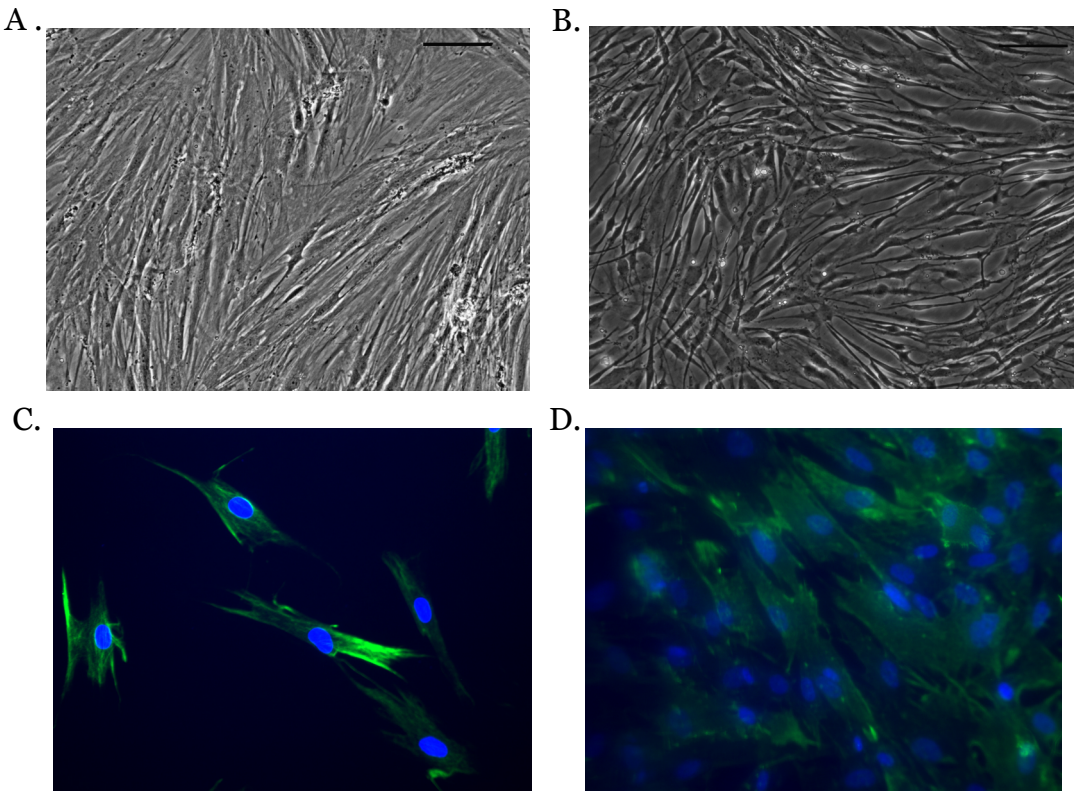


Figure 5.1 Primary healthy and asthmatic fibroblasts are morphologically indistinguishable in culture. Representative photomicrographs of healthy (A & C) and asthmatic (B & D) fibroblasts. All donor cells showed the archetypal spindle shape of fibroblasts in culture. Positive staining (in green) for vimentin (C & D, at x40 original magnification) confirmed a mesenchymal phenotype; nuclei are shown in blue (DAPI) (Scalebar = 50μm).

Gross examination of ongoing cultures did not reveal differences in cell phentoype. Mesenchymal cell type was confirmed by positive vimentin immunostaining. There is no archetypal fibroblast protein marker, so instead cells were confirmed negative for desmin protein (smooth muscle marker), von Willebrand factor (endothelial marker) and cytokeratin (epithelial marker)(results not shown). Both healthy and asthmatic cells displayed the same marker expression profile.

Initial assessment of fibroblast proliferation was carried out following 48h in culture with and without TGF-β2 (Figure 5.2). A preliminary experiment using 1, 5 and 10ng/ml TGF-β2 showed the lower limit of 1ng/ml TGF-β2 was sufficient to increase collagen protein production. Previous publications by other groups using asthmatic fibroblasts demonstrated significant phenotypic changes using 500pg/ml TGF-β2 (Thompson *et al.*, 2006; Wicks *et al.*, 2006). In addition, NHBEC were shown to secrete up to 600pg/ml total TGF-β2 in the previous chapter (Section 4.2.6). For all fibroblast stimulations it was decided to use 1ng/ml as standard.

Assessment of proliferation rate was carried out 48h after seeding in 12 well plates, with and without TGF-β2. Results are shown as proliferation rate (Figure 5.2). Baseline fibroblast proliferation did not differ between healthy and asthmatic donor cells used in this study. TGF-β2 exposure led to a decrease in proliferation, more apparent in healthy samples tested.

Donor cells used in this study showed no significant increases in cell morphology, baseline proliferation or proliferative response to TGF-β2.

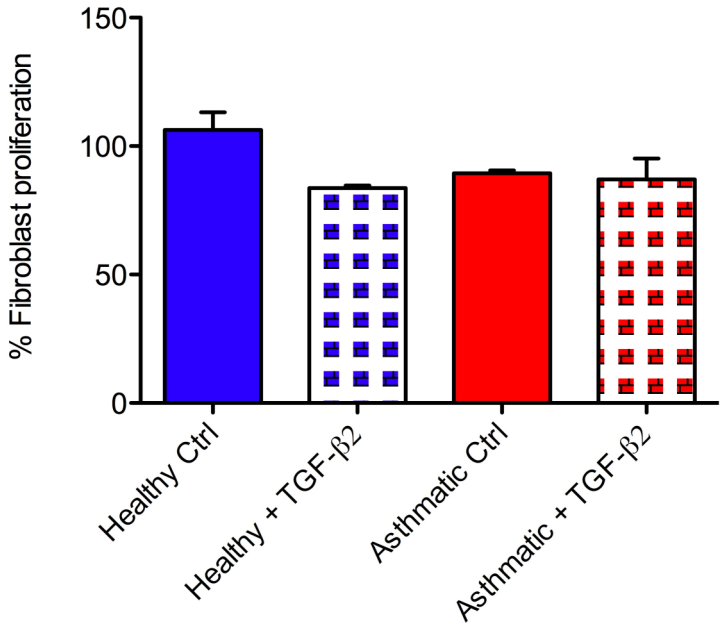


Figure 5.2 Fibroblast proliferation *in-vitro* following TGF-β2 stimulation
No significant difference in proliferation was observed following 1ng/ml TGF-β2 exposure for 48h, or between healthy and asthmatic cell basal or TGF-β2 stimulated rates. (2 subjects/group; n=6 for each subject). Data is expressed as % fibroblast proliferation (cell no. at 48h/(initial seeding cell no./100).

5.3.2 Timecourse gene expression analyses

Quantitative PCR (q-PCR) was used to determine the expression *of COLIα1* (pro-collagen Iα1)*, α-SMA* and *TGF-β2* in healthy NHLF over time. The expression pattern of these genes thought to be upregulated in asthma and following TGF-β2 stimulation. mRNA was extracted at timepoints between 0 and 72h. Cells were between 70-80% confluency at 0h to avoid contact inhibition affecting mRNA transcription. Gene expression results were normalised to *GAPDH* and *ATP5B*, and shown as a fold change from baseline expression at timepoint 0h (Figure 5.4). Results were analysed by one-way ANOVA, followed by Dunnett's post-hoc test comparing values to that of the time zero control.

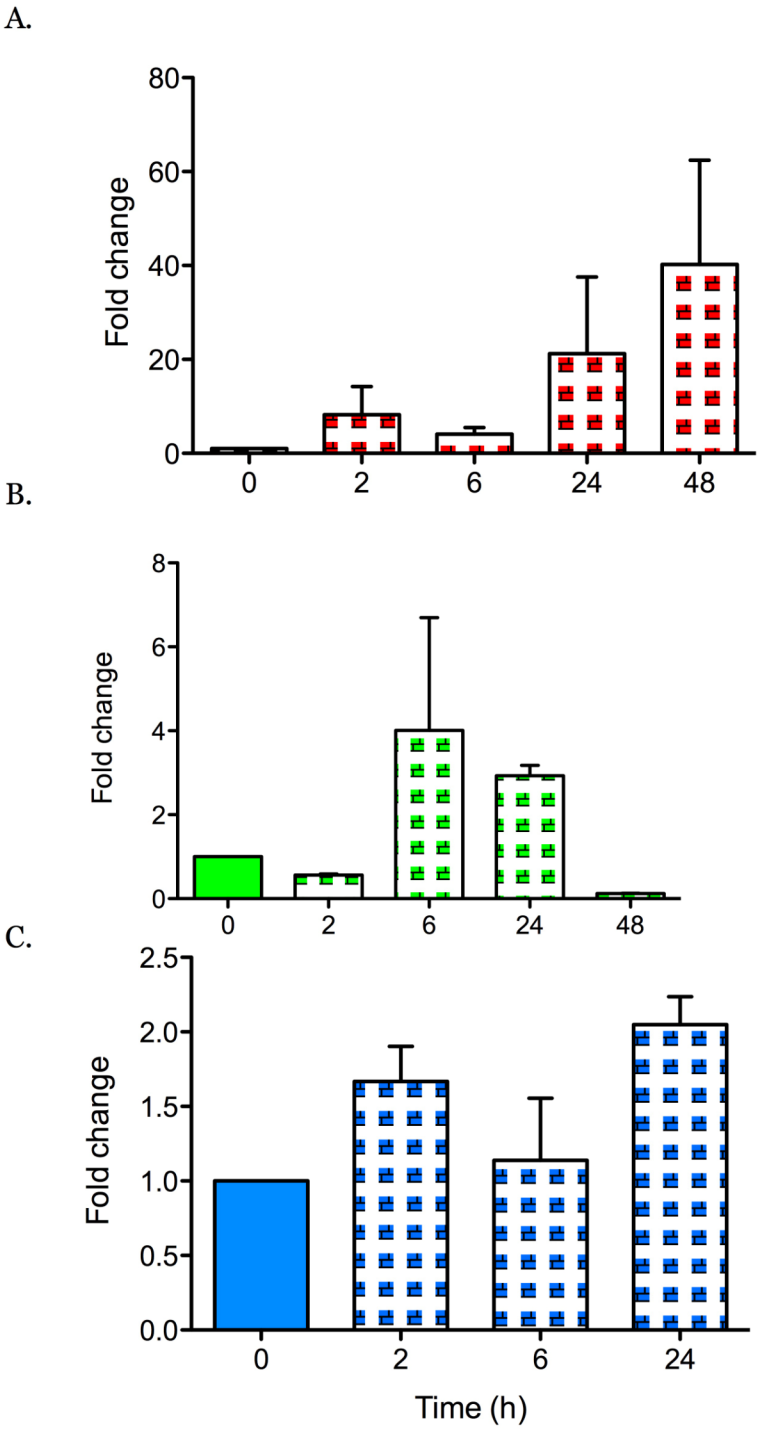


Figure 5.3 Timecourse of mRNA expression levels in NHLF following TGF-β2 stimulation. Fibroblast cultures were stimulated with 1ng/ml TGF-β2, mRNA harvested and reverse transcribed, then q-PCR analysis of A. *COLIα1*, B. *α-SMA* and C. *TGF-β2* performed. Values were normalised to *GAPDH* and *ATP5B*, and are shown as a fold change from time zero, +/- SEM. n=5 independent samples at each timepoint. Data analysis showed no statistical significance.

Expression of *COLIα1* was variable over the timecourse, showing greatest increases at 24h and 48h after stimulation. At 72h following stimulation cultures had become quiescent and did not yield sufficient RNA for analysis. Up to 48h, fold change increases in *COLIα1* expression were consistently observed, but did not show statistical significance were statistically insignificant due to wide variation within each timepoint.

α-smooth muscle actin mRNA expression was nominally greatest 6h following stimulation, however variation within the sample group meant it was not significant. At 24h, there was a consistent trend towards increased *α-SMA* expression, that fell to below baseline levels by 48h. *TGF-β2* expression was marginally increased over the timecourse, but again showed wide within-timepoint variability, and was not statistically significant.

In the healthy bronchial fibroblasts used results were not statistically significant, however increases in expression were observed at 24h for the three genes analysed. Pro-collagen Iα1 showed greatest increases following TGF-β2 exposure. This timepoint was used in all further q-PCR analyses.

5.3.3 Comparison of collagen production by healthy and asthmatic bronchial fibroblasts in culture

The previous section established that 24h was an optimal timepoint for mRNA analyses using commercially obtained healthy fibroblasts. In the following set of experiments, fibroblasts isolated from 1 healthy and 2 mildly asthmatic donors were challenged with TGF-β2 at 1ng/ml. Cultures were harvested at 24h, mRNA extracted and reverse transcribed, and q-PCR performed (Figure 5.5). Only one healthy donor sample was available for qPCR analysis due to limited sample availability.

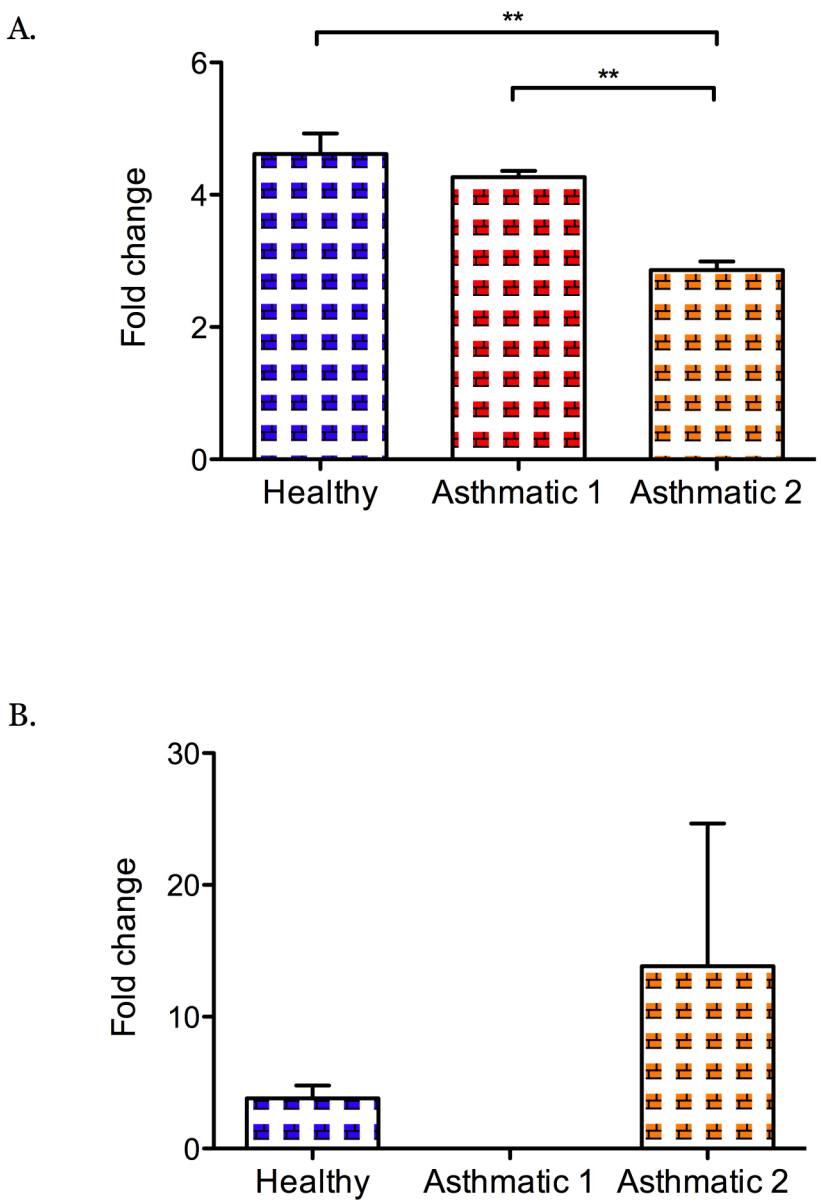


Figure 5.4 mRNA expression levels of *COLIα1* (A) and *COLIII (B)* in healthy and asthmatic fibroblasts at 24h post TGF-β2 stimulation. mRNA was harvested 24h after challenge with 1ng/ml TGF-β2. mRNA levels are expressed relative to *GAPDH* and *ATP5B,* and as a fold change from time-matched unstimulated controls. ** p=<0.01. N=3 for each subject.

Pro-collagen Iα1 expression was increased in all samples following 24h TGF-β2 exposure. There was a significant increase in *COLIα1* between asthmatic sample 2 and healthy, and between asthmatic sample 2 and asthmatic sample 1. Greatest increases from time matched controls were observed in healthy fibroblasts. Results here suggest that the 2 asthmatic fibroblast samples assessed did not have an increased TGF-β2 initiated collagen response compared with healthy fibroblasts.

The mRNA levels indicate up- or downregulation of the gene, but not the successful secretion of the protein. Determination of collagen protein production *in-vitro* in this study was by Sircol™ assay. This contains Sirius red dye that binds to the Gly-X-Y helical structure of mammalian collagen fibrils types I-V. It does not detect mature fibrils of covalently cross-linked collagen without prior acid-pepsin digestion, and so measured only newly synthesised collagen. To gauge complete collagen production, both soluble secreted levels in conditioned media and cell layer-associated collagen were measured in 2 healthy and 2 asthmatic-derived fibroblast donors. The results were normalised to cell number (Figure 5.6).

Results illustrate increased total (media + cell layer) collagen protein production within each donor cell set. Figure 5.6A shows a significant response ($p<0.001$) to TGF-β2 stimulation by a healthy donor, whilst the healthy donor 2 in Figure 5.6B did not reach statistical significance. Both asthmatic donor samples 1 and 2 significantly increased collagen production when exposed to TGF-β2 ($p<0.05$). To assess whether these changes were significantly different between groups, collagen protein levels were converted to percentage increases (Figure 5.7).

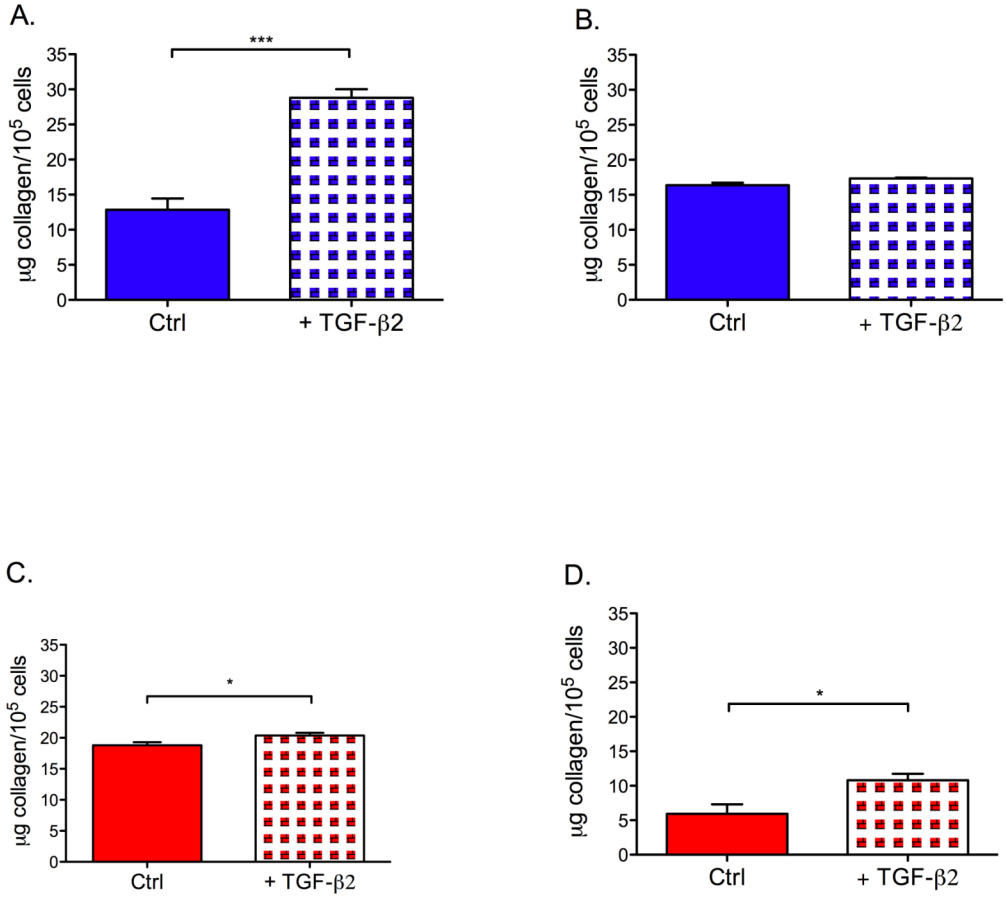


Figure 5.5 Analysis of total collagen secretion at 48h in healthy and asthmatic fibroblasts, with and without TGF-β2 stimulation.

Total collagen produced by healthy (A & B) and asthmatic (C & D) fibroblasts, 48h following TGF-β2 stimulation, in the presence of L-proline and ascorbic acid. Collagen production is shown normalised to cell number. Within donor data (stimulated *vs*. unstimulated) were analysed by t-test. *$p<0.05$, n=5 for each donor and condition.

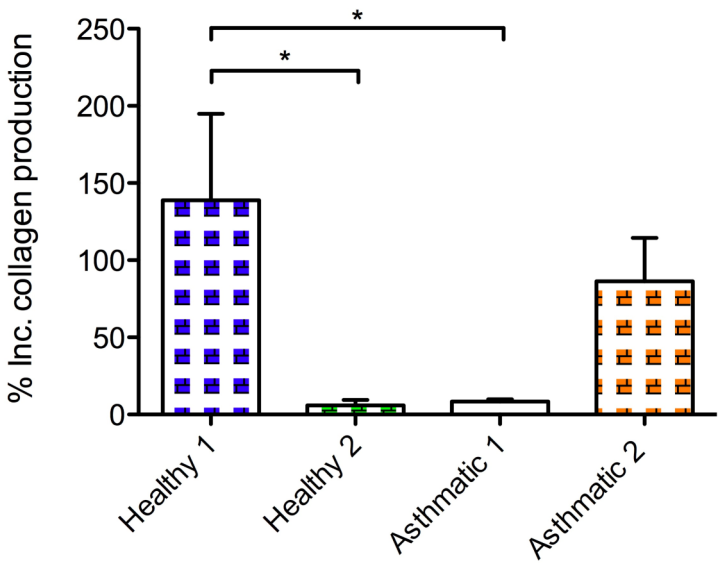


Figure 5.6 Percentage collagen production increase following TGF-β2 stimulation. Results were analysed by ANOVA followed by Dunnett's test.

Collagen protein production was variable both within and between donors. Healthy donor 1 showed a significant increase ($p<0.05$) in % collagen production compared with healthy donor 2 and asthmatic donor 1 fibroblasts.

In the preceding results, no difference was attributable to the asthmatic status of cells. It was shown that primary cell responses are highly variable, and no distinction can be made between mildly asthmatic and healthy fibroblast collagen production. This is in agreement with previously published studies (Dubé *et al.*, 1998).

5.3.4 Comparison of myofibroblast differentiation in healthy and asthmatic bronchial fibroblasts in culture

Differentiation of the bronchial fibroblast to a more secretory, smooth muscle-like cell type – the myofibroblast, may be an important facilitator of AWR phenomena. Conventionally this differentiation is identified by the expression of α-smooth muscle actin (Darby *et al.*, 1990), and may be increased in allergic asthma (Gizycki *et al.*, 1997). TGF-β2 stimulated and control fibroblasts were harvested for mRNA and protein, and α-smooth muscle actin expression and production analysed.

Asthmatic donor samples (n=2) showed a significant increase in *α-SMA* mRNA compared to healthy fibroblasts at 24h. Similar results were reported by Wicks *et al.*, who also showed asthmatic fibroblasts responded with smooth muscle transcript increase at a ten-fold lower concentration of TGF-β2 than healthy (Wicks *et al.*, 2006).

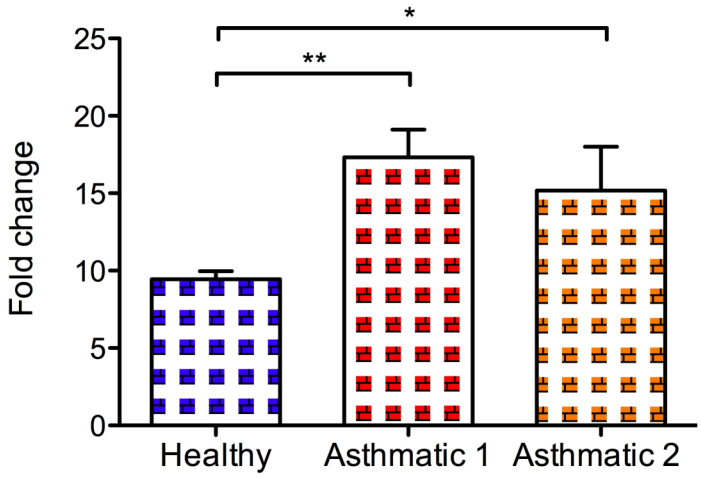
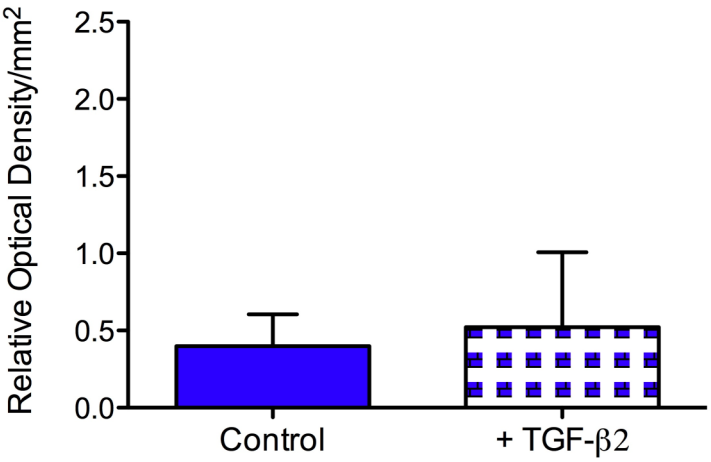


Figure 5.7 Expression level of *α-SMA* mRNA in healthy and asthmatic fibroblasts 24h following TGF-β2 stimulation. mRNA levels are expressed relative to *GAPDH* and *ATP5B,* and as a fold change from time-matched controls +/- SEM. *p<0.05, **p<001, n=3 for each subject. ANOVA followed by Tukey's.

For protein analysis, initially 24h, 48h and 72h timepoints were assayed and 48h chosen as most representative timepoint. One healthy and one asthmatic donor were available for assay due to limited sample availability. TGF-β2 challenge experiments were repeated three times in each set of donor fibroblasts.

Fibroblast cultures were harvested at 48h in RIPA buffer, cell lysates seperated on 4-12% gradient SDS-PAGE gels and blotted onto nitrocellulose membrane. Membranes were probed for α-SMA protein. To ensure equal loading of gels and to normalise α-SMA band densities, membranes were stripped and re-probed for vimentin. Results from healthy donor fibroblasts are shown in Figure 5.9. α-SMA protein levels did not differ significantly from control at 48h. TGF-β2 challenge was repeated on one set of asthmatic donor fibroblasts (Figure 5.10).

A.



B.

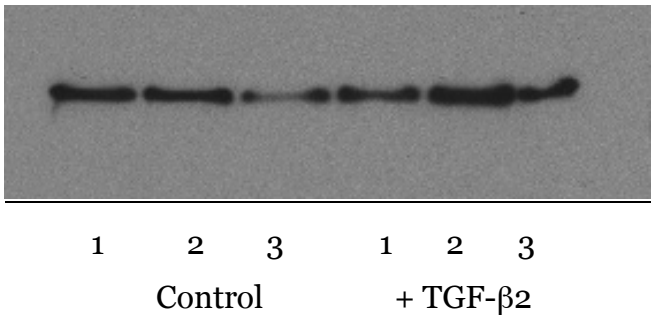
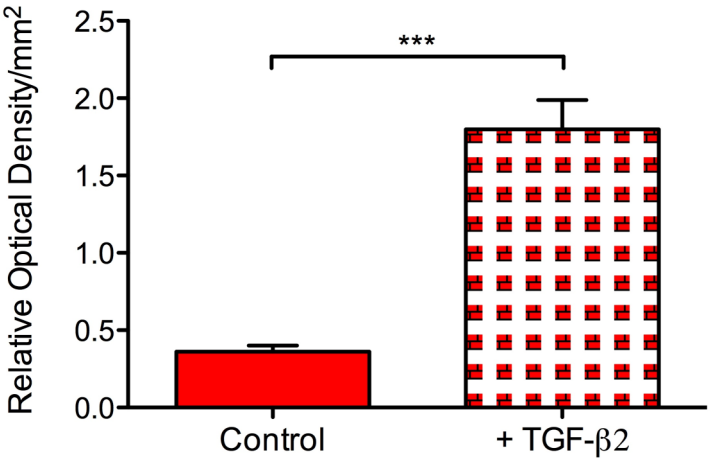


Figure 5.8 Healthy fibroblast α-SMA protein expression.

Relative optical density of western blotted α-SMA protein bands were measured on ?? (A). A representative scanned image of α-SMA is shown in (B). α-SMA was normalised to vimentin expression. No significant difference was found (n=3).

A.



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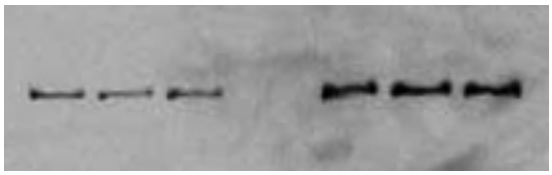

C.

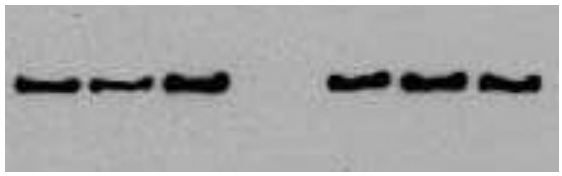

Figure 5.9 Asthmatic fibroblast α-SMA protein expression.
Band densitometry (A) shows significant increase in α-SMA protein (+/- SEM, analysed by t-test, $p<0.001$). Representative α-SMA protein bands shown in (B) were measured and normalised to vimentin levels (C) (n=3).

As seen in Figure 5.10B, α-SMA was consistently increased following TGF-β2 stimulation in asthmatic fibroblasts from one donor. This is consistent with qPCR results shown in Figure 5.8. Baseline protein expression did not differ between healthy and asthmatic donors. A conclusion cannot be drawn from

these results as to whether asthmatic cells are intrinsically more sensitive to TGF-β2, due to insufficient donor sample number. Results do confirm however, the variability inherent in primary cell responses.

5.3.5 Healthy bronchial fibroblast remodelling responses modulated by HBEC soluble secreted factors

As the barrier between underlying lung tissue and the environment, bronchial epithelial cells are thought to respond to environmental challenge with a cascade of actions that could affect many underlying cells including bronchial fibroblasts. In Chapter 4 Section 4.2.6 it was shown that TGF-β2 secretion can be modulated in HBEC in reponse to environmental challenge. The following section addresses the hypothesis that healthy bronchial fibroblasts would show increased remodelling responses (collagen production and myofibroblast differentiation) when exposed to soluble secreted factors present in bronchial epithelial cell conditoned media. It was also posited that exposure to conditioned media derived from asthmatic bronchial epithelial cells would generate a greater response.

5.3.5.1 HBEC conditioned media effects on ECM production by healthy fibroblasts

Bronchial epithelial cell conditioned media was prepared by collecting from healthy and asthmatic HBEC cultures between d7-21 of ALI culture. Media from single donors was pooled and frozen at -20°C until use. Control fibroblast cultures were exposed to fresh BEDM, and challenged cultures received half fresh BEDM, half HBEC conditioned BEDM. Only healthy fibroblasts were challenged in these experiments.

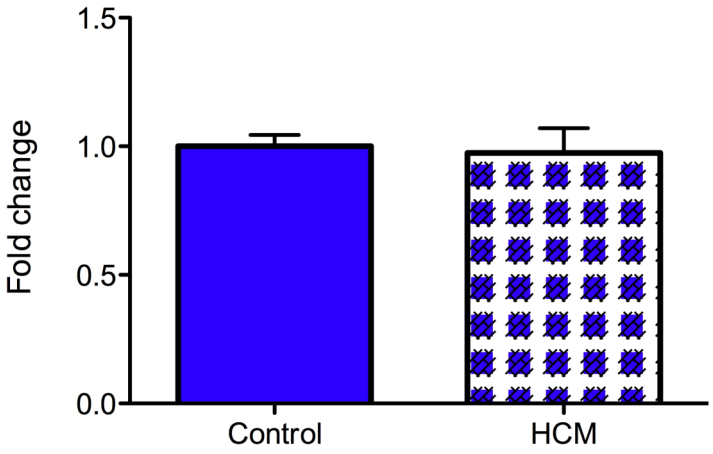

Figure 5.10 mRNA expression levels of *COLIα1* in healthy fibroblasts at 24h. Cultures were exposed to BEDM (control) or 50% healthy bronchial epithelial cell conditioned media (HCM). mRNA levels are expressed relative to the geometric mean of *GAPDH* and *ATP5B*. No significant difference was found (n=2).

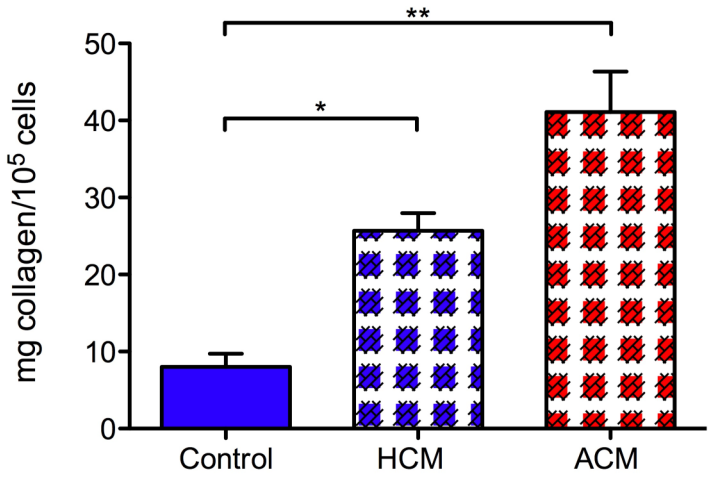

Figure 5.11 Total collagen secretion by healthy fibroblasts following conditioned medium exposure. Healthy (HCM) and asthmatic (ACM) HBEC CM exposed fibroblast cultures were analysed by Sircol assay at 48h.Results are shown normalised to cell number. Results were analysed by ANOVA followed by Dunnett's post-hoc test. (*p<0.05, **p<0.01, n=5).

No difference was observed in pro-collagen Iα1 mRNA expression in healthy fibroblasts following healthy BEC conditioned media exposure (Figure 5.11). Insufficient asthmatic conditioned media was available at the time of the experiments, and so was not included. Exposure to HBEC CM significantly increased healthy fibroblast collagen protein production compared with control BEDM alone (Figure 5.12). Asthmatic-derived CM exposure had an even greater effect than healthy CM.

Asthmatic CM was assayed by ELISA at 1036pg/ml total TGF-β2, and 34.3pg/ml active TGF-β2, in comparison to healthy CM that had 1191pg/ml total TGF-β2 and 16.3pg/ml active. As the active (and therefore available) TGF-β2 levels are so low, it is highly likely that other secreted factors are affecting collagen production.

5.3.5.2 HBEC conditioned media effects on myofibroblast differentiation by healthy bronchial fibroblasts

α-SMA protein expression was assessed as an indicator of myofibroblast differentiation following conditioned media exposure. HBEC CM exposure significantly increased healthy fibroblast α-SMA production compared with control media (Figure 5.10). As with collagen protein production, asthmatic-derived CM exposure had an even greater effect than healthy CM.

Results suggest that in the asthmatic CM used, there is either an increase in one factor that can drive collagen production and myofibroblast differentiation, or that a combination of soluble factor alterations are having a direct effect on fibroblast phenotype and function, that could then contribute to AWR.

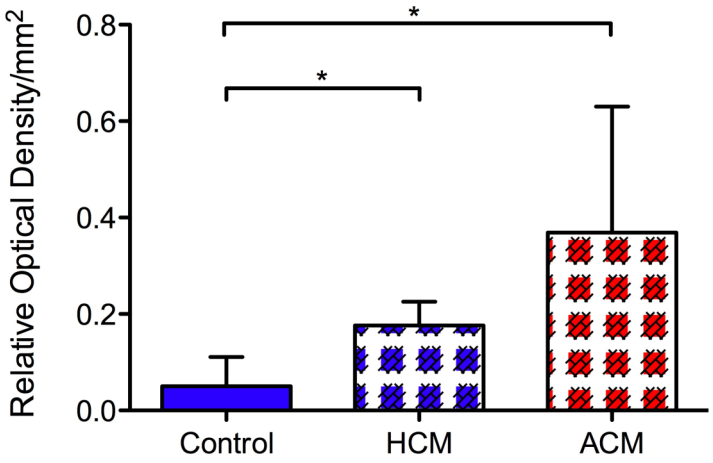

Figure 5.12 Healthy fibroblast α-SMA protein expression following 48h conditioned media exposure. Healthy (HCM) and asthmatic (ACM) HBEC CM exposed fibroblast cell lysates were western blotted. Band densitometry showed variable yet significant ($p<0.01$) increases in α-SMA protein expression. Results were analysed by ANOVA followed by Dunnett's test (n=3).

5.3 Summary

Fibroblasts isolated from healthy and asthmatic donors appeared phenotypically similar. All donor cells responded to TGF-β2 stimulation.

Collagen mRNA and protein levels varied between donors. TGF-β2 stimulation resulted in increased expression and protein production, but this did not differ significantly between healthy and asthmatic donor fibroblasts. A significant increase in α-SMA mRNA was observed following TGF-β2 stimulation in both healthy and asthmatic donor cells. Asthmatic cells showed a statistically greater increase than healthy cells, and this was also shown at the α-SMA protein level.

Conditioned media derived from asthmatic HBEC had a greater effect upon collagen production and α-SMA by NHLF than healthy HBEC CM, and was not attributable solely to TGF-β2 levels.

The aims of this chapter were conceived to test the hypothesis that bronchial fibroblasts derived from mildly asthmatic donors are more sensitive to the pro-remodelling effects of TGF-β2 in comparison to healthy donor cells. Experimental results showed α-SMA expression was increased in asthmatic-derived fibroblasts to a greater degree than in healthy fibroblasts. In the limited sample sizes used, the hypothesis may be partially accepted; asthmatic fibroblasts were more susceptible to the pro-myofibroblast differentiation effect of TGF-β2, but not to the pro-secretory effects.

5.4 Discussion

There is a wealth of published literature that attempts to identify intrinsic differences in asthmatic structural cells, often employing *in-vitro* studies. There appears no consensus as to whether mildly asthmatic fibroblasts are morphologically distinct from their healthy counterparts. Kotaru *et al.* assessed fibroblasts from bronchial and distal regions of asthmatic lung, and asserted that increased cytoplasmic projections on bronchial fibroblasts was indicative of a resident secretory cell type, rather than a more spindle-shaped proliferative phenotype (Kotaru, 2006). This study did not compare asthmatic derived cells with healthy controls however. Nihlberg *et al.* used this method to compare healthy and mildly asthmatic cells in the same manner, finding that asthmatic bronchial fibroblasts had significantly (1.4x, $p<0.001$) more extensions than healthy. This was taken by the authors to indicate an ECM secretory phenotype (Nihlberg *et al.*, 2010). This method was not used in this study as it relatively untried.

The Nihlberg study above described baseline proliferation as 24% lower in mildly asthmatic fibroblasts, which disagrees with the finding shown in Figure 5.2. Other studies have found increased proliferative rates in asthma, although their results are taken following stimulation with the Th2 cytokines IL-4 and Il-13 (Kraft *et al.*, 2001). However, the results presented here, finding no significant difference between healthy and asthmatic cells, are in agreement with other authors (Ward *et al.*, 2008). These contrasting results may be due to differences in cell passage number, or confluence prior to seeding making

cells more quiescent and therefore increasing time taken to enter the cell growth cycle. Additionally, these reports use different methods to calculate proliferation, including MTT assay, crystal violet absorbance and cell counts.

Validation of the stability of expression of housekeeping genes is an important pre-requisite of q-PCR studies as results may otherwise be misleading. The geNorm™ analysis shown in Figure 5.3 was undertaken following publication of an increase in expression of commonly used housekeeping genes in bronchial fibroblasts exposed to TGF-β2 (Wicks *et al.*, 2006). Furthermore, the authors reported a 10-fold lower threshold of smooth muscle-related gene transcript activation in asthmatic fibroblasts, a result which broadly in agreement with the finding of this study that asthmatic cells were more predisposed to increase α-SMA protein production upon TGF-β2 stimulation.

Increased collagen production in asthmatic lung was previously demonstrated by measurement of collagen peptides in induced sputum of asthmatics (Nomura *et al.*, 2002). Other studies have used analysis of amino acid hydroxyproline levels by HPLC to determine collagen deposition *in-vivo* and *in-vitro* (Marshall *et al.*, 2004), however the Sircol™ assay is now a well-validated and reproducible method of collagen measurement. Dube *et al.* compared baseline pro-collagen I and III production by healthy and asthmatic bronchial fibroblasts, and reported no difference between diseased and control cells. The authors did not assess collagen production following TGF-β isoform stimulation (Dubé *et al.*, 1998). A study using fibroblasts isolated from lung resection tissue did not find a significant increase in collagen I or collagen III mRNA or protein following TGF-β2 or TGF-β1 stimulation (Batra *et al.*, 2004). This could be attributable to fibroblast source, as cells were isolated from lung parenchyma and not bronchial tissue. Contrastingly, Malavia *et al.* reported TGF-β2 derived from HBECs stimulated soluble collagen production by NHLF as measured by Sircol™ , a finding repeated in this study (Malavia *et al.*, 2008). Alteration in MMP:TIMP levels is another mechanism by which collagen homeostasis may be altered in the asthmatic lung (Cataldo *et al.*, 2004), and it was reported that asthmatic bronchial fibroblasts in culture exhibited a decreased capacity to degrade collagen, through decreased

production of MMP-2, than healthy control fibroblasts (Laliberté *et al.*, 2001). Subepithelial collagen deposition is a feature of AWR thought attributable to increased numbers of myofibroblasts in the lamina propria (Zhang *et al.*, 1994).

During myofibroblast differentiation cells generate an intra-cellular contractile apparatus containing microfilaments of α-SMA, and other smooth muscle related proteins. In a study comparing asthmatic and healthy bronchial fibroblast responses to TGF-β2, Wicks *et al.* showed increased α-SMA mRNA and protein expression in both cell types. Asthmatic cell α-SMA expression was not significantly greater than healthy, however expression of the smooth muscle related protein calponin 1 was higher in asthmatic fibroblasts. This work also showed asthmatic fibroblasts to be maximally responsive to TGF-β2 at a 10-fold lesser concentration than healthy fibroblasts (500pg/ml vs. 5ng/ml) (Wicks *et al.*, 2006). Similar α-SMA expression results with a single (1ng/ml) concentration of TGF-β2 were reported here, and subsequently it may be of interest to challenge healthy and asthmatic fibroblasts with a range of TGF-β2 concentrations for a more informative comparison of TGF-β2 responsiveness.

Bronchial epithelial cells have been shown to secrete increased amounts of TGF-β2 following injury, and TGF-β2 is increased in asthmatic tissue and BAL fluid (Balzar *et al.*, 2005a; Thompson *et al.*, 2006; Zhang *et al.*, 1999). Exposure of fibroblasts to bronchial epithelial cells or conditioned medium has been used to investigate cellular responses, and here produced increases in collagen production and α-SMA expression that were greater than TGF-β2 levels alone could account for. These results suggest that in the HBEC CM used, there is altered secretion of a soluble factor or factors that drive collagen production and myofibroblast differentiation, and so subsequently contribute to AWR. These alterations appear more pronounced in cells challenged with asthmatic HBEC CM than healthy HBEC CM. Identification of this activity could begin by fractionation of conditioned media by molecular weight prior to

fibroblast challenge to establish those weight fractions that produce the greatest increases in the chosen remodelling markers.

Previous work by other groups has identified a number of soluble secreted factors from HBECs that affected bronchial fibroblast remodelling responses. Hastie *et al.* reported that asthmatic epithelial cell CM stimulated collagen III production, but not collagen I by fibroblasts (Hastie *et al.*, 2002). In this Chapter, it was reported that the *ColIII* gene was lowly expressed and highly variable. Individual collagen subtypes were not assayed, instead the Sircol™ assay used measured soluble collagens I-V, and so any increase in collagen III will be represented in the results given. A co-culture model of fibroblasts cast in type I collagen gels floating above a submerged monolayer of HBECs identified TGF-β2 production by HBECs as the causative factor leading to increased gel contraction. Similar results were reported with HBEC CM alone. Of note, contraction was further increased when HBEC cells were exposed to LPS (Mio *et al.*, 1998). TLR-4 activation via LPS and Der p 2 is seen following HDM stimulation of cells. In Chapter 4, Section 4.2.6, it was shown that LPS exposure increased active TGF-β2 significantly in one of two primary HBEC donors challenged. Additionally, connective tissue growth factor (CTGF), a known downstream effector of TGF-β, could have been secreted by HBECs following autocrine TGF-β signalling and thereby increased fibroblast collagen production as seen (Nishioka *et al.*, 2011).

Several studies report HBEC control of fibroblast proliferative responses. Xu *et al.* reported that MMP-2 was secreted by HBECs and induced bronchial fibroblast proliferation (Xu *et al.*, 2002), whilst McAnulty *et al.* identified modulation by bronchial epithelial prostaglandin E2 (PGE2) production . Furthermore, proliferation was increased in a study by Matsushima *et al.* showing human airway trypsin-like protease (HAT) produced by HBECs activated PAR-2 on bronchial fibroblasts (Matsushima *et al.*, 2006). Following injury of 16HBE14o- cells, increased levels of TGF-β2, basic Fibroblast Growth Factor (b-FGF), platelet-derived growth factor (PDGF) and endothelin-1 levels were assayed in culture supernatants (Zhang *et al.*, 1999). Exposure of fibroblasts to this CM increased proliferation significantly, and blocking these

growth factors inhibited fibroblast proliferation by 76%. Remodelling markers were not assessed however.

Finally, of relevance to the *in-vitro* culture system used TβR expression can be affected i*n-vitro* by cell density, with a low cell density maintaining high receptor levels and an enhanced response to TGF-β stimulation in comparison to high cell density cultures (Petridou *et al.*, 2000; Raghu *et al.*, 1989). Although cultures were time-matched, it is possible that small differences in confluency could have affcted the capacity of cells to respond to TGF-β2. Additionally, assessing cells *in-vitro* separate from the inflammatory context of the asthmatic airway could affect TGF-β responsiveness, suggested by the finding that GM-CSF stimulation increased expression of TGF-β receptors TβRI, TβRII and TβRIII on airway smooth muscle cells (Chen *et al.*, 2003).

Assessment of fibroblast responses made in complete isolation can provide insight into remodelling mechanisms, however the conditioned media challenge results confirm that crosstalk via soluble mediators between cell types is important in mediating cellular behaviour. Furthermore, fibroblasts can influence epithelial cell behaviour, shown by fibroblast CM challenge of bovine bronchial epithelial cells causing a proliferative response, attributed to fibroblast production of hepatocyte growth factor (Skibinski *et al.*, 2007). Evidence presented here and from accumulated published data suggests a role for TGF-β2 produced by HBEC in driving the responses of underlying fibroblasts, although other mediators act alongside the cytokine to produce the sub-epithelial fibrosis of airway wall remodelling.

5.5 Future Work

The work presented in this thesis aimed to test whether alterations in active TGF-β2 levels contribute to airway wall remodelling in the asthmatic lung. Some progress was made towards this, however several points remain upon which this hypothesis may be further tested.

Improvements to the assessement of TGF-β2 protein expression in biopsy tissue could be made through use of a fluorescently labelled secondary antibody. Subsequent measurement of fluorescent counts in a defined area of tissue rather than the more subjective scoring method employed in this study could yield more confident grading of TGF-β2 expression. Additionally, TGF-β activity (rather than protein) could be enumerated by intracellular staining of the downstream signalling protein, phospho-Smad 2.

In TGF-β activation studies, phospho-Smad2 levels could be analysed by western blot of cell lysates, indicating whether TGF-β2 was activated and signalled within a short time frame not observable *via* ELISA of conditioned media. The HDM FP preparation could be fractionated into serine and cysteine proteinase fractions according to the protocol of Winton *et al.* (Winton *et al.,* 1998), and used individually in cell challenges.

This study examined tissue and cellular responses from mild, steriod naïve atopic asthmatics *vs.* non-atopic healthy controls. Inclding new sample groups of non-atopic asthmatics and atopic healthy controls could provide information about basal TGF-β2 regulation in an atopy as well as allergy. Most importantly, the study could be extended to include to include cells and tissue from moderate/severe asthmatics. Phenotypic differences between healthy and severely diseased cells are potentially larger, and therefore more easily identifiable. This could be of use in future experiments seeking to modulate the TGF-β2 response.

Appendix A

Preparation of solutions and protocols for histology (all adapted from "Theory and Practice of Histological Techniques, Bancroft & Stevens, 1990).

D.1 Haematoxylin and Eosin stain

The basic dye haematoxylin stains acidic structures a purple-blue. Nuclei, ribosomes and rough endoplasmic reticulum have a strong affinity for this dye due to high content of DNA and RNA respectively. Eosin is an acidic dye which stains basic structures pink to pink-red.

Harris' Haematoxylin

Haematoxylin monohydrate	5g
Aluminium potassium sulphate 12 hydrate	85g
100% alcohol (IMS)	50ml
Glacial acetic acid	40ml
Sodium iodate	1g
dH_2O	900ml

Eosin

Eosin y	5g
dH_2O	500ml

Protocol

1. Filtered Harris' haematoxylin	4 minutes
2. Running water to "blue"	5 minutes
3. Eosin	30 seconds

4. Brief rinse in water
5. Dehydrate through increasing concentrations of alcohol, to xylene ready for mounting.

D.2 Masson's Trichrome

Nuclei and other basophilic structures are stained blue, collagen green and cytoplasm and erythrocytes red.

Weak picric acid (1% picric acid in 70% alcohol)

Picric acid	6g
100% alcohol (IMS)	350ml
dH_2O	150ml

Biebrich scarlet (0.1%)

Biebrich scarlet	0.5g
Glacial acetic acid	5ml
dH_2O	500ml

2.5% phosphomolybdic acid (PMA)/ 2.5% phosphotungstic acid (PTA)

Phosphomolybdic acid	12.5g
Phosphotungstic acid	12.5g
dH_2O	500ml

Acids seperately dissolved in 250ml dH_2O, then two solutions combined.

Fast green (2.5% fast green in 2.5% acetic acid)

Fast green	12.5g
Glacial acetic acid	12.5ml
dH_2O	487.5ml

1. Harris' haematoxylin	4 minutes
2. Running water to "blue"	5 minutes
3. 1% Picric acid	30 seconds
4. Running water until faint yellow tint remains	
5. 0.1% Biebrich scarlet	1 minute
6. Brief rinse in water	
7. 50% PMA/PTA	10minutes
8. Fast green	6 minutes
9. Dehydrate through increasing alcohols, to xylene ready for mounting.	

D.3 Periodic acid-Schiff's (PAS)

Stains glycogen and other reactive carbohydrates magenta, and nuclei blue.
All stocks are bought from Sigma Aldrich, Dorset at ready to use concentrations.

1. 1% periodic acid	10 minutes
2. Rinse in running water	2 minutes
3. Schiff's reagent	10 minutes
4. Rinse in running water	5 minutes
5. Harris' haematoxylin	2 minutes
6. Dehydrate through increasing alcohols, to xylene ready for mounting.	

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