University of Southampton

The Differentiating and Proliferative Effects of Transforming Growth Factor beta (TGFβ) and Epidermal Growth Factor (EGF) on Asthmatic Fibroblasts

Volume 1 of 1

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ABSTRACT

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By Christine Barbara Boxall

Rationale: Airway remodelling is a well-characterised component of asthma and is thought to contribute to increased resistance to breathing and persistence of bronchial hyperresponsiveness in asthmatics. It has been proposed that Transforming Growth Factor beta (TGFβ), a key fibrogenic growth factor released in response to epithelial damage, can promote remodelling by inducing synthesis of extracellular matrix components from (myo)fibroblasts. Epidermal Growth Factor (EGF) is a potent mitogen for epithelial and mesenchymal cells and is also overexpressed in asthma. The balance between EGF driven proliferation and TGFβ mediated differentiation of fibroblasts is of potential importance in the pathogenesis of airway remodelling in asthmatics.

Hypothesis 1: Asthmatic bronchial fibroblasts are more responsive to the effects of TGFβ2 induced differentiation and proliferation than normal fibroblasts.

Hypothesis 2: EGF antagonises the effects of TGFβ2 induced differentiation and proliferation in bronchial fibroblasts.

Methods: Fibroblasts were obtained as outgrowths from bronchial biopsies from normal (n=23) and asthmatic (n=19) subjects. Cultures were treated with TGFβ2 and EGF, alone or in combination to drive proliferation and/or differentiation which was monitored by methylene blue elution assay or αSMA expression. RNA was extracted from the cells for measurement of EGF and TGFβ receptor and ligand expression by Taqman PCR; data were normalised to 18S rRNA. Media were collected for determination of ligand release in response to TGFβ2 and EGF, and measured by ELISA. Binding of TGFβ to its receptors was measured by flow cytometry. Expression and control of amphiregulin (AR) and Heparin Binding EGF-like Growth Factor (HB-EGF) release by ADAM 12 and 17 were measured by Taqman PCR and ELISA. As there were no commercial ELISAs available to measure HB-EGF I developed an HB-EGF and optimised conditions for detection of the growth factor in conditioned media.

Results: TGFβ2 caused growth arrest in fibroblasts and initiated their transformation into myofibroblasts, as shown by induction of αSMA. EGF induced fibroblasts to proliferate but did not induce their differentiation. In combination, EGF and TGFβ promoted fibroblast proliferation. Initially αSMA expression was suppressed, suggesting a dominant EGF effect. Upon prolonged culture, TGFβ2 and EGF promoted fibroblast transformation into myofibroblasts indicating a shift towards TGFβ dominance. Changes in TGFβ receptor and ligand expression did not appear to be disease dependent. There was a trend for down regulation of the TGFβ signalling pathway (TGFβ RI, CTGF, αSMA) in quiescent asthmatic fibroblasts, although no difference was seen after TGFβ2 treatment.

EGF induced mRNA expression of AR and HB-EGF in normal and asthmatic fibroblasts. The increase in AR in response to EGF was higher in the asthmatic fibroblasts ($p=0.019$) compared to the normals. TGFβ had no effect on AR expression but caused a marked dose-dependent induction of HB-EGF expression which was significantly higher in the normal compared with asthmatic cultures ($p=0.024$). AR was detected in conditioned media and its release was promoted by cellular activation with phorbol ester. HB-EGF was detected in conditioned medium after stimulation with phorbol ester, TGFβ2 and hexadimethrine. There was a trend for lower HB-EGF release from asthmatic fibroblasts. There was also a lower release of AR from asthmatic fibroblasts, although this was attributed to utilisation resulting in a small but significant mitogenic effect apparent only in the asthmatic fibroblasts.

Conclusion: The study has revealed novel observations surrounding the complex control of TGFβ and EGF signalling in bronchial fibroblasts. In a TGFβ and EGF rich environment, akin to conditions in inflamed asthmatic airways, fibroblasts can undergo both proliferation and differentiation, potentially contributing to the fibrosis associated with airway remodelling. The suppressed release of AR and HB-EGF, growth factors with potent paracrine activity, lead to a proposal that there is a decline in signalling between the epithelium and asthmatic fibroblasts which may lead to an imbalance in bidirectional communication within the Epithelial-Mesenchymal Trophic Unit (EMTU), leading to decreased epithelial proliferation and an increase in mesenchymal proliferation and differentiation.

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DECLARATION

All the studies presented in this thesis were performed by the author unless otherwise stated.

PUBLICATIONS AND CONFERENCE PRESENTATIONS (CHRONOLOGICAL ORDER)

Review: The Contribution of Transforming Growth Factor Beta and Epidermal Growth Factor Signalling to Airway Remodelling in Chronic Asthma. Boxall C, Holgate ST and Davies DE. Submitted to European Respiratory Journal, July 2004.

Oral Presentation: American Thoracic Society International Conference, Orlando, USA, 2004. Boxall C, Powell RM, Davies DE and Puddicombe SM (2004): Altered regulation of amphiregulin processing in normal and asthmatic fibroblasts Am. J. Resp. Crit. Care. Med. 169 (7), A868.

Poster Discussion: American Thoracic Society International Conference, Orlando, USA, 2004. Boxall C, Powell RM, Davies DE and Puddicombe SM (2004): Regulation of HB-EGF production by TGFβ in normal and asthmatic fibroblasts Am. J. Resp. Crit. Care. Med. 169 (7), A741.

Oral Presentation: Faculty of Medicine, Health and Biological Sciences Postgraduate Conference, University of Southampton, UK, June 2003. Induction of autocrine Epidermal Growth Factor Receptor (EGFR) ligand expression in normal and asthmatic fibroblasts

Poster Discussion: American Thoracic Society International Conference, Seattle, USA, 2003. Boxall C, Powell R, Puddicombe S, Richter A, Davies DE and Holgate ST (2003): Co-ordinate effects of EGF and TGFβ on the growth and differentiation of Bronchial (Myo)fibroblasts. Am. J. Resp. Crit. Care. Med. 167 (7), A959.

Poster Discussion: American Thoracic Society International Conference, Seattle, USA, 2003. Boxall C, Powell R, Puddicombe S, Richter A, Davies DE and Holgate ST (2003): Induction of autocrine Epidermal Growth Factor Receptor (EGFR) ligand expression in normal and asthmatic fibroblasts. Am. J. Resp. Crit. Care. Med. 167 (7), A959.

Poster Presentation: Faculty of Medicine, Health and Biological Sciences Postgraduate Conference, University of Southampton, UK, 2002. Regulation of Transforming Growth Factorβ (TGFβ) receptor expression in normal and asthmatic fibroblasts

Poster: American Thoracic Society International Conference, Atlanta, USA, 2002. Boxall C, Powell R, Puddicombe S, Richter A, Davies DE and Holgate ST (2002): Regulation of Transforming Growth Factorβ (TGFβ) receptor expression in normal and asthmatic fibroblasts. Am. J. Resp. Crit. Care. Med. 165 (8), A77.

Oral Presentation: 8th Annual Yamanouchi Research Institute Conference, Oxford, UK, 2001. TGFbeta and EGF signaling in the epithelial-mesenchymal trophic unit in asthma.

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ABBEVIATIONS

7-AAD	7-aminoactinomycin D
ADAM	a disintegrin and metalloproteinase
AMD	age related macular degeneration
αSMA	α smooth muscle actin
AR	amphiregulin
ALK	activin like kinase
BAL	bronchoalveolar lavage
BECs	bronchial epithelial cells
BMP	bone morphogenetic protein
BSA	bovine serum albumin
BTC	betacellulin
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
cCAF	chicken chemotactic and angiogenic factor
CDKI	cyclin dependent kinase inhibitor
cDNA	copy deoxyribonucleic acid
CO_2	carbon dioxide
CRE	cAMP response elements
CT	cycle threshold
CTGF	connective tissue growth factor
LTC_4	cysteinyl leukotriene C_4
LTD_4	cysteinyl leukotriene D_4
DABCO	1,4-diazabicyclo(2,2,2)octan
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DPP10	dipeptidyl peptidase 10
EAR	early asthmatic response
ECM	extracellular matrix
ECP	eosinophil cationic protein
ED-A	extra domain A
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked-immunosorbant assay
EMTU	epithelial-mesenchymal trophic unit
EPR	epiregulin
ERK	extracellular regulated protein kinase
ET-1	endothelin-1
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FAM	6-carboxyfluorescein
FBS	foetal bovine serum
FcεRI	high affinity IgE receptor
FEV1	forced expiratory volume (in one second)
FITC	fluorescein isothiocyanate

FGF-2	fibroblast growth factor-2
FKBP-12	FK506 binding protein-12
FPPH	familial primary pulmonary hypertension
GAG	glycosaminogylcan
GINA	the global initiative for asthma
GPRA	G-protein coupled receptor for asthma susceptibility
GM-CSF	granulocyte-macrophage colony-stimulating factor
GR	glucocorticoid receptor
HB-EGF	heparin binding EGF
HBSS	hanks buffered saline solution
HBDs	heparin binding domains
HCl	hydrochloric acid
HER	human EGF receptor
HHT	hereditary hemorrhagic telangiectasia
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IL	interleukin
IgE	immunoglobulin E
IP3	inositol 1,4,5-trisphosphate
IPF	idiopathic pulmonary fibrosis
IFNγ	interferon γ
JNK	Jun N-terminal kinase
LAP	latency associated peptide
LAR	late asthmatic response
LLP	large latency complex
LPA	lysophoshatidic acid
MAD	mothers against decapentaplegic (ddp)
MAPKK	Map Kinase Kinase
MAPKKK	Map Kinase Kinase Kinase
MBP	major basic protein
MCP	Monocyte chemoattractant protein
MFI	mean fluorescent intensity
$MgCl_2$	magnesium chloride
MIS	Müllerian Inhibiting Substance
MLCK	myosin light chain kinase
M-MLV	Moloney Murine Leukemia Virus
MMP	Matrix metalloproteinase
MMPI	Matrix metalloproteinase inhibitor
MP1	MEK partner 1
mRNA	messenger ribonucleic acid
NAC	National Asthma Campaign
NHBLI	National Health Blood Lung Institute
NHS	National Health Service
NRG	Neuregulin
NRK	Normal rat kidney
NSBH	non-specific bronchial hyperresponsiveness
OVA	ovalbumin
PAI-1	plasinogen activator inhibitor-1
PAR	protease activated receptor
PBS	phosphate buffed saline

PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipase γ
PMA	4β-phorbol 12-myristate 13-acetate
RANTES	Regulated on activation normal T cell expressed and secreted
Ras	rat sarcoma
RGD	R: arginine; G: glycine; D: aspartic acid
RIN1	Ras interaction/interference
RKIP	Raf kinase inhibitor protein
RPE	retinal pigment epithelium
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
SA-HRP	Streptavidin – horseradish peroxidase
SARA	Smad anchor for activation
SBE	Smad binding elements
SFD	Sorsby's Fundus Dystrophy
SFM	serum free medium
SMAD	Sma- and Mad-related protein
SM MHC	Smooth Muscle Myosin Heavy Chain
SOD	superoxide dismutase
SOS	son of sevenless
SPRED	Sprouty-related EVH1 domain-containing protein
SRE	serum response element
STAT	signal transducer and activator of transcription
STRAP	serine-threonine kinase receptor-associated protein
TAB1	TAK1 binding protein 1
TAK1	TGFβ activated kinase 1
TAMRA	6-carboxy-N,N,N',N',-tetramethylrhodamine
TBE	Tris buffered EDTA
TBST	Tris buffered saline/Tween 20
TCE	TGFβ controlling element
TCR	T cell receptor
TE	Tris-EDTA
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
TGFβR	transforming growth factor β receptor
Th	T helper cell
TIMP	tissue inhibitor of matrix protease
TGIF	tumour growth interacting factor
TMB	tetramethyl benzidine
TNF	Tumour necrosis factor
TRAP-1	TGFβ receptor associated protein-1
TSP1	thrombospondin
UV	ultraviolet
VEGF	vascular endothelial growth factor
WHO	World Health Organisation

CHAPTER 1

The Contribution of Transforming Growth Factor beta and Epidermal Growth Factor Signalling to Airway Remodelling in Asthma

CHAPTER 1

THE CONTRIBUTION OF TRANSFORMING GROWTH FACTOR BETA AND EPIDERMAL GROWTH FACTOR SIGNALLING TO AIRWAY REMODELLING IN ASTHMA

1.1. The problem of Asthma

Asthma is a chronic inflammatory disease of the airways characterized by recurrent respiratory symptoms such as wheezing, breathlessness, chest tightness and coughing. It poses a serious health problem, affecting approximately 10% of the world's population. As prevalence has increased substantially over the last few decades, disease related expenditure has increased proportionately. For example, in 2001 an Asthma Audit by the National Asthma Campaign (NAC) quoted a total figure of £850 million spent on NHS treatment, with a further estimated £1.2 billion in lost productivity and £161 million in social security costs (1). These figures illustrate the financial implications of asthma, but for the individual sufferers, control of symptoms and long term decline of lung function can only be improved by a better understanding of the underlying pathogenesis of the disease.

Asthma is defined as *"a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment."* (2).

The Global Initiative for Asthma (GINA) guidelines comprise a set of procedures for the management of asthma that are recognised around the world by clinicians. These guidelines separate asthma severity into four groups, ranging from intermittent to severe persistent, where physical activity is limited (table 1.1). It

should be noted however, that all patients regardless of their grouping could be subject to severe asthma attacks.

	Symptoms	Night time symptoms
Intermittent	Less than once a week. Normally asymptomatic	Less than twice a month
Mild Persistent	More than once a week, less than once a day	Less than twice a month
Moderate Persistent	Daily β2 agonist use	More than once a week
Severe Persistent	Continuous limited physical activity	Frequent

Table 1.1: GINA classification of asthma severity (adapted from (3)).

There is a wealth of evidence suggesting that the incidence of asthma is increasing worldwide. Surveys carried out in Leicestershire, U.K. in 1990 and eight years later in 1998, on the prevalence of respiratory symptoms in Caucasian children aged 1-5 years, showed both an increase in wheeze and an increase in the diagnosis of asthma (11% to 19%) over the time period (4). A similar U.K. study based in Nottingham in 1988 and 1995 of children between 4 and 11 years of age showed an increase of 7.7% for reported wheeze ever and a 2.7% increase in the number of asthmatic children (5). Furthermore, the NAC Asthma Audit of 2001 reported that general practitioners in the UK in 2000 were presented with over 18,000 new cases associated with asthma attacks every week.

Although there is variation in the prevalence of asthma and atopy between populations, there is a trend towards a higher incidence in westernised countries as opposed to developing countries. This increase cannot be explained solely by changes in genetic factors, due to the time frame involved, or by improvements in diagnostic procedures. It is noteworthy, however, that those countries with the highest disease prevalence of asthma and atopy have close links with the U.K. (New Zealand, Australia, Canada and South Africa) through population migration, suggesting that environmental influences are acting on a strong genetic background.

1.2. Asthma - a Chronic Inflammatory Disease of the Airways

Cytokines are chemical messengers that control immune responses and are secreted mainly by T cells. Cytokines (comprising interleukins and interferons) are divided into two groups, Th1 and Th2 (although the distinction between the two groups is clearer in mice than in humans). Th1 (T-cell Helper type 1) promote cell-mediated immunity whilst Th2 (T-cell Helper type 2) induce humoral immunity (antibodies). Th1 cells are characterised by the predominant secretion of interleukin-2 (IL-2), IL-12, Interferon γ (IFNγ) and Tumour Necrosis Factor β (TNFβ). Th2 cytokines include IL-4, IL-5, IL-9 and IL-13. Atopic asthma has traditionally been viewed as a predominantly Th2 polarized inflammatory disease of the central and peripheral airways.

In utero, the developing foetus grows in a Th2 polarized environment (to reduce the risk of miscarriage, a strong Th2 response is necessary to modify the Th1 cellular response *in utero*). After birth, there is a postnatal transition from a Th2 skewed state to a Th1 polarized state. It is postulated that individuals who are predisposed to develop atopy reach the Th1 state more slowly (for review see Holt *et al*, (6)). There also appears to be some correlation between microbial exposure and development of atopy. In the early 1990s it was discovered that certain invasive or repeated infections during early life could selectively enhance the development of Th1 cells, preventing the proliferation of Th2 cells responsible for allergic sensitisation and atopy (7).

Strachan suggested in 1989 (8) that the prevalence of allergic diseases could be explained *"if allergic diseases were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally"*. The resultant "hygiene hypothesis" generated much interest as it included factors such as the marked improvements in personal and household hygiene standards in industrialised nations since the 1950's, as well as the decline in family size. The reduction of natural childhood immunity to pathogens experienced in the last few decades' has added weight to the hypothesis. However, while a number of studies lend support to the hygiene hypothesis in asthma ontogeny, the evidence still

remains inconclusive. Although exposure to a reduced number or different spectra of micro-organisms may continue to play a role in the increasing prevalence of asthma, a number of other factors have been implicated in the onset of disease. For example, intrauterine exposure to the effects of maternal smoking and nutritional status can increase the risk of developing atopy prenatally.

1.2.1. Allergic sensitisation

The airways of a genetically susceptible individual may be sensitised if exposed to aeroallergen. This process occurs *in utero*, due to low exposure of allergen through the placenta. Initially, allergen is recognised by allergen presenting cells such as dendritic cells, which are located throughout the bronchial epithelium (fig 1.1). The allergen is processed by the cell and displayed on the cell surface in the context of MHC Class II molecules. The dendritic cells then migrate to the lymph nodes where they in turn present the processed allergen to naïve T cells. In the presence of IL-4, this interaction induces the naïve T cells to differentiate into Th2 cells.

Following antigen presentation, the Th2 cells express T cell receptors (TCR) specific to the processed allergen. The Th2 cells can then interact with B lymphocytes, which synthesise and release antibody specific to the allergen of interest. Specifically the TCR, CD40L and CD28 on the Th2 cells bind to the HLA DRII, CD40 and CD80/CD86 respectively, on the B lymphocytes. This interaction induces the release of IL-4 from the Th2 cells, which binds to the IL-4 receptor on the B lymphocyte. IL-4 then activates the promoter of the ε germline gene to initiate transcription and also leads to isotype switching of the antibody heavy chain from IgM to IgE. The released IgE cross links and forms complexes, attaching to the high affinity Fcε receptor present on mast cells and macrophages, thus priming them for subsequent exposure of allergen.

1.2.2. Early Asthmatic Response (EAR)

Subsequent exposure to inhaled allergen such as dust mite faecal pellets or pollen can trigger an attack in a susceptible individual. The early asthmatic response

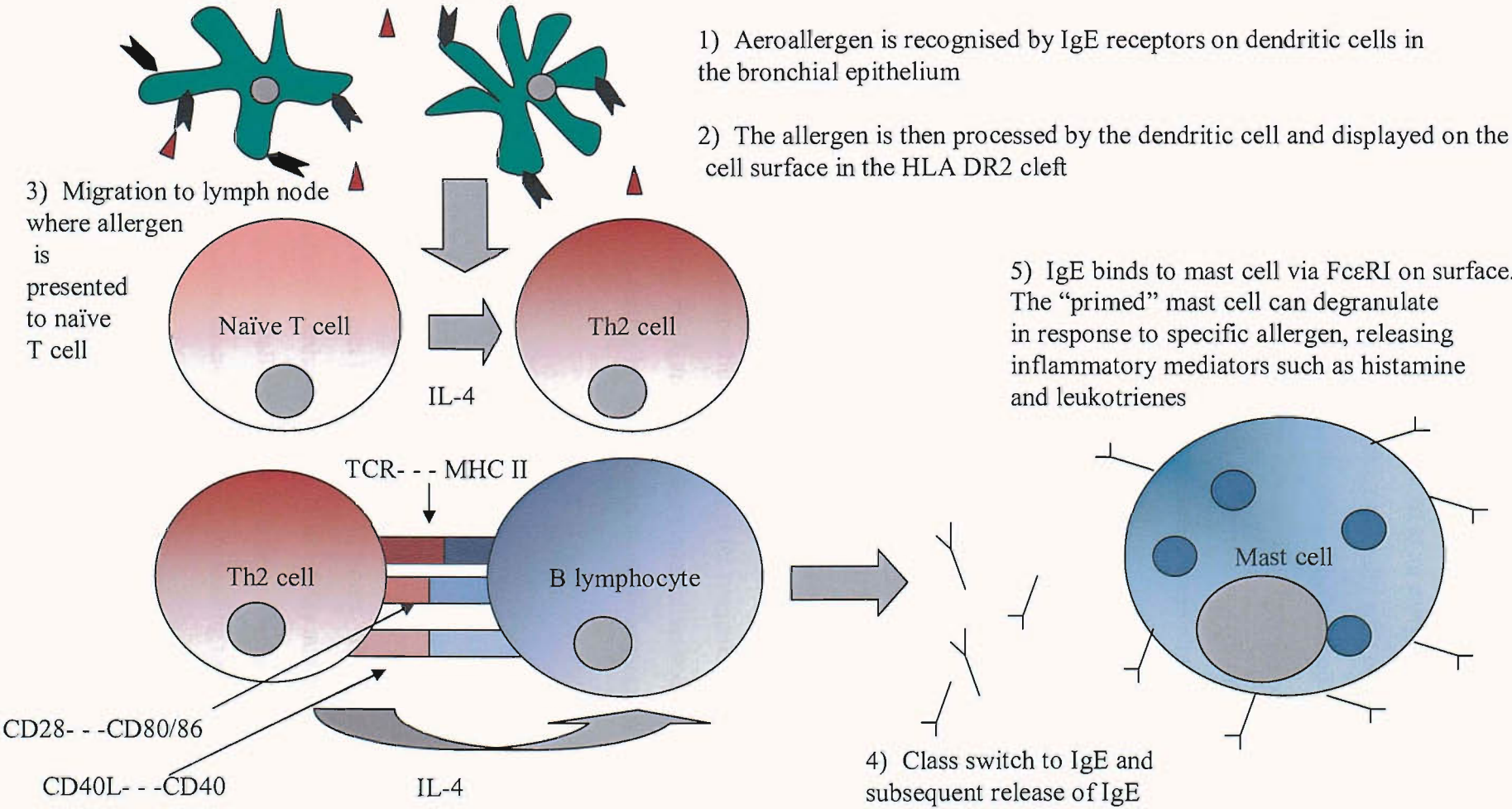


Figure 1.1: Sensitisation to allergen in pre-asthmatic airways

Allergens are processed by dendritic cells in the airways (1+2). The dendritic cells migrate to the lymph nodes where they are involved in the differentiation of Th0 cells (3). The differentiated Th2 cells can then interact with B lymphocytes, promoting release of IgE and subsequent priming of mast cells with allergen specific antibodies (4+5).

(EAR) occurs in atopic individuals 5-10 minutes after exposure to sensitised allergen, and can persist for up to one hour. It is clinically characterised by obstruction of the airways due to contraction of the underlying bronchial smooth muscle. The airways of asthmatics are restructured at an early stage of the disease resulting in excessive narrowing of the bronchi, especially with exogenous stimuli such as exercise, cold air, fog, irritants and allergens. The reduced calibre throughout the 23 generations of conducting airways becomes especially apparent during an exacerbation and in the early hours of the morning (diurnal variation). The extra work of breathing through these narrowed airways along with lung hyperinflation results in the sensation of chest tightness. Vocally airway obstruction is manifested by wheeze, which is caused by turbulent flow through the narrowed bronchi during expiration.

Immunologically, allergen binds to IgE on primed airway mast cells, causing degranulation and release of preformed inflammatory mediators such as histamine and tryptase as well as newly generated autocoids such as oxidative products of arachidonic acid. Histamine is a potent bronchoconstrictor and vasodilator. Tryptase, another bronchoconstrictor, can additionally activate eosinophils as well as increasing microvascular permeability. Cysteinyl leukotrienes such as LTC_4 and LTD_4 and prostinoids such as PGD_2 and TXA_2 are also synthesised and released from mast cells, resulting in further bronchoconstriction, microvascular leakage and mucus hypersecretion.

1.2.3. Late Asthmatic Response (LAR)

The late phase response occurs in about 50% of asthmatic subjects. It starts 2-3 hours after the initial attack and can last between 3-12 hours. The likelihood of initiating a late phase reaction is enhanced by the time of day and by viral upper respiratory tract infections. Clinically, the LAR manifests itself as airway narrowing due to inflammation, microvascular leakage leading to oedema and mucus hypersecretion.

The LAR is typified by the infiltration of eosinophils, although monocytes, lymphocytes, basophils, mast cells (9) and neutrophils (10) also play a role.

Eosinophils are recruited to the airways in response to the release of IL-3, IL-5 and GM-CSF as well as chemokines (eotaxin, RANTES and MCP3) from activated T cells and mast cells. Eosinophils also release leukotrienes, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-5, IL-8, TNFα, RANTES and eotaxin, which promotes further inflammation. Eosinophils can also release eosinophil cationic protein (ECP) and major basic protein (MBP), both of which have the potential to damage the epithelium. To magnify the response, the bronchial epithelium can prolong the inflammatory response by the release and synthesis of many cytokines and chemokines, including GM-CSF and eotaxin.

1.3. Susceptibility genes in asthma

A number of genes have been implicated in the development of asthma and atopy, including the β2 adrenoreceptor, human leukocyte antigen (HLA) complex, high affinity IgE receptor (FcεRI) and the T-cell receptor αβ complex. IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF are all encoded on the 5q31-q33 region of the genome and are implicated in asthma pathogenesis and regulation of IgE. Dipeptidyl peptidase 10 (DPP10) (11) and PHF11 (12) were identified by positional cloning and are also associated with the allergic inflammatory phenotype.

Several other genes have been implicated in asthma pathogenesis and are distinguished by their cellular expression in structural cells within the airways, rather than being involved in immune cell function. Examples of these genes include ADAM 33 (13) which is selectively expressed in mesenchymal cells and GPRA (G-protein coupled receptor for asthma susceptibility) which is expressed in both bronchial epithelium and smooth muscle (14). Polymorphic variation in genes within structural cells may help to explain the end-organ expression of allergic diseases such as asthma.

1.4. Preventative Medications

Although there is no known cure for atopic asthma, pharmacologic therapies are highly effective in controlling symptoms of asthma. The treatment of asthma

involves the avoidance of known triggers, the use of bronchodilators to reverse the airflow obstruction due to bronchoconstriction, and the use of drugs to reduce narrowing occurring as a result of inflammation. If necessary, treatment can involve complete ventilatory support.

Asthmatic patients are treated according to the severity of their disease, although inhaled β2 adrenoceptor agonists are given to all asthmatic subjects, as both a preventative measure and to provide immediate relief during an attack. Inhaled β2 adrenoceptor agonists allow high concentrations of drug to be distributed locally, mediating short-term relaxation. Patients with intermittent asthma are usually prescribed β2 adrenoceptor agonists as required for symptom relief. The recommended treatment for mild persistent asthmatic subjects is the daily use of an inhaled corticosteriod (such as beclomethasone, budesonide or fluticasone) as an anti-inflammatory agent, or cromoglycate (such as nedocromil sodium) for the treatment of children. Corticosteroids are hypothesised to suppress the inflammation associated with asthma by binding to the cytoplasmic glucocorticoid receptor thereby depriving NFκβ and AP-1 of cytokine activation (15).

Moderate persistent asthmatics are prescribed inhaled corticosteroids at doses of 500mcg or higher. Long acting inhaled β2 adrenoceptor agonists are prescribed if additional control is needed. Anti-leukotrienes are becoming increasingly popular especially in children and in the treatment of aspirin-sensitive and exercise-induced asthma where cysteinyl leukotrienes play an important role. A higher dose (800-2,000mcg) of inhaled corticosteroids is given to those with severe persistent asthma, in addition to inhaled long-acting β2 adrenoceptor agonists. Occasionally oral corticosteroids such as prednisone or prednisolone are prescribed in the short-term to control severe asthma episodes and help speed recovery, although in the long term they can have potentially serious metabolic and cardiovascular side effects.

It should be noted that a subpopulation of asthmatic subjects exist who have reduced responsiveness to even the highest prescribed doses of corticosteroids. This insensitivity has been linked to a reduced number of glucocorticoid receptors

(GR), altered affinity of the GR for the corticosteroid, reduced ability of the GR to bind to the DNA and an enhanced expression of inflammatory cytokines (16) which compete for DNA binding sites (for reviews see (17;18)).

1.5. Morphology of the airways

The architecture of the airways is complex, consisting of a number of histologically distinct layers (fig 1.2). The airways are lined by a protective layer of ciliated columnar epithelial cells. This is the first physical barrier separating the antigens inhaled into the lumen of the upper airways and the underlying lung tissue. The ciliated epithelial cells are interspaced by goblet cells whose primary function is to secrete a protective film of mucus over the epithelial layer to help prevent entry of particulates. Adjacent to the ciliated epithelial cells and goblet cells are the basal cells, which play a structural role and can differentiate in response to injury.

A basement membrane, comprising the *lamina rara* and *densa*, separates the epithelium from the mesenchyme. The principal constituents of basement membranes are collagen IV, laminin, collagen VII and nidogen. Collagen III, collagen V, collagen I, fibronectin and tenascin are the major components of the *lamina reticularis*, the layer just below the basement membrane.

The interstitial matrix under the *lamina reticularis* is termed the submucosa. The matrix is a hydrated polysaccharide gel containing fibrous proteins such as collagen and elastin as well as structural and adhesive proteins such as fibronectin and proteoglycans. Fibroblasts and myofibroblasts are found in the submucosa and are responsible for the synthesis and release of these interstitial matrix proteins. Below the submucosa lies the smooth muscle, which provides structural support and in asthma and is the major contractile cell type leading to the marked airflow limitation.

1.6. Airway Remodelling

Atopic asthma, the most common variant comprising 85-90% of asthma worldwide, has traditionally been viewed as a predominantly Th2 polarized inflammatory

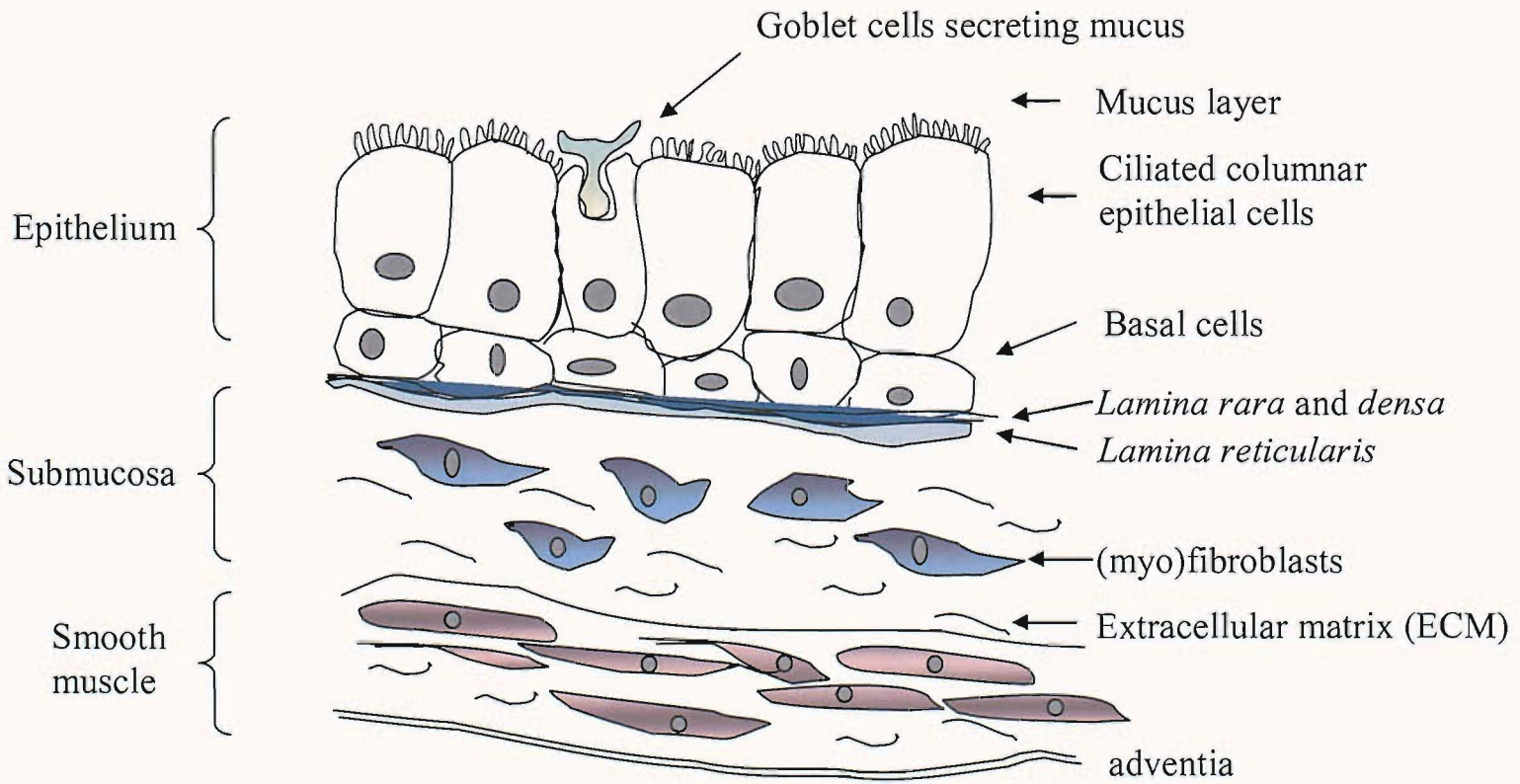


Figure 1.2: Morphological features of normal airways

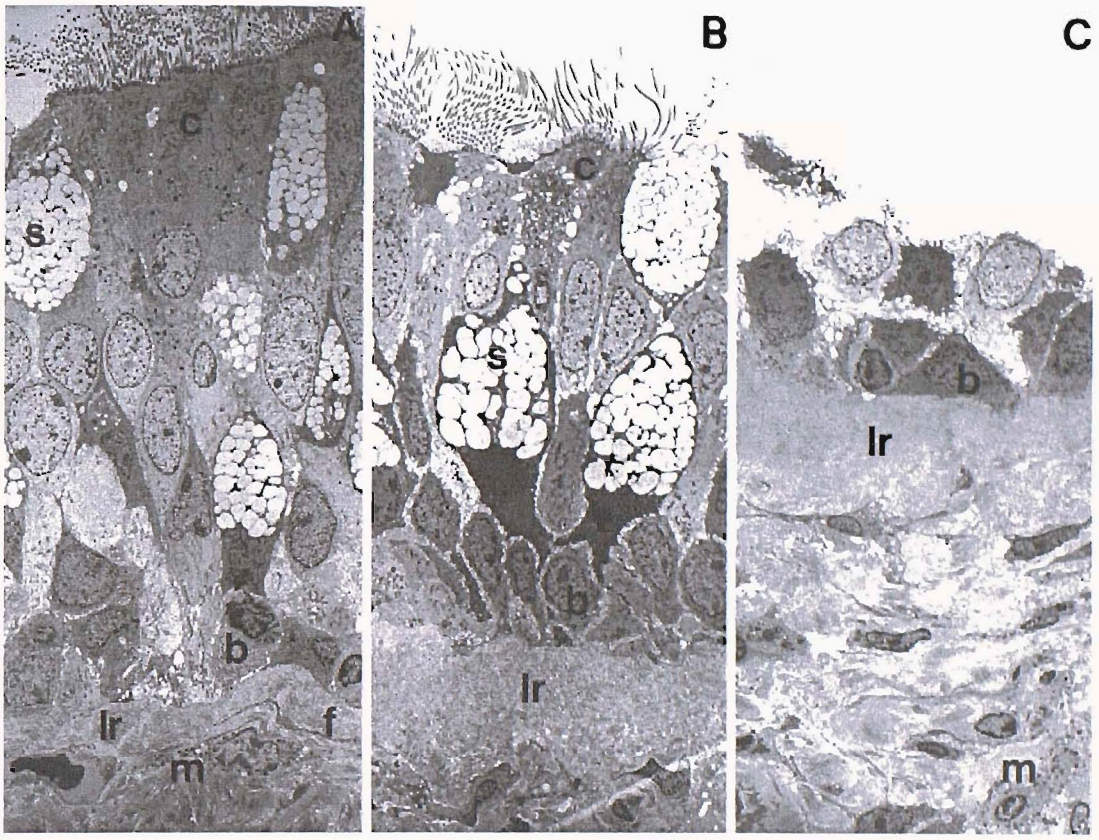
The epithelium is the first line of defence in the airways. Underneath the ciliated epithelial cells are the undifferentiated basal cells which lie on the basement membrane. (Myo)fibroblasts reside in the submucosa below the *lamina reticularis*. The structural support of the airways is mainly provided by the layer of smooth muscle situated below the submocosa. This pictorially shows the airways as being stratified. However, there is ongoing debate as to whether the airways are stratified or pseudostratified (i.e. the ciliated epithelial cells are attached directly to the basement membrane).

disease of the central and peripheral airways. Two interlinked processes take place in asthmatic airways: chronic inflammation and airway remodelling.

Airway remodelling is the general description for the thickening and restructuring of the airways seen in asthma patients (fig. 1.3 and 1.4). The characteristics of airway remodelling include subepithelial fibrosis, fibroblast and myofibroblast hyperplasia, myocyte hyperplasia and hypertrophy together with epithelial damage, goblet cell metaplasia and increased vascularity. Mathematical models suggest that the remodelling and thickening observed in the airway of asthmatic subjects will enhance airway narrowing in response to bronchospasm during an acute asthma exacerbation (19;20). Remodelling is also considered to be responsible for the more rapid decline in lung function over time experienced in the asthmatic compared to non-asthmatic population. Furthermore, it has been reported that airway hyperresponsiveness is associated with features of both remodelling and airway inflammation (21).

1.6.1. Epithelial changes

The lung epithelium is continually subjected to environmental insults such as air pollution, viruses and bacteria, as well as allergens such as grass pollen and house dust mite. Some irritants such as house dust mite allergens exert protease activity and can degrade the tight junctions of the epithelium (22). It has been suggested that the epithelium of asthmatics is more susceptible to environmental insults compared to that of normal subjects. Indeed, clumps of attached ciliated epithelial cells (Creola Bodies) have been observed in bronchoalveolar lavage (BAL) fluid from asthmatics (23). Bucchieri *et al* (24) showed that asthmatic bronchial epithelial cells are more susceptible to apoptosis in response to oxidant stress than normal epithelial cells. Additionally, Puddicombe *et al* (25) reported that $p21^{waf}$, a cyclin dependent kinase inhibitor (CDKI), is expressed at a higher level in asthmatic epithelium compared to healthy controls. Expression of $p21^{waf}$ was also induced by TGFβ and H_2O_2, possibly contributing to the slower repair response associated with airway inflammation and remodelling.



(A) Normal (B) Mild Asthma (C) Severe Asthma

Figure 1.3: Epithelial changes demonstrative of remodelled asthmatic airways

The epithelium is frequently damaged in asthmatic airways, revealing denuded areas where the basal cells are exposed (C). (B) shows an increase in goblet cells in the epithelial layer. Both transmission electronmicrographs of the asthmatic airway show thickening of the *lamina reticularis*. **b** Basal cell, **c** columnar epithelial cells, **f** (myo)fibroblast, **lr** lamina reticularis, **m** mast cell, **s** secretory goblet cells. (reprint by kind permission from Holgate *et al*, 2000)

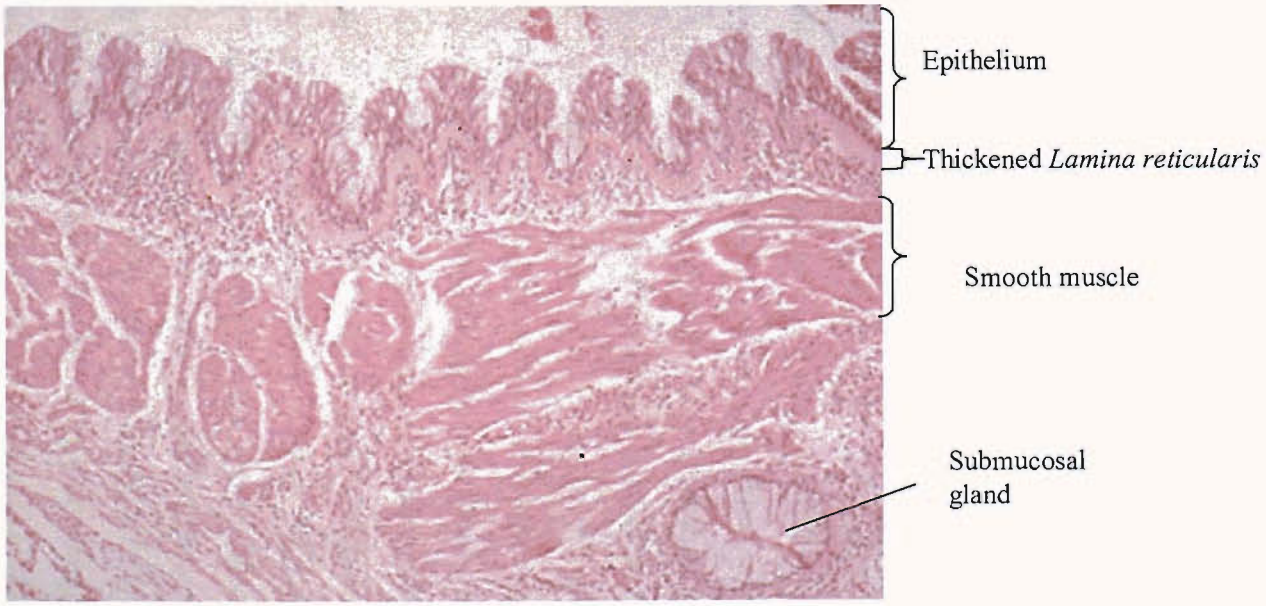


Figure 1.4: Increased smooth muscle mass in remodelled asthmatic airways (reprint by kind permission of Davies *et al*, 2003).

There is an increase in the size and number of goblet cells (fig. 1.3) and submucosal glands in asthmatic airways, resulting in an excessive release of mucus. The increased expression of mucus can lead to the formation of plugs that can extend to the membranous bronchioles and contribute to airway obstruction. Mucin glycoproteins are the major macromolecular component of mucus and are expressed as two major forms: the membrane-tethered mucins and the secreted mucins. Immunohistochemical staining for MUC5AC, the predominant secreted mucin, has revealed abundant staining in goblet cells situated in the epithelial surface lining and glandular ducts of tissues from patients with fatal asthma (26). MUC2 and 4 mRNA expression has also been reported to be increased in asthmatic bronchial biopsies (27).

1.6.2. Thickening of the *lamina reticularis*

A basement membrane, comprising the *lamina rara* and *lamina densa*, separates the airway epithelium from the mesenchyme. The *lamina rara* and *densa* in asthmatic subjects are not reported to differ from that of normals. The *lamina reticularis*, the layer composed of collagen I, collagen III, collagen V, fibronectin and tenascin situated just below the basement membrane, is altered in asthma (fig. 1.3). The overall thickness of the *lamina reticularis* is approximately 3-4μm in non-asthmatics while in asthma this is increased two- to three-fold (28) (29). James *et al* (30) demonstrated that *lamina reticularis* thickening correlates with other remodelling phenomena. The group showed that basement membrane thickness was correlated to smooth muscle mass, submucosal gland and inner wall area in both large and small cartilaginous airways but was not related to airway wall dimensions in the membranous airways. Remodelling is considered to be a potentially harmful event, although the thickening of the *lamina reticularis* may actually serve as a protective mechanism by increasing the stiffness of the airways to attenuate the sporadic bronchoconstriction (31) (19).

1.6.3. Increased numbers of structural mesenchymal cells

Fibroblasts and myofibroblasts are the main structural cells of the mesenchyme that mediate the majority of the events contributing to the subepithelial fibrosis in remodelled airways. A number of studies have shown that there is an increase in myofibroblasts (28) in the submucosal layer of asthmatics. Benayoun *et al* (32) demonstrated that there were significantly greater numbers of fibroblasts in bronchial biopsy samples from severe asthmatics compared to normals. The number of fibroblasts in the submucosa of asthmatic subjects has also been significantly correlated with thickening of the subepithelial *lamina reticularis* (33).

In addition to an increase in the number of myofibroblasts, there are a number of reports describing smooth muscle hyperplasia in asthmatic airways. For example, Woodruff *et al* (34) demonstrated a 50-83% ($p < 0.005$) increase in the amount of smooth muscle in the submucosa of asthmatics. It was reported that tritiated thymidine incorporation was increased in asthmatic airway smooth muscle cells *in vitro* compared to normal cells, indicating that airway smooth muscle proliferation may be increased in asthma, thus contributing to the hyperplasia (35). In 2004, Johnson *et al* (36) suggested that the increased proliferation may be attributable to the altered matrix deposition by asthmatic smooth muscle cells. Specifically, extracellular matrix rich in perlecan and collagen I, but with lower amounts of laminin $\alpha 1$ and collagen IV deposited by asthmatic cells enhanced the proliferation of non-asthmatic smooth muscle *in vitro*. There are conflicting reports as to whether there is also a difference in the size (hypertrophy) of asthmatic smooth muscle cells. Benayoun *et al* (32) described hypertrophy with an accompanied increase in myosin light-chain kinase expression from severe persistent asthmatic subjects compared to those with milder disease. Conversely, in mild to moderately severe subjects, Woodruff *et al* (34) did not detect any differences in cell size.

Dulin *et al* (37) noted that smooth muscle isolated from asthmatic tissues exhibits normal sensitivity to constrictor agonists when studied during isometric contraction. The sheer increase in smooth muscle mass was suggested to be responsible for generating more contractile force than that experienced in the non-

asthmatic airways. The increased muscle bulk can also reduce the luminal circumference and alter the pattern of mucosal folding, resulting in a reduced number of folds and enhanced airway narrowing.

1.6.4. Increased deposition of Extracellular Matrix (ECM)

In asthma there is an altered profile of ECM proteins present in the submucosal layer below the *lamina reticularis*. It is proposed that this is due not only to the increased number of synthetic cells present but also due to differences in the degradation of the existing proteins. The ECM is a dynamic environment. Indeed it is estimated that the physiological turnover of total ECM in the human lung is 10-15% per day (38). Therefore it is easy to envisage how small changes in enzyme levels can alter the equilibrium of protein levels in the submucosal layer.

The asthmatic *lamina reticularis* contains less collagen IV than normal, although the submucosa of asthmatic airways contains more collagen I, III and V (33;36;39;40). Additionally, the orientation and fibre thickness of collagen I appears to be altered in asthmatics (29;41). Dube *et al*, (42) suggested that differences in collagen expression were due to posttranslational modifications, as baseline procollagen production was not found to differ between normal and asthmatic fibroblasts.

There are a number of reports demonstrating increased levels of ECM proteins in the submucosa of asthmatics. For example, tenascin (43), lumican, biglycan and versican (44) are all present at elevated levels in the submucosa of asthmatic subjects. Westergren-Thorsson (45) reported that subjects with the most hyperresponsive airways produced up to four fold more total proteoglycan than subjects with less responsive airways. Laminin $\beta 2$ and $\alpha 1$ staining (46) was also higher in biopsies from asthmatic subjects. Elastin fibres are needed to maintain bronchial patency and contribute to the elastic recoil of the lungs. Bousquet *et al* reported (47) that biopsy samples from asthmatics showed altered elastin staining. In addition, Vignola *et al* (48) showed that the enzyme responsible for elastin

degradation, elastase, was increased in sputum samples from asthmatics compared to healthy controls.

Matrix metalloproteinases (MMPs) are responsible for the degradation of many ECM proteins, including collagens, fibronectin, laminin, proteoglycans and elastin. An altered expression profile of ECM degrading enzymes has been reported in asthma. For example, asthmatic bronchial fibroblasts produce less MMP-3 (stromolysin) than normal fibroblasts (49). Laliberté *et al*, (50) demonstrated that baseline MMP-2 (gelatinase A) secretion was lower in asthmatic fibroblasts when compared to those from normal airways. Furthermore, the group showed that asthmatic fibroblasts displayed a decreased capacity to degrade collagen by phagocytosis.

MMP-9 (gelatinase B), a metalloproteinase responsible for cleaving collagen IV and degrading denatured collagen, is reportedly expressed at higher levels in eosinophils isolated from asthmatics compared to normals (51). A significant increase in the level of circulating MMP-9 was also seen in patients after exacerbation compared with patients with stable asthma (52). Additionally, MMP-9 staining in the subepithelial basement membrane region was reportedly higher in severe asthmatic biopsies than control subjects (53). These findings may account for the decreased expression of collagen IV in the submucosa of asthmatic subjects. Tissue specific inhibitors of metalloproteinases (TIMPs) can inhibit activation of the MMPs by binding an MMP in a 1:1 stoichiometric fashion. Laliberté *et al*, (50) hypothesised that a compromised balance between TIMP-1 and MMP-1 levels may be responsible for the increase in collagen deposition observed in asthmatic airways. Indeed, TIMP-1 mRNA and protein levels were elevated in asthmatic alveolar macrophages isolated from BAL fluid, a finding demonstrated by Ohno *et al* (51).

1.6.5. Remodelling in childhood asthma

The chronology of events leading to the onset of disease is debatable. It is widely accepted that susceptible subjects go on to develop atopic asthma after sensitisation to a particular allergen. The precise initiation of remodelling events in the airways

is less certain, although recent studies suggest that remodelling may be an early event in the aetiology of disease (reviewed by McKay and Hogg, (54)). Indeed, the impact of remodelling may be greater during the childhood years as children's lungs grow rapidly in the first few years of life.

In addition to the more complex ethical issues surrounding bronchoscopies performed on children, it is often very difficult to differentiate between wheeze induced by viral infections and bronchial asthma in the pre-school child. In the studies that have been carried out in paediatric asthma, it appears that a number of phenomena associated with remodelling are present at an early age. For example, Cokugras *et al*, (55) studied biopsy samples from ten children with moderate asthma and demonstrated thickening and hyalinisation of the sub-basement membrane region in nine patients. Although eosinophils were present in only one sample, 60% had degranulating mast cells in the submucosa. Najafi *et al*, (56) performed bronchoalveolar lavage on 39 newly diagnosed wheezy children. The study found that children under 2 years of age had larger amounts of ciliated columnar and goblet cells in the BAL fluid compared to older children, suggesting acute inflammation of the airways early in life. A study comparing the degree of remodelling in asthmatic children prescribed with 1,600 μg/day inhaled steroid to adult asthmatics revealed that *lamina reticularis* thickening is present in children with difficult asthma at a similar level to that observed in biopsies obtained from adult asthmatic subjects (57).

1.6.6. Is Remodelling positively correlated to disease severity?

The majority of studies carried out to compare the effect of disease severity suggest that tissue remodelling is more advanced in subjects with severe asthma. For example, Harmanci *et al* demonstrated that bronchial wall thickening was more prominent with increased severity, decreased FEV(1) values and the duration of asthma (58). The submucosa of asthmatic subjects contained more blood vessels which was also correlated with the severity of the disease (59).

Benayoun *et al*, (32) revealed that mucosal eosinophilia, neutrophilia, epithelial damage, and subepithelial basement membrane thickness were not releated to disease severity. However, a number of observations distinguished patients with severe persistent asthma from patients with milder disease. These included increased numbers of fibroblasts, larger areas of mucous gland and smooth muscle, smooth muscle hypertrophy, increased collagen type III deposition, and higher myosin light-chain kinase expression. Chakir *et al*, (40) described an association between disease severity and collagen deposition. In contrast, Chu *et al* (60) found no identifiable differences in collagen deposition or TGFβ expressing cells in the large airways of mild when compared to severe asthmatics.

1.6.7. Reversibility of remodelling?

Airway remodelling has been considered to be an irreversible process although there is evidence to suggest that early use of corticosteroids can delay or even reverse the structural changes (61). A five year follow-up study comparing subjects treated with corticosteroid early in the course of their disease to a delayed treatment group showed that early use of corticosteroids (budesonide) significantly improved lung function and increased exercise tolerance. Furthermore, the early treatment group used reliever medication less, had fewer exacerbations and after 5 years 17% were able to stop using budesonide when compared to 3% in the delayed treatment group (62).

A number of studies have directly examined the effect of corticosteroids on remodelling by performing bronchoscopies. A study carried out by Sont *et al* (63) showed that subjects treated with corticosteroids over a period of two years experienced a lower rate of mild exacerbations, an improved FEV1 and a reduction in the thickness of the subepithelial layer compared to the control group. In a double blind study of 28 asthmatic subjects, Hoshino *et al* (64) demonstrated that treatment with the inhaled steroid beclomethasone dipropionate significantly reduced the vessel number and vascularity in the airways of steroid treated patients. Further study showed that biopsy samples taken before and after treatment with an inhaled steroid revealed a decrease in inflammatory cells and a reduced amount of

epithelial damage after ten years, although bronchial hyperresponsiveness remained the same (65). The majority of investigations carried out do suggest that early and prolonged use of corticosteroids can ultimately improve lung function and reverse, or at least minimise, some of the remodelling events, although it should be remembered that long-term use of corticosteroids can in itself bring about other adverse effects such as adrenal suppression and arterial hypertension.

1.7. The Epithelial-Mesenchymal Trophic Unit

It is proposed that the epithelium and the underlying fibroblasts communicate through autocrine and paracrine mechanisms, effectively forming the epithelial-mesenchymal trophic unit (EMTU). The EMTU is set up during development and is crucial to the process of branching morphogenesis. It is postulated that reactivation of developmental pathways contributes to the structural alterations of airways in asthma (66). During exacerbations, activated inflammatory cells interact with the epithelium to produce an array of Th2 cytokines and chemokines. As well as having a protective role, the inflammatory cells can have a detrimental effect by causing damage to the epithelium through the release of free radicals and proteases. In asthma it has been proposed that the repair of the wounded bronchial epithelium is ongoing due to perpetuation of a chronic inflammatory cycle (66).

The epithelium can also communicate with the underlying mesenchyme (fig. 1.5). Transforming growth factor beta (TGFβ) is released from damaged epithelial cells (67) and can mediate its effects by interacting with TGFβ receptors on fibroblasts. This interaction can promote the transformation of fibroblasts into myofibroblasts. Myofibroblasts are more biosynthetic in nature and can synthesis and release an array of growth factors and cytokines, resulting in remodelling of the airways and continuation of the chronic inflammatory events. This is amplified by release of various factors such as endothelin-1 (ET-1), a co-mitogen for smooth muscle, eotaxin, a chemoattractant for eosinophils and VEGF, an angiogenic factor which promotes the growth of new blood vessels (68), (69). EGFR ligands can also be released from epithelial cells, fibroblasts and smooth muscle cells. EGFR ligands can promote proliferation. It is proposed that ligands of the EGF family are

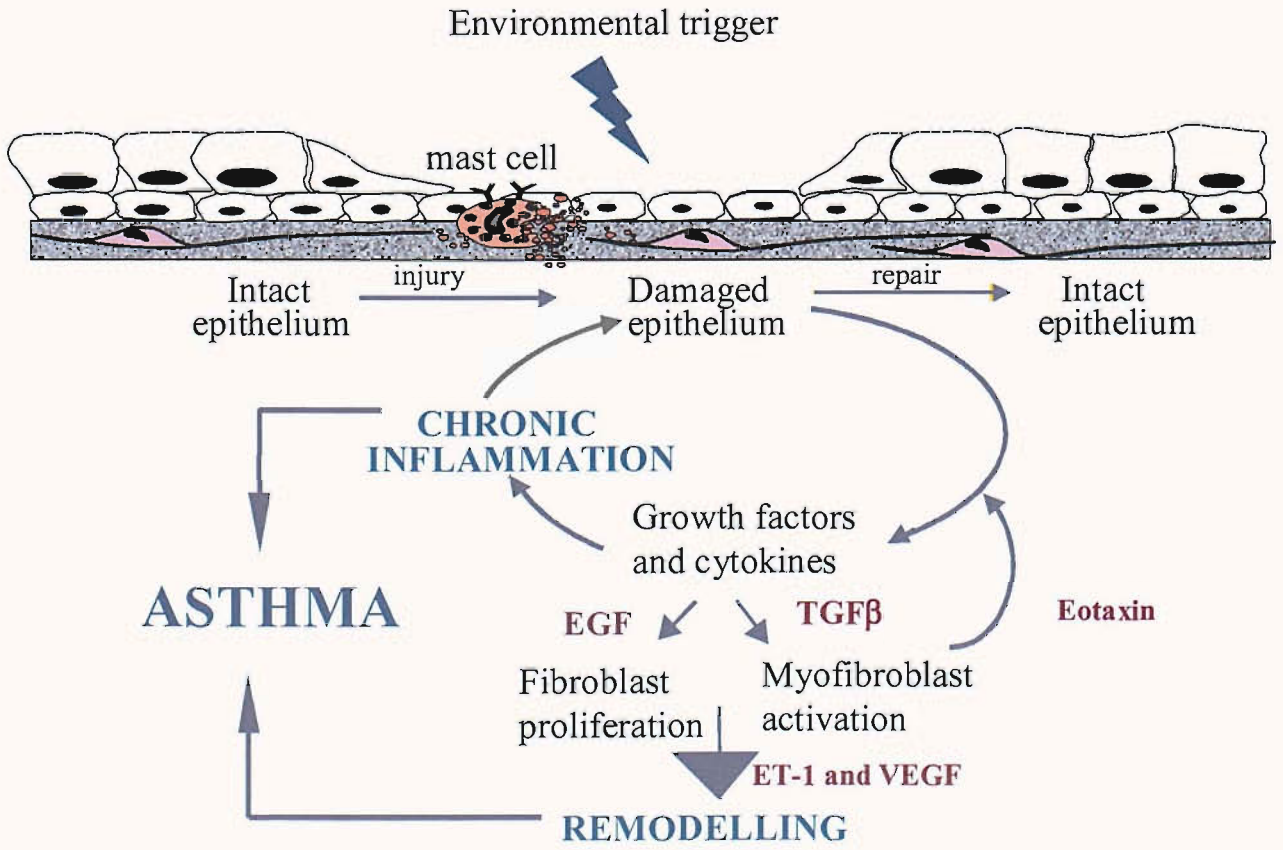


Figure 1.5: Reactivation of the Epithelial-Mesenchymal Trophic Unit in asthma (adapted from Holgate *et al*, 2000).

The epithelium and the mesenchyme can communicate in asthmatic airways due to the release of soluble growth factors. The epithelium can release proinflammatory and profibrogenic growth factors that can mediate remodelling and perpetuate the chronic inflammatory cycle. Activated fibroblasts can in turn release factors which promote inflammation as well as remodelling processes such as smooth muscle hyperplasia and angiogenesis. It is postulated that the remodelling and inflammation attenuate the normal repair of the damaged epithelium and ultimately lead to the development of asthma.

released from the damaged epithelium and cause the proliferation of the underlying mesenchymal cells, as demonstrated in TGFα transgene mice (70).

1.8. Transforming Growth Factor beta (TGFβ) signalling and its role in asthma

In asthma, it has been hypothesised that an enhanced expression of TGFβ in the airways may have far reaching effects in the pathophysiology of the disease. TGFβ released from inflammatory cells, the epithelium and (myo)fibroblasts can promote remodelling by inducing expression of a wide range of ECM components. Although TGFβ is anti-inflammatory and can promote cellular migration (71), an excess of TGFβ can inhibit subsequent epithelial cell proliferation that is required during early wound healing. The net result of excessive TGFβ in the airways in asthma may explain many of the morphological changes that occur in airway remodelling (fig. 1.6).

More than thirty members of the TGFβ superfamily have been identified (72). The superfamily is divided into four main families: TGFβ family, Bone Morphogenetic (BMP) family, inhibin/activin family and the Müllerian Inhibiting Substance (MIS) family. There are three TGFβ isoforms identified in mammals, namely TGFβ1, TGFβ2 and TGFβ3. Although TGFβ was first isolated and characterised from human platelets (73) it is produced by most cell types, including fibroblasts, smooth muscle, epithelial and inflammatory cells.

The genes for TGFβ1, 2 and 3 are located on chromosomes 19q13 (74), 1q41 (75) and 14q24 (76) respectively. The gene encoding TGFβ1, in contrast to TGFβ2 and 3 lacks both the TATA and CAAT boxes. TGFβ1 has two promoter regions containing AP-1, NF-1, SP-1 and Erg-1 response elements (77). The promoters for TGFβ2 and –3 contain the responsive elements for ATF and CREB, suggesting hormonal and developmental control (78).

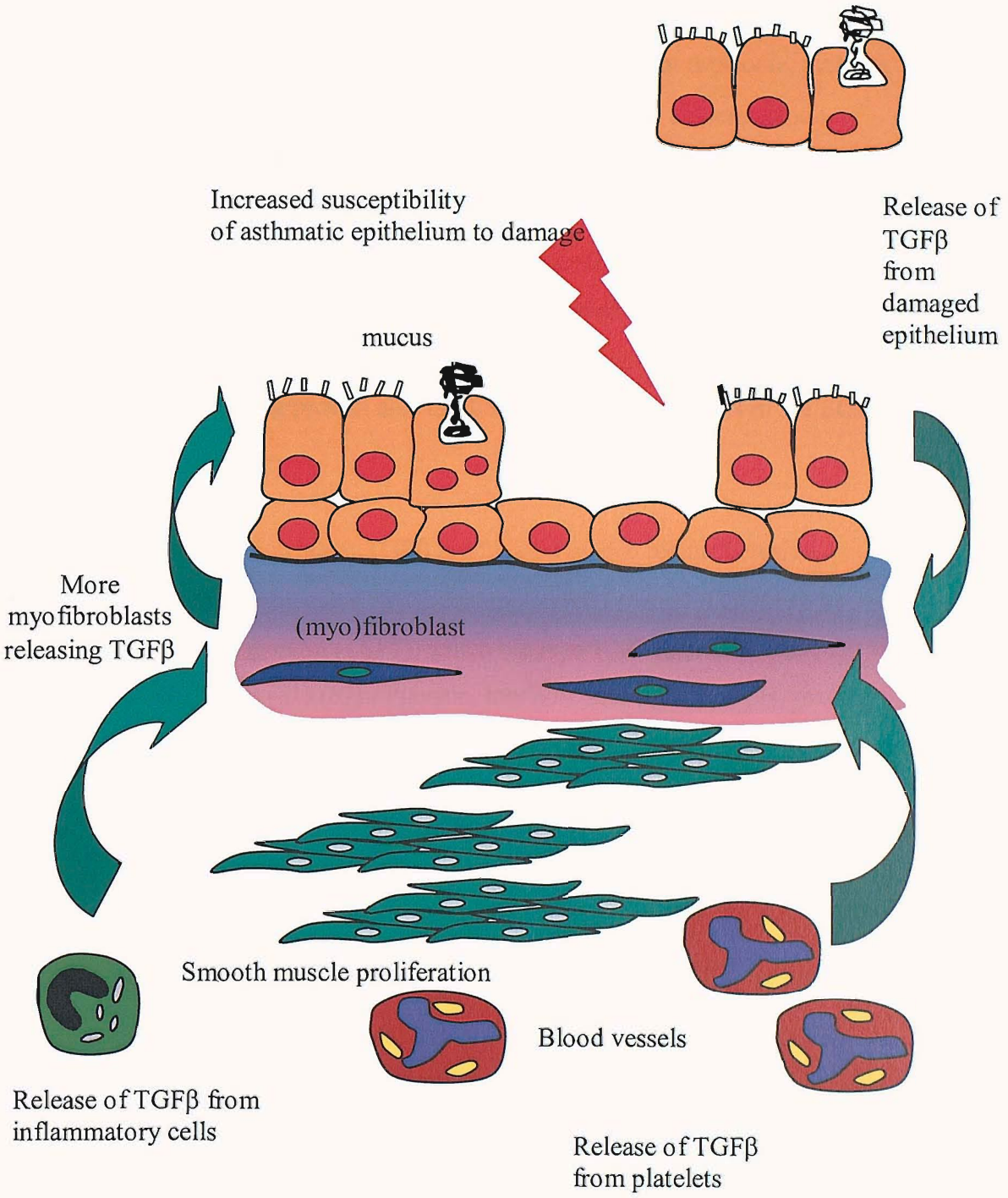


Figure 1.6: Increased release of TGFβ in asthmatic airways

Studies have revealed that there is an increased expression and release of TGFβ in asthmatic airways. TGFβ is released from the epithelium, fibroblasts, smooth muscle cells and inflammatory cells and is proposed to have a major role in the fibrosis associated with airway remodelling.

TGFβ is encoded as a biologically inactive large precursor peptide termed pre-pro-TGFβ. Endopeptidases catalyse the cleavage of the precursor molecule, yielding the mature 25kDa protein. The mature TGFβ dimerises and associates non-covalently with latency associated peptide (LAP), the precursor cleavage product, to form small latent TGFβ. TGFβ is secreted from the cell as either small latent TGFβ or more often as large latent TGFβ (small latent TGFβ bound to latent TGFβ binding protein). TGFβ can then be rapidly sequestered by a number of different binding proteins including decorin, biglycan, type IV collagen, fibronectin and elastin (79). The binding protein also has the ability to bind to sites in the ECM (80).

The mechanism by which latent TGFβ is activated *in vivo* remains elusive, although a number of molecules have been implicated *in vitro*. TGFβ can be activated by thrombospondin (TSP1), which binds to LAP and induces a conformational change (81) (82). Plasmin, low dose radiation, low pH, reactive oxygen species (83) and nitric oxide nitrosylation (84) have all been suggested to play a role in TGFβ activation.

1.8.1. TGFβ Receptors

There are six types of TGFβ receptor, RI-RVI. Most of the information in the literature concerns RI, RII and RIII. TGFβ RIV (85) and RVI (86) bind TGFβ but are not known to propagate down stream signalling. TGFβ RV is a serine/threonine kinase like RI and RII and further investigation may reveal its importance in controlling downstream signalling events (87). Betaglycan and endoglin comprise the two types of TGFβ RIII. Endoglin is expressed only on endothelial and myeloid cells. Betaglycan is expressed on most cell types and has a variable molecular weight due to the heparan sulphate and glycosaminoglycan (GAG) chains attached to it. The receptor is responsible for binding TGFβ and presenting the ligand to the type II receptor. This enables the local concentration of ligand to be increased and may also have a role in stabilising TGFβ in the optimal conformation for binding to the type II receptors. TGFβ2 has a weak affinity for

the type II receptor alone and can only signal via interaction with betaglycan (88) where the short cytoplasmic tail of the receptor acts to enhance TGFβ2 signalling (fig. 1.7). In contrast, TGFβ1, can signal directly through TGFβRII.

The type II receptor is a 70kDa constitutively active serine/threonine kinase. Upon ligand binding the receptor recruits and phosphorylates the type I receptor in the GS domain and juxtamembrane regions. Whilst the type II receptor can bind TGFβ1 independently of betaglycan, recruitment of the type I receptor is still required for downstream signalling. The type I receptor is a 55kDa protein, responsible for downstream signalling via a cascade of SMAD proteins. There are seven type I receptors. ALK-1 (mainly associated with endothelial cells) and ALK-5 are type I receptors for TGFβ (89), ALK-4 is the receptor for activin and ALK-2, 3 and 6 are receptors for the BMPs (90) (91). TGFβ receptor-associated protein 1 (TRAP-1) binds to the inactive type I receptor but is released when the receptor is activated by ligand binding (92). The activated receptor complex is a heteromeric complex consisting of two type II and two type I receptors (fig. 1.7).

1.8.2. Downstream signalling

TGFβ mediates intracellular signalling via the Smads. Mad (mothers against dpp) was the first member of the Smad family identified in *Drosophila.* Its discovery led to the identification of Mad homologues in *C. elegans* called Sma-2, -3 and –4, due to their small size. Smad (Sma- and Mad-related protein) were later identified as TGFβ signalling molecules in vertebrates (fig. 1.8). Receptor Smads i.e. Smad-2 and -3 reside in the cytoplasm in their inactivated state. Smad-2 exists as a monomer due to a structural element in the MH1 domain that inhibits protein-protein interactions in the basal state. Smad-3 has multiple oligomeric states and smad-4 most likely exists as a trimer (93). They are serine phosphorylated by the type I receptor after ligand dependent activation. The interaction of the Smad anchor for activation (SARA) protein (94) with the receptor allows interaction with Smad-2 and Smad-3. Smad 2 signalling is dependent upon SARA but Smad 3/SARA interaction is not required for downstream signalling (95). The phosphorylated receptor Smads bind Smad-4 (a co-Smad), and the complex

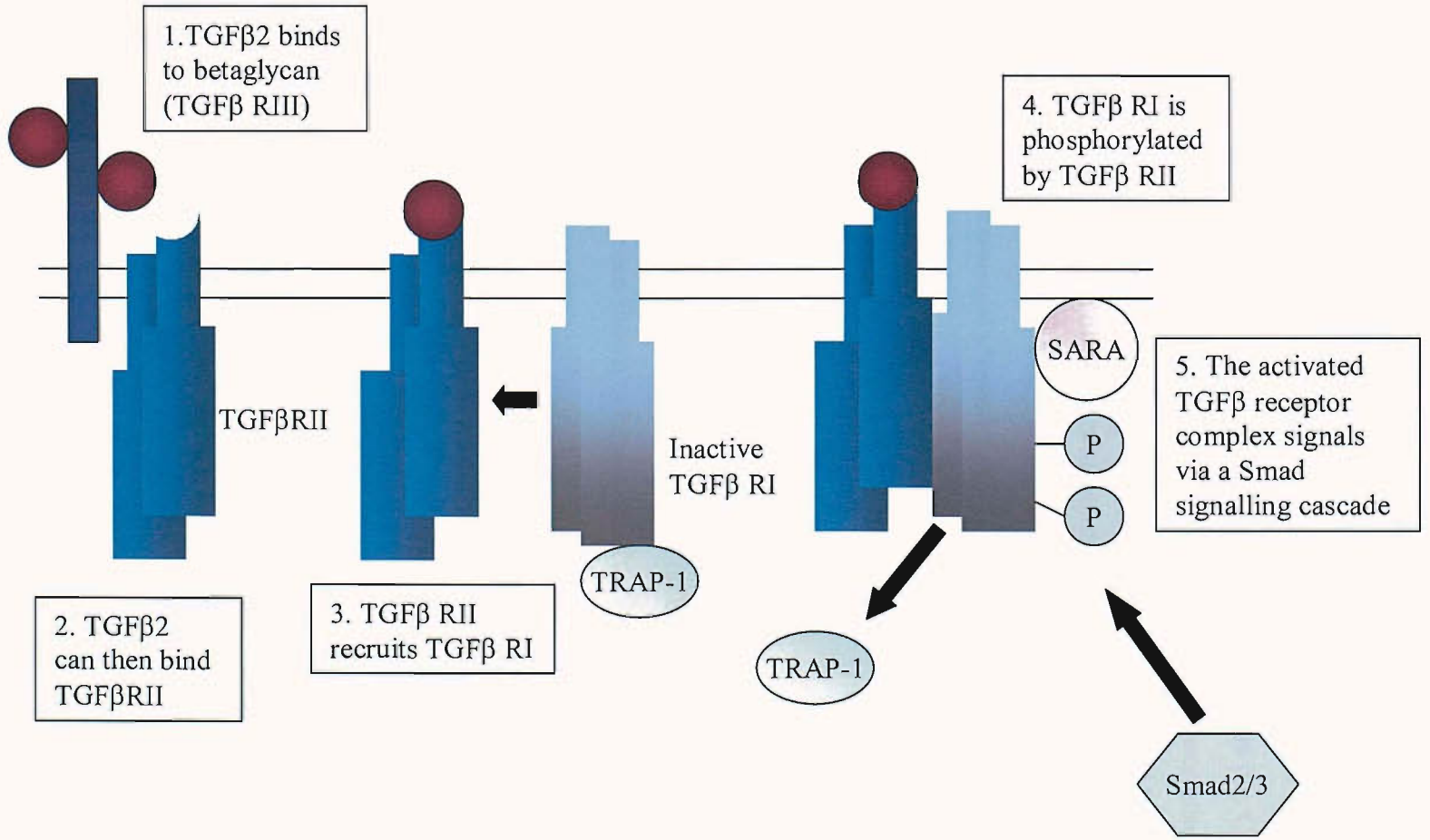


Figure 1.7: Binding of TGFβ2 to its receptors

Betaglycan binds TGFβ2, enhancing the affinity of the growth factor for TGF βRII. Once bound, TGFβ RII recruits and phosphorylates TGFβ RI thus promoting down stream signalling.

translocates to the nucleus. There is evidence to suggest that TRAP-1 interacts with smad-4 (fig. 1.8) and may act as a chaperone protein (96).

The Smads can bind directly to the Smad Binding Element (SBE) of various genes such as junB, c-jun and IgA (97) (98). Smads can also mediate gene transcription via functional co-operation with other transcription factors such as FAST-1 (99). TGFβ activated Smad-2 and -3 can also bind to co-repressors e.g. CREB-binding protein (CBP) and p300, which can in turn interact with transcription factors, to modify gene transcription (100). Hocevar *et al* (101) described Smad-4 independent activation of TGFβ with resultant induction of fibronectin synthesis. The mechanism involves TGFβ activation of Map Kinase Kinase 4 (MKK4), which activates Jun N-terminal kinase 1 (JNK1). JNK1 phosphorylates c-jun, promoting the formation of the c-jun-ATF 2 heterodimer. These heterodimers can bind to cAMP response elements (CRE) in the fibronectin promoter and thus promote fibronectin synthesis.

There are other possible pathways by which TGFβ receptors can transmit signals. Protein farnesyltransferase-α (102) and FK506 binding protein-12 (FKBP-12) (103) have both been shown to bind to the TGFβ receptors, although FKBP-12 might act as a negative regulator of TGFβ receptor endocytosis (104). It is also possible that a MAP kinase-like pathway is involved in the propagation of signals from the membrane to the nucleus, via TAB1 and TAK1 (a MAP kinase kinase kinase) (105), (106).

1.8.3. Regulation of TGFβ signalling

After ligand binding a process of endocytosis typically down regulates growth factor receptors. In fibroblasts, heteromeric TGFβ receptors are internalised whereas homomeric receptors are recycled back to the membrane (107) (108). The diversity of TGFβ signalling is controlled in part by the residues phosphorylated on the receptor in response to ligand stimulation. Saitoh *et al* (109) observed that serine 172 and threonine 176 of RI are dispensable for ECM protein production but essential to epithelial growth inhibition mediated by TGFβ. Luo and Lodish, (110)

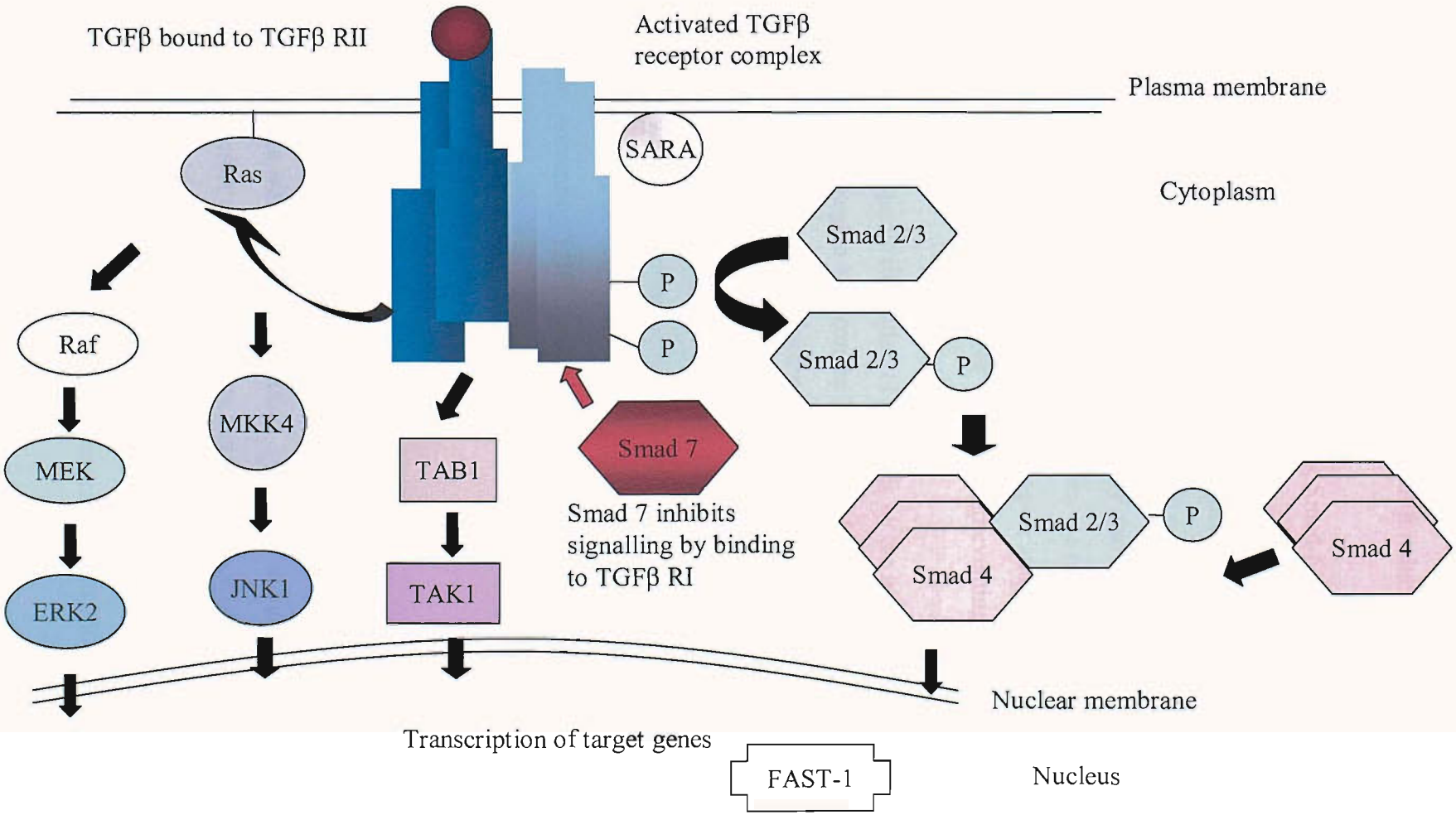


Figure 1.8: Intracellular signalling pathways activated by TGFβ

TGFβ can signal via Smads or a MAP kinase-like pathway, resulting in transcription of target genes. Nuclear transcription of target genes occurs either by Smads binding directly to SBEs of target genes or by functional co-operation with transcription factors such as FAST-1. Phosphorylation changes are only indicated on the Smad pathway for ease of interpretation.

demonstrated that autophosphorylation of Ser213 on RII is essential for activation of the TGFβ RII kinase, activation of RI and TGFβ induced growth inhibition.

There are two mammalian antagonistic Smads, Smad-6 and Smad-7. BMP signalling can be inhibited by Smad-6 and –7, and TGFβ signalling by Smad-7. Smad-6 inhibits BMP/Smad-1 signalling by competing with Smad-4, forming a Smad-1/Smad-6 inactive complex (111). Smad-7 acts as an antagonist of TGFβ RI (112) and is induced by TGFβ. Conversely, EGF can induce the mRNA levels of smad-7, providing evidence of cross talk between the pathways (113). STRAP stabilizes the association between the type I receptor and smad-7, preventing the receptors association with Smad-2 or –3 and thus preventing signalling (114).

Another form of negative regulation of Smads is by the interaction with MAP Kinase. The linker region between the MH1 and MH2 domain of the receptor Smads can be phosphorylated which prevents the receptor Smad/Smad-4 complex from entering the nucleus and eliciting gene transcription (115). There are also a number of inhibitory transcription factors such as Evi-1 (116), c-ski (117) and SnoN (118), which can prevent activated Smads from binding to SBEs on DNA. TGIF is an example of a co-repressor that can compete with p300 for Smad-2 association, preventing TGFβ mediated signalling (119).

1.8.4. TGFβ and the epithelium

TGFβ regulates the migratory phase of epithelial repair. Boland *et al* (120) reported that 1-10ng/ml TGFβ1 inhibited the proliferation of primary epithelial tracheal cells but enhanced cell migration. TGFβ also induced cell spreading, reduced the number of cell-cell contacts and increased cell-substratum anchorage, thus favouring a migratory phenotype. Furthermore, Howat *et al* (121) showed that TGFβ1 but not TGFβ2 could progressively increase the migration of damaged bronchial epithelial monolayers at concentrations down to 250pg/ml.

TGFβ can inhibit cell proliferation of epithelial cells by a number of different mechanisms involving the down-regulation of c-myc (122), and the up-regulation

of the cyclin dependent kinase inhibitors (CDKIs), $p15^{INK4B}$, $p21^{waf1}$ and $p27^{kip1}$ (123). P21 is overexpressed in asthmatic epithelium strongly suggestive of cell stress and growth arrest (25).

1.8.5. TGFβ, fibroblasts and myofibroblasts

Fibroblasts comprise the major structural cell in the mesenchyme in its resting state. Quiescent fibroblasts are involved in the baseline secretion of proteins such as collagen, contributing to the normal turnover of ECM in the airways. Upon insult to the tissue, fibroblasts become activated and capable of migrating to the site of injury. In order to carry out this function, the fibroblasts secrete collagen and fibronectin fibrils to enable orientation of the cell, thus creating both a migratory meshwork as well as lines of stress and mechanical tension.

Myofibroblasts are generally viewed as morphological intermediates of fibroblasts and smooth muscle cells (fig 1.9). They are the predominant cell type found in wound granulation tissue but unregulated they play a major role in fibroproliferative diseases. Myofibroblasts are more biosynthetic in nature than the fibroblast and are capable, due to activation of myosin light chain kinase (MLCK) and Rho kinase, of both rapid contraction and a more sustained contraction respectively. *In vivo*, the myofibroblast is a transient cell type and is normally activated upon connective tissue injury. A combination of TGFβ and Extra Domain-A (ED-A) fibronectin (124) (125) (126) induces the transformation of proto-myofibroblasts into myofibroblasts. TGFβ induces αSMA and SM22α via interactions with the TGFβ controlling element (TCE) and two serum response element (SRE) regions in the αSMA promoter of fibroblasts (127). Thannickal *et al*, (128) reported that expression of a stable myofibroblast phenotype is dependent upon both TGFβ and adhesion dependent signals. Inhibition of TGFβ1 induced phosphorylation of focal adhesion kinase (FAK) on Tyr-397, a cell adhesion dependent event, resulted in inhibition of αSMA expression. TGFβ1 was found to increase integrins α4, α5 and β1 as well as fibronectin in human lung fibroblasts. The authors propose that TGFβ induced fibroblast differentiation occurs via a Smad mediated adhesion-independent signalling pathway followed by a delayed

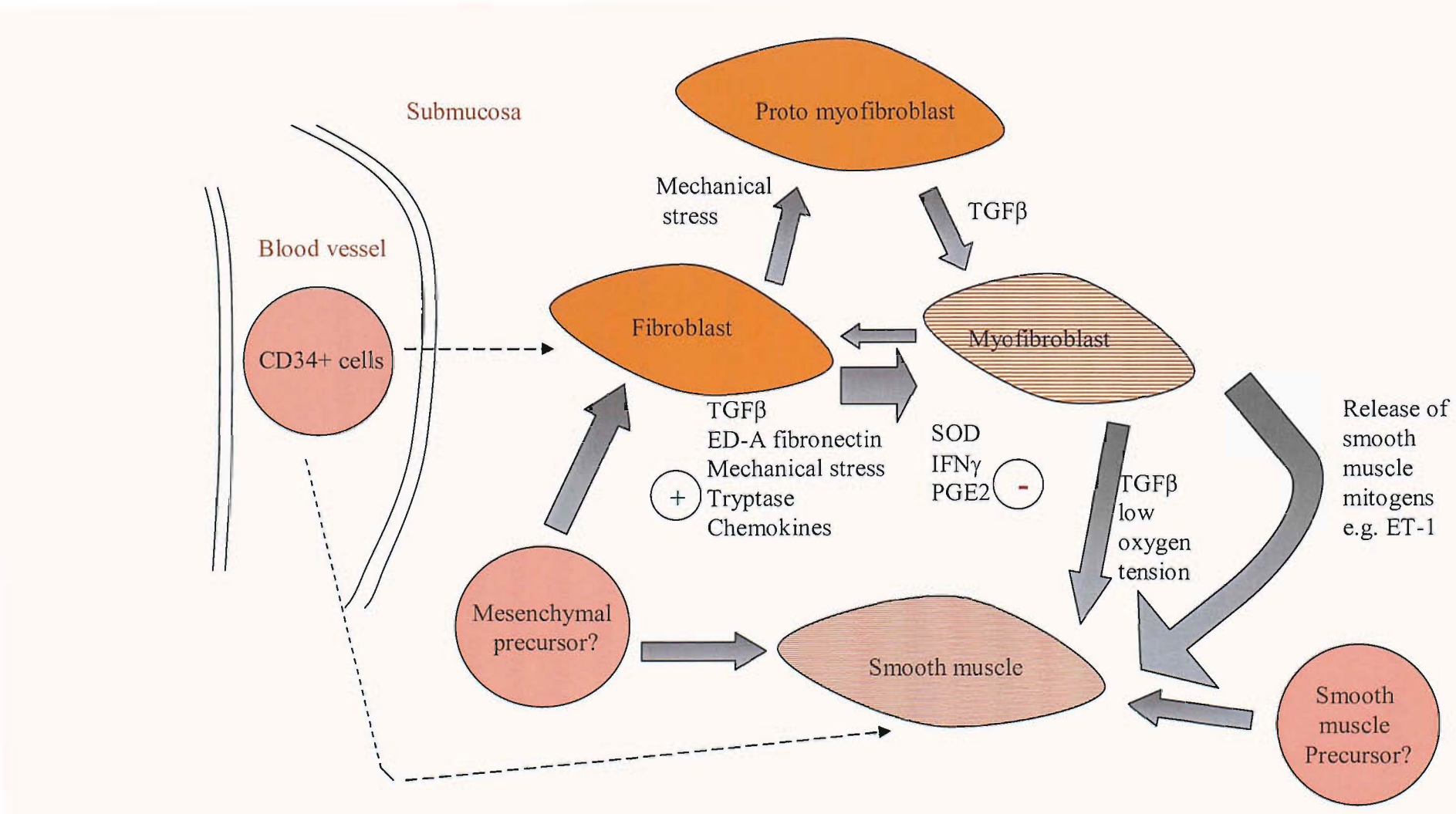


Figure 1.9: Potential plasticity of mesenchymal cells

Although the established dogma describes fibroblasts transforming into myofibroblasts under the influence of TGFβ, there are reports of other precursor cells and a number of other inducers of the myofibroblast phenotype. There is also some evidence to suggest that myofibroblasts may act as smooth muscle precursors (144-146). CD34+ cells circulating in the blood have also been identified as potential sources of fibroblasts (137) and smooth muscle cells (147).

adhesion dependent pathway involving focal adhesions that are essential for maintenance of the phenotype. Studies of wound healing suggest that myofibroblast disappearance occurs via apoptosis (129). TGFβ promotes the transformation from fibroblast to myofibroblast as well as enhancing the survival of myofibroblasts by inhibiting IL-1β induced apoptosis (130).

The cytokines IL-4 and IL-13 have been reported to induce the myofibroblastic phenotype in a time and dose dependent manner (131) (132). However, Richter *et al*, (68) demonstrated that IL-4 and IL-13 were relatively ineffective in promoting myofibroblast transformation directly, although transformation was effective through IL-4 or IL-13 mediated TGFβ2 release from epithelial cells (68). *In vivo* the myofibroblast is only present transiently. Vozenin-Brotons *et al*, (133) reported that addition of superoxide dismutase (SOD) to porcine dermal myofibroblasts reduced the levels of αSMA and collagen production, an event not associated with an increase in cell death. IFNγ has been implicated in preventing the generation of myofibroblasts and can also moderately inhibit the production of αSMA in TGFβ induced myofibroblasts (134) (135). PGE2, signalling via E prostanoid receptor 2 (EP2), has also been reported to prevent the TGFβ induced fibroblast to myofibroblast transition in primary fetal and adult lung fibroblasts (136).

The accepted view is that myofibroblasts are derived from fibroblasts. A recent study, however, has identified another potential source of myofibroblasts. Schmidt *et al* (137), suggest that bronchial myofibroblasts can originate from fibrocytes, blood-borne cells that can home to sites of tissue damage (fig. 1.9). These cells are CD34-positive, express both collagen I and αSMA and localize to areas of collagen deposition below the epithelium. By tracking labelled circulating fibrocytes in a mouse model of allergic asthma, the authors showed that fibrocytes are recruited into the bronchial tissue following allergen exposure and differentiate into myofibroblasts.

In addition to induction of myofibroblast differentiation and deposition of extracellular matrix proteins, TGFβ can influence fibroblast proliferation. Some studies have shown TGFβ to cause fibroblast proliferation by indirectly inducing

FGF-2 (138) and its receptors (139). McAnulty *et al*, (140) demonstrated that the concentration of TGFβ determined whether growth was stimulated or inhibited. In another study by Dube *et al* (42), it was reported that TGFβ1 had no significant effect on bronchial fibroblast proliferation. Therefore, the overall mitogenic effect of TGFβ appears to be dependent on the cell type, the location within the body and the dose used.

TGFβ treated fibroblasts also have indirect effects on inflammation by releasing a number of chemoattractants. For example, release of eotaxin in response to TGFβ and IL-13 can act as a chemoattractant for eosinophils (141). TGFβ induced CCL-2 (Monocyte Chemoattractant Protein-1) acts as a chemoattractant for monocytes (142). TGFβ is also reported to induce fibroblast-like synoviocytes from rheumatoid arthritis and osteoarthritis patients to induce CXCL-8 (IL-8), a neutrophil attractant, and CCL-3 (macrophage inflammatory protein 1α) mRNA which can recruit monocytes, macrophages, and T-cells (143).

1.8.6. Relationship between TGFβ and Airway Smooth Muscle

Smooth muscle is primarily under the control of the autonomic nervous system and can develop an isometric force per cross-sectional area equal to skeletal muscle, although the speed of contraction is much slower than skeletal muscle. Airway smooth muscle plays a major role in the pathophysiology of a number of airway diseases, including asthma. As described previously, the smooth muscle layer in asthma is significantly increased in size due to hyperplasia and possibly hypertrophy. In addition to its role as regulator of bronchomotor tone, smooth muscle can also secrete cytokines and growth factors, thus contributing to the inflammation and remodelling of the airways.

It has been proposed that TGFβ1 and TGFβ2 are responsible for the conversion of myofibroblasts into smooth muscle cells (fig. 1.9). Microarray analysis of fetal lung fibroblasts have shown that TGFβ can induce expression of a number of smooth muscle specific genes including smooth muscle myosin heavy chain, calponin, and smoothelin (144). Furthermore, there is evidence that rabbit bladder

myofibroblasts are able to transform into smooth muscle cells after treatment with TGFβ for 21 days (145). The smooth muscle transition has also been reported to occur independently of TGFβ. For example, a study by Jones and Jacobson (146) identified interstitial rat lung fibroblasts as the source of perivascular smooth muscle cells in response to low oxygen levels.

It is possible that the role of TGFβ is to induce the expression of smooth muscle mitogens such as endothelin-1 (68) and thus play a more indirect role in smooth muscle differentiation. Alternatively, Yeh *et al*, (147) demonstrated that adult peripheral blood CD34+ cells can transdifferentiate into smooth muscle cells *in vivo*, although this is augmented significantly by local tissue injury.

1.8.7. Role of TGFβ and Extracellular Matrix formation

Activated smooth muscle cells and fibroblasts can release extracellular matrix (ECM) proteins in response to TGFβ. For example, TGFβ induces α1 collagen I mRNA and α2 collagen protein in human lung fibroblasts (148). Eickelberg *et al* (149) showed that the percentage of collagen deposited in the ECM was higher in TGFβ treated human lung fibroblasts compared to controls. TGFβ has also been shown to stabilise tropoelastin mRNA in human fetal lung fibroblasts (150).

TGFβ also has indirect effects through the up-regulation of inhibitors of ECM proteases such as plasminogen activator inhibitor-1 (PAI-1) (151), tissue inhibitor matrix protease-1 (TIMP-1) (152) and TIMP-3 (153), and the down regulation of degrading ECM proteases such as interstitial collagenase (154).

1.8.8. Involvement of TGFβ in fibrosis

Altered regulation of TGFβ signalling has been implicated in a number of fibrotic diseases. For example, Coste *et al* (155) reported significantly higher levels of all three TGFβ isoforms in both nasal polyp tissue as compared to mucosa in affected subjects, and nasal mucosa from nasal polyposis subjects as compared to healthy controls. Nasal polyposis has similarities to asthma; it is a chronic inflammatory

disease of the upper airways characterised by remodelling events such as basement membrane thickening, fibrosis and epithelial hyperplasia. Furthermore, human lung fibroblasts derived from subjects with idiopathic pulmonary fibrosis (IPF) have an increased TGFβ1, pro-α1-(I) collagen, MMP-9, TIMP-1, TIMP-2 and TIMP-3 profile (156). TGFβ can also cause fibrosis in other organs such as the skin (157), bowel (158) and liver (159).

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder. The disorder is associated with frequent bleeding due to a failure of vessel repair, a phenomenon which increases with the age of the patient. Type I HHT is linked to mutations in the extracellular region of the endoglin protein (160;161) leading to a reduced number of endoglin receptors on the surface of the endothelium (for review see van den Driesche *et al*, (162). The type II condition is associated with mutated ALK-1 (163;164). Lane *et al*, (165) highlighted a genetic defect in BMP RII, which can lead to familial primary pulmonary hypertension (FPPH). FPPH is an autosomal dominant, fatal disorder of the arteries involving remodelling and occlusion of small pulmonary arteries and plexiform lesions. Both FPPH and HHT have similar phenotypes, possibly as both BMP RII and ALK-1 signal via Smad 1, 5 and 8.

1.8.9. Involvement of TGFβ in asthma

TGFβ1 is released in higher amounts in basal and allergen-challenged sites in bronchio-alveolar lavage (BAL) fluid of asthmatic patients than controls (166). Increased TGFβ immunoreactivity is found in mucosal bronchial biopsies from asthmatic subjects compared to controls (167) (168). Indeed a significant correlation was found between the number of epithelial or submucosal cells expressing TGFβ in asthma and the thickness of the basement membrane and fibroblast number. Elevated phosphorylated Smad-2 levels have been demonstrated in bronchial biopsies obtained from asthmatic subjects (169), indicating that TGFβ signalling downstream from the receptor is also enhanced in asthma. TGFβ1 mRNA levels in eosinophils are significantly increased in severe asthmatics compared to mild asthmatics (170) (171). Vignola *et al*

(167)demonstrated that alveolar macrophages from asthmatics released greater amounts of TGFβ compared to control subjects.

TGFβ has been implicated as a key mediator in asthma, responsible for a number of remodelling events. Four polymorphisms of TGFβ have been identified. A single polymorphism (C/T at position –509) in the TGFβ1 promoter has been linked to elevated TGFβ1 plasma levels (172) and IgE levels (173). A later study by Silverman (174) has revealed that the T allele of C-509T is associated with the diagnosis of asthma and may increase transcription by altering TGFβ promoter-reporter activity by interacting with the transcription factor Yin Yang 1. Pulleyn *et al* (175) demonstrated that there is a greater relative frequency of homozygosity for this allele in severe asthmatics compared to a control group. Conversely, Bučkova (176) showed that the polymorphism was not linked to elevated IgE levels in the Czech population.

A mouse model has been described where BALB/C mice were intratracheally instilled with either TGFβ1 or buffered saline (177). An increase in collagen I and III mRNA was seen in the airways after one week and an increase in total collagen was detected after a month after TGFβ treatment. There was also a detectable increase in airway hyperreactivity in the growth factor treated mice, thus demonstrating the importance of TGFβ in the pathogenesis of remodelling.

1.8.10. TGFβ as a therapeutic target

Fetal wounds heal without the deposition of scar tissue. Experiments on adult skin revealed that TGFβ mRNA is induced in response to wounding, whereas no upregulation was detected in human fetal skin under similar conditions (178). Shah *et al*, (179) demonstrated that treating dermal wounds in adult rats with a neutralising TGFβ antibody prevented formation of scar tissue. In a later study, Shah *et al* (180) showed that administration of anti-TGFβ1 or TGFβ2 antibodies to wounds could increase wound-healing time and reduce scarring, as can addition of TGFβ3 directly to wounds. Anti-TGFβ2 antibodies have also been utilised in the

field of conjunctival scarring and significantly improved glaucoma filtration surgery outcome in animal models (181).

Inhibition of TGFβ signalling may be a beneficial target in asthma. In theory it would have far reaching effects in reducing remodelling by lifting the inhibition on epithelial proliferation, preventing myofibroblast transformation, repressing the proliferative effect on myofibroblasts and inhibiting the excessive production of ECM components. A potentially negative effect would be the alleviation of immunosuppression and thus the possible accentuation of inflammation. Epithelial cells release TGFβ in order to initiate cellular migration in the process of epithelial repair. Therefore an inhibition of this process may be detrimental to airway remodelling.

1.8.11. Modulation of TGFβ and downstream effects

Nogami *et al* (182), reported that TGFβ1 decreased the number of β-adrenoceptors on human tracheal smooth muscle cells. TGFβ treatment partially suppressed the β2 agonist stimulated increase in cAMP, an observation that may explain the defective relaxation to β2 agonists shown by some asthmatics. Ishikawa and colleagues (183) showed that IFN-γ could suppress this TGFβ response. Interestingly, mucosal adenoviral IFN-γ gene transfer can effectively attenuate established allergen-induced airway inflammation and airway hyperresponsiveness in ovalbumin (OVA)-sensitized mice (184) although human trials have not been so successful (185).

Glucocorticosteroids are the mainstay therapy for the majority of mild to severe-persistent asthmatic subjects. Glucocorticoids assert an anti-inflammatory effect but may also have an anti-fibrotic action (for review see Shukla *et al* (186)) by decreasing collagen synthesis. Dexamethasone, for example, has been shown to block the fibrogenic effect of TGFβ *in vivo* at the cellular and molecular level (187). Wen *et al* (188) reported a significant inhibition of TGFβ1 and TGFβ2 production and a reduced expression of auto-induced TGFβ1 and TGFβ2 mRNA in response to glucocorticoid treatment in human fetal lung fibroblasts.

A novel approach to combating the TGFβ induced fibrotic response is the use of the human pregnancy hormone, relaxin. Relaxin has been shown to inhibit the TGFβ induced expression of both collagen and fibronectin in human lung fibroblasts, as well as inducing levels of MMP-1 (189). The hormone also stimulated wound healing of bronchial epithelial cells *in vitro* (190) and in a murine model, relaxin restored bleomycin-induced collagen accumulation back to normal levels (189).

1.8.12. A Question of Balance

This thesis has, as yet, appraised the actions of TGFβ in the airways and other tissues. It would be a naive however to suppose that TGFβ is the only fibrotic mediator in the airways or even that in the milieu of growth factors present in the lung that it was solely responsible for fibrosis associated with remodelled asthmatic airways. The actions of TGFβ are controlled not only by the expression levels of this growth factor in the lung and the ability of cells to respond to this signal but also on the balance of fibrotic versus anti-fibrotic factors produced as a result of wound healing.

1.9. Epidermal Growth Factor Receptor (EGFR) signalling and its role in asthma

There are four structurally related epidermal growth factor receptors: namely EGFR (HER, c-ErbB1), c-ErbB2 (HER2), c-ErbB3 (HER3) and c-ErbB4 (HER4). Each receptor consists of a cysteine rich extracellular ligand binding portion, a transmembrane spanning segment, an intracellular tyrosine kinase domain and a C-terminal region that contains a number of tyrosine residues. The extracellular ligand binding portion (or ectodomain) is divided into four domains, namely L1, S1, L2 and S2. Ligand can bind between L1 and L2; while domains S1 and S2 are cysteine rich and play a role in receptor dimerization (191;192). Upon ligand binding, receptors dimerize to form homo- and heterodimers. Cross

phosphorylation of tyrosine residues on the receptors enables the signal to be transduced.

1.9.1. ErbB receptors

EGFR (ErbB1) is a 170kDa transmembrane growth factor receptor with tyrosine kinase activity. EGFR has been extensively studied, although the majority of the studies have been carried out in the field of oncology. The receptor is overexpressed in a number of cancers including tumours of the breast, colon and ovaries. ErbB2 is the preferred binding partner (193) of the other ErbB receptors although it is an orphan receptor. Recruitment of ErbB2 to form a heterodimer can increase the binding affinity of the ligand for the receptor (194-196). Binding of ErbB2 to EGFR has also been reported to reduce the rate of internalisation and subsequent degradation of EGFR (197). ErbB3 is the least related family member and has little or no associated kinase activity. As a result, the receptor can only signal as a heterodimer, although it still serves as a docking protein to recruit a wide spectrum of downstream effectors. ErbB4 is present on a wide number of tissues, although expression tends to be skewed towards the differentiated compartments (for review see Carpenter (198)). There are four potential isoforms of ErbB4 due to mRNA splicing, although not all have been detected.

1.9.2. Epidermal Growth Factor Receptor Ligands

Mammalian ligands for the ErbB family include epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin (AR), betacellulin (BTC), heparin-binding epidermal growth factor-like growth factor (HB-EGF), epiregulin (EPR), epigen (199) and the neuregulins 1-4 (NRG1-4). Soluble EGF, AR, epigen and TGFα bind to ErbB1 to promote activation of the receptor. BTC, HB-EGF and EPR can bind to both ErbB1 and ErbB4. Neuregulin-1 and -2 bind to both ErbB3 and ErbB4 where as neuregulin-3 and –4 bind to ErbB4 only.

The process of ligand binding to an ErbB receptor promotes dimerization of the receptor. The complex formed can be either a homo- or heterodimer. The type and

number of ErbB receptors expressed by a cell, and ligand preferences towards certain dimers will dictate the composition of dimer that is likely to form upon ligand binding. The choice of ErbB receptor to which a ligand binds can also determine the functional response to the ligand. For example, HB-EGF induced activation of ErbB1 induces migration and proliferation whereas ErbB4 activation results only in cellular migration (200).

The ligands show no significant homology outside of the 40-50 amino acid EGF-like motifs that is the shared bioactive and structural feature of this family. The salient feature of the EGF-like motif is six characteristically spaced cysteine residues that form three intramolecular disulphide linkages and define a three loop secondary structure. There is a substantial amount of overlap and redundancy in the functions of the EGFR ligands. The cellular response elicited is dependent upon cell type, competition with other EGFR ligands, different receptor binding affinities and composition of the heterodimer or homodimer formed in response to ligand binding. The precise composition of the complex formed also determines the duration of signalling and the degree of receptor recycling.

1.9.3. Restricted expression patterns

EGFR ligands are present in all cell types, although the expression profile of the individual ligands varies. HB-EGF, amphiregulin and EGF have a wide spread tissue distribution pattern. TGFα is expressed at high levels in epithelial cells and macrophages. Epigen expression has a restricted tissue distribution, being present only in the liver, heart and testis (199). Epiregulin transcripts have been detected in the placenta, macrophages and heart tissue, although constitutive expression has been demonstrated in a number of cell lines and in the developing mouse embryo (201;202).

A number of ligands are preferentially secreted to either the apical or basolateral surface of a polarised cell, thus restricting the local distribution pattern. In colonic epithelial cells, amphiregulin sorting is restricted to the basolateral side of the cell (203). EGF, however is sorted equally to the apical and basolateral membranes in

polarized epithelial cells. The receptors also have a restricted expression patterns. In polarised lung epithelial cells, ErbB receptors are expressed basolaterally and are only exposed after epithelial damage. Immediately following a mechanical injury, EGFR ligands such as neuregulin 1 (204) can activate ErbB2 at the edge of the wound, thus hastening the restoration of epithelial integrity.

1.9.4. Binding Affinities

The affinity of a ligand for its receptor can influence its effectiveness at transducing intracellular signals. It has been reported that TGFα and EGF have very similar binding affinities although the binding affinity of amphiregulin for EGFR is several orders of magnitude less than that of EGF (205;206). The binding affinity of a ligand for a receptor can be influenced by the co-expression of accessory molecules that present the ligand to the receptor. HB-EGF and AR both contain amino terminal heparin binding domains (HBDs). The HBDs allows heparin and heparin sulphate proteoglycans on the cell surface to bind and present the soluble form of AR to EGFR, and HB-EGF to EGFR and ErbB4. This increases the local concentration of ligand and aids in stabilising its functional association with the receptor. The ErbB ligand family also exhibit different isoelectric points, which in part explains their differences in stability, intracellular processing as well as binding to both receptors and accessory molecules.

1.9.5. Cellular responses to EGFR ligands

Proliferation is the most well studied function of EGFR signalling. All of the EGFR ligands are capable of promoting mitogenesis although their potency is dependent on cell type. For example, amphiregulin induces proliferation of epithelial cells whereas HB-EGF is a potent smooth muscle mitogen.

There are a few studies demonstrating an anti-proliferative role of EGFR ligands. Kato *et al*, (207) demonstrated that amphiregulin stimulates growth of carcinoma cells, fibroblasts, smooth muscle cells, mammary epithelial cells and keratinocytes but inhibits growth in a number of normal or neoplastic cell lines. Although

historically, amphiregulin acquired its name from its' ability to stimulate or inhibit proliferation, the name could easily be applied to either EGF or epiregulin. The actual response to the growth factor is dependent upon the concentration of growth factor, competition with other EGFR ligands and the composition of the matrix the cells are plated on. Other factors include the type of cell and the profile of receptors expressed by that cell.

EGFR ligands can promote a migratory phenotype in cells, a response clearly illustrated by EGF. For example, EGF can enhance the repair of the mechanically damaged monolayers of bronchial epithelial cells (208). EGF-dependent cell motility is regulated by phospholipase C γ (PLCγ) activation (209;210) and MAP kinase activation (211). EGF can stimulate the disassembly of focal adhesions and alter components of the adherens (212) and desmosomal junctions, thus decreasing cell-cell contact and contributing to cell motility (213).

Although, EGF can induce mitogenesis of epithelial cells, expression patterns *in vivo* are generally associated with non-proliferative or differentiated cell populations (214). Signalling via the EGFR can alter the differentiation status of the cell. EGFR ligands can promote the differentiation of epithelial cells but induce de-differentiation of smooth muscle cells. For example, treatment of visceral smooth muscle cells isolated from chicken embryo gizzards with EGF, HB-EGF, TGFα, epiregulin and betacellulin caused a morphological change to a fibroblast-like phenotype (215). The cells lost their ability to contract and a decrease in expression of smooth muscle markers was reported. Conversely, Yamane *et al*, (216) reported that TGFα could also promote the early differentiation of tongue myoblasts. The study showed that addition of exogenous TGFα to E10 mouse embryonic cells increased both desmin mRNA and protein, as well as increasing myoD and myogenin mRNA.

1.9.6. A Disintegrin And Metalloprotease (ADAM) Proteins

All the EGFR ligands are synthesised as membrane anchored precursors that are enzymatically cleaved to release the soluble form. The proteolytic processing

induces a change in function from juxtacrine signalling to autocrine or paracrine signalling. For example, soluble HB-EGF can act as a mitogen and chemoattractant. The membrane bound precursor can also act in a juxtacrine manner to induce proliferation. Additionally, it can act as a diptheria toxin receptor (217) and in mice, as a blastocyst adhesion molecule (218).

The A Disintegrin And Metalloprotease domain proteins, or ADAMs, have been implicated in the processing of EGFR ligands and belong to the metzincin subgroup of zinc-dependent proteases. The ADAM family encompass a large number of proteins, thirty-four to date (for review see Seals and Courtneidge, (219)), although only nineteen are expressed in humans. ADAM 9, 10, 15 and 17 are ubiquitously expressed in human tissue, whilst the majority of the ADAMs display restricted expression in the testis and are involved in spermatogenesis and egg fertilization.

Approximately half of the ADAMs contain a zinc-binding consensus motif in their metalloprotease domain which is essential for enzymatic activity. In regard to the processing of EGFR ligands, ADAM 9, 10, 12, 17 and 19 have all been reported to play a role in pro-growth factor cleavage. Proteolytic ADAM activity can be inhibited *in vivo* by Tissue Inhibitors of MetalloProteases (TIMPs). For example, TIMP-3 can inhibit ADAM 17 (220) and ADAM 12 (221), whilst ADAM 10 activity can be inhibited by both TIMP-1 and TIMP-3 (220).

1.9.7. Mechanism of EGFR ligand cleavage

EGFR ligands are cleaved from the membrane in response to a diverse range of stimuli. Evidence in the literature suggests that cleavage is cell type dependent, with different proteases involved in cleavage of the same ligand in different cell types. The processing of membrane bound betacellulin, epiregulin and epigen by ADAMs has not been fully elucidated as yet. Similarly, the regulation of EGF shedding has not been clarified, although Le Gall *et al*, (222) demonstrated that cleavage is zinc metalloprotease dependent.

1.9.8. Transforming Growth Factor α

Serum (223), EGF (224), FGF and PDGF (225) have all been reported to increase TGFα shedding. Fan and Derynck (225) showed that p38 MAP Kinase signalling was responsible for the baseline cleavage of TGFα in CHO cells, whilst growth factor mediated cleavage occurred via a MEK/ERK pathway. TGFα is proteolytically cleaved at both N-terminal and C-terminal sites to release species with molecular weights ranging from 6-25kDa (226). ADAM 17 has been implicated in TGFα cleavage since the discovery that both TGFα and ADAM 17 (or TACE) knockout mice exhibit similar characteristics, including open eyes at birth and wavy hair (227). Hinkle *et al*, demonstrated (228) that recombinant ADAM 17 cleaved N-terminal TGFα peptides fifty times more efficiently than C-terminal peptides. The authors suggested that cleavage at the N-terminal site by ADAM 17 could remove any steric hinderance due to N-glycosylation sites (229;230) at an adjacent site upstream, enabling C-terminal cleavage by an as yet undetermined protease. Hinkle *et al* (228) also reported that ADAM 10 could cleave a soluble precursor of TGFα (proTGFecto) at the N-terminal site, although this activity was ninety fold less efficient than ADAM 17.

1.9.9. Amphiregulin

Amphiregulin mRNA is up-regulated by both amphiregulin and α-thrombin in vascular smooth muscle cells (207). Gschwind *et al* reported (231) that stimulation of squamous cell carcinoma cells with G-protein coupled receptor agonists LPA or carbachol resulted in release of amphiregulin, an event involving ADAM 17. Tobacco smoke has also been linked to cleavage of amphiregulin via oxygen radical activation of ADAM 17 (232) and transactivation of EGFR.

1.9.10. Heparin Binding-Epidermal like Growth Factor (HB-EGF)

Ectodomain shedding is induced by stimuli such as 4β-phorbol 12-myristate 13-acetate (PMA) (233), lysophosphatidic acid (LPA) (234) and the Ca^{2+}-ionophore, ionomycin (235). HB-EGF shedding is regulated by protein kinase C (PKC),

especially PKC–δ activation. Pharmalogical inhibition of PKC has been shown to diminish PMA-stimulated HB-EGF release (218;233).

The exact mechanism whereby HB-EGF is shed from the membrane is unknown although a number of proteases have been suggested to play a role in the cleavage. ADAM 9 has been reported to process proHB-EGF upon PMA treatment of Vero-H cells (236). Asakura *et al* (237) reported that KB-R7785, a specific HB-EGF inhibitor, prevented HB-EGF processing by ADAM 12, inducing subsequent cardiac hypertrophy in cultured rat neonatal cardiomyocytes. In 1997 Suzuki *et al* (238) showed that MMP3 releases soluble HB-EGF by cleavage at a specific juxtamembrane site in Vero, MC2 and U937 cells. Yu *et al* (239) reported that CD44 recruits active MMP7 with the HB-EGF precursor to ErbB4 forming a complex at the cell membrane in a number of tumour cell lines. The expression of the adhesion molecule CD44 also enhances the presentation of heparin binding EGFR ligands to the receptor (240) and is up regulated by EGF (241).

1.9.11. Signalling downstream from EGFR

In the absence of ligand binding, EGFR can exist as both monomers and dimers (242;243). EGFR dependent tyrosine kinase activity is however only activated by ligand-induced dimerisation, resulting in formation of either hetero- or homodimers. Dimerisation results in either autophosphorylation or transphosphorylation (by Src or Jak-2) of five tyrosine residues in the cytoplasmic domain of the receptor.

The phosphotyrosine acts as a SH2 docking domain for Grb2, which is constitutively bound to SOS (son of sevenless). Grb2-SOS can bind directly or indirectly to EGFR via Shc (fig 1.10). SOS then interacts with membrane-bound Ras (rat sarcoma). The interaction causes Ras-GDP to be converted to Ras-GTP, promoting its activation. Raf, a Mitogen Activated Protein Kinase Kinase Kinase (MAPKKK), is phosphorylated by activated Ras. This triggers a further cascade of phosphorylations whereby Raf phosphorylates Mek (a Mitogen Activated Protein

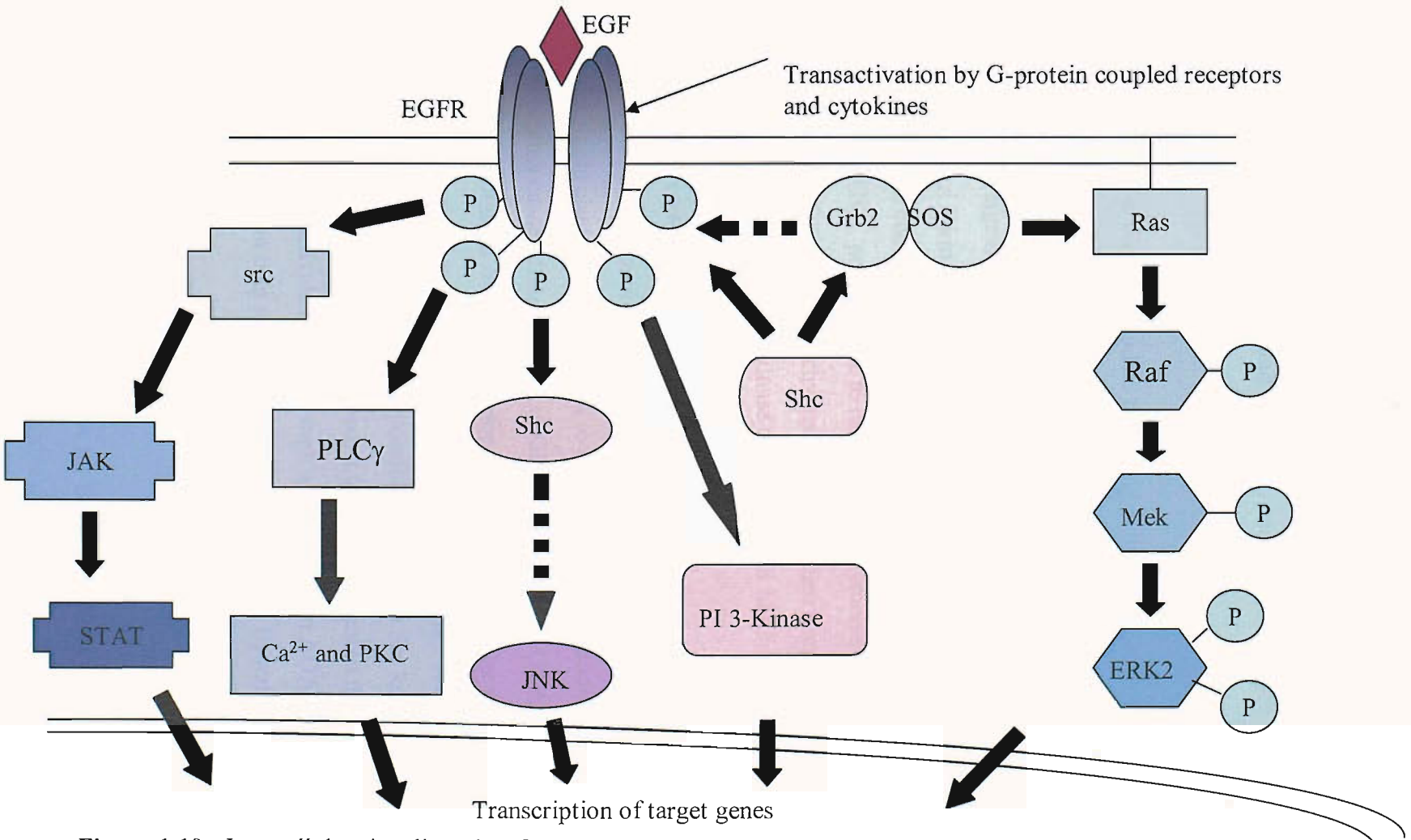


Figure 1.10: Intracellular signaling via EGFR

Ligand induced receptor activation results in phosphorylation of the EGFR and signaling via a number of possible downstream signalling pathways. Phosphorylation changes are only indicated on the most studied Erk-2 cascade for ease of interpretation.

Kinase Kinase), which phosphorylates extracellular regulated protein kinase (ERK), a MAPK. Activated MAP kinases can translocate to the nucleus and interact with transcription factors such as fos, jun, Sp-1, PEA3, E2F and Elk-1. Alternatively, transient activation of ERKs is usually associated with activated ERKs remaining in the cytoplasm and the subsequent phosphorylation of cytoplasmic proteins.

The above pathway is the most studied signalling pathway although EGFR can transduce signals from the membrane via a number of different pathways. The SH2 domain on the EGFR can act as a docking domain for a number of different proteins e.g. PLCγ, signal transducers and activators of transcription (STAT) (244) and phosphoinositol 3-kinase. Non-ligand-induced activation of EGFR has been described in stressed cells. Stimuli such as H_2O_2 and ultraviolet light induce activation of the EGFR, possibly due to inactivation of tyrosine phosphatases (245) or activation of PKCδ (246). The activated EGFR can then transduce signal via phosphorylation of Jun N-terminal kinase (JNK) and p38 MAP kinase.

G protein coupled receptors can transactivate the EGFR (fig. 1.11). Stimuli such as ET-1, LPA (247) and thrombin (248) can activate G protein coupled receptors permitting soluble cleaved ligand to bind to the receptor. Activation of G protein coupled receptors causes disassociation of GDP and binding of GTP. Release of α and βγ G protein subunits from the receptor can act as intracellular signalling proteins and cause an increase in Ca^{2+} levels. The elevated Ca^{2+} levels can indirectly cause activation of the EGFR, possibly involving a Src kinase (249) or MMP (250) dependent mechanism. Indeed, it has been suggested that G-protein coupled receptors can activate EGFR through activation of an ADAM protein, such as ADAM 12, which can then promote cleavage of an EGFR ligand (251).

1.9.12. Regulation of EGFR signalling

EGF can increase the production of mRNA for EGFR as well as increasing the half-life of the mRNA. Jinno *et al* (252) showed that the half-life was extended from 1.6 hours to more than 6 hours in human KB epidermoid carcinoma cells.

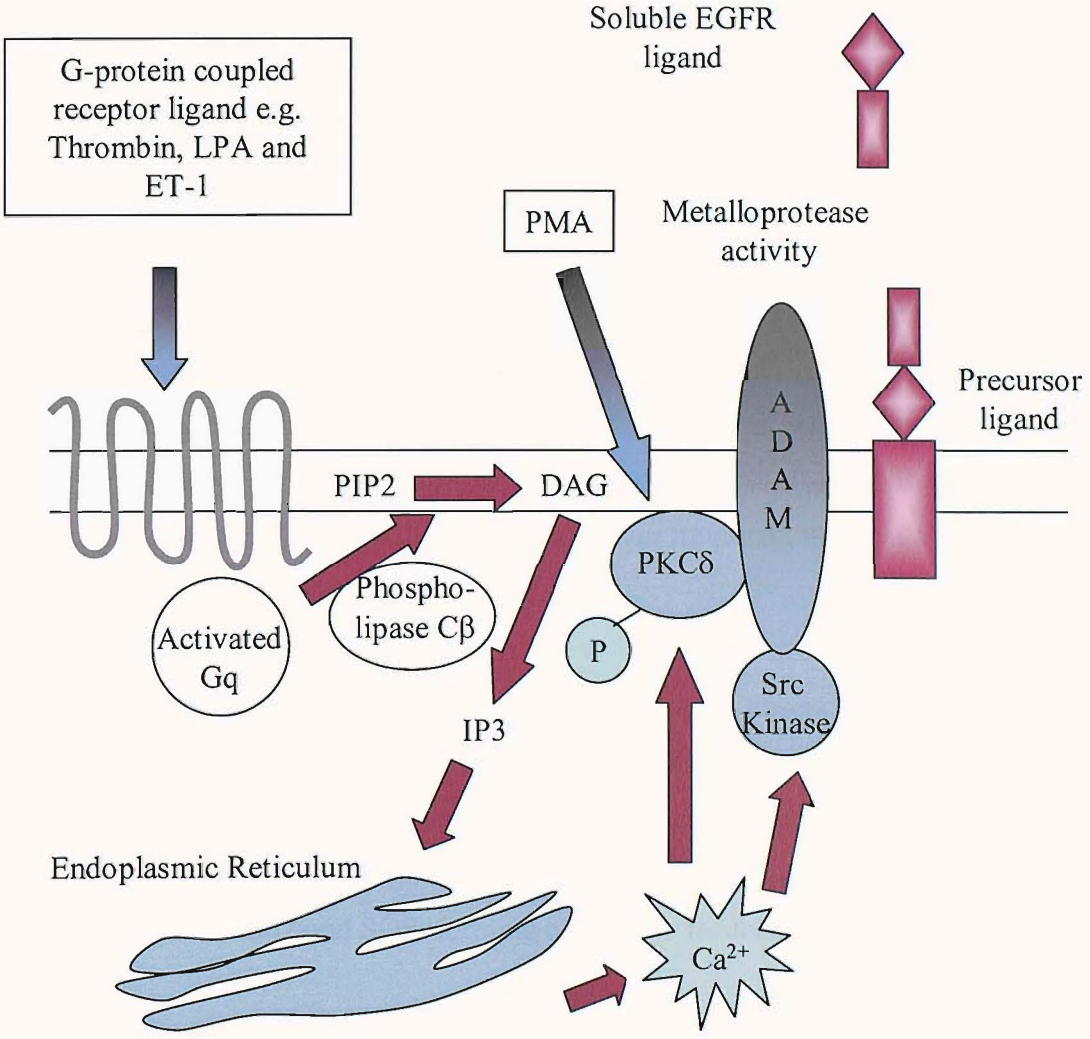


Figure 1.11. Transactivation of the EGFR
G protein coupled receptor ligands such as thrombin and LPA can indirectly activate the EGFR by promoting the cleavage of EGFR precursor ligands by ADAMs, such as ADAM 12 or ADAM 17.

EGF also promotes the down regulation of EGFR kinase activity within 5-90 minutes of stimulation, depending on cell type. This occurs due to internalisation of 80-95% of the EGFR, loss of bound ligand and ultimately to the lysosomal destruction of receptors. EGFR is rapidly trafficked to coated pits, internalised, and either degraded when endosomes fuse with lysosomes or recycled. The type of ligand receptor interaction determines the fate of the receptor. For example, continued EGF binding in the acidic late endosomal compartment (pI 4.6) leads to degradation of the receptor whereas reduced binding of TGFα (pI 6.8) to EGFR results in recycling due to disassociation of ligand and receptor in early endosomes (253).

ErbB2, ErbB3 and ErbB4 are not rapidly internalised following ligand binding, although different regulation mechanisms may be involved in controlling signal transduction. For example, cells treated with PMA (254) or neuregulin (255) induced the proteolytic cleavage of ErbB-4 via an ADAM 17 dependent event (256). The proteolytic activity resulted in the release of a 120-kDa ectodomain fragment and a remaining 80-kDa cytoplasmic domain fragment, that could be further processed by γ-secretase (257;258).

The carboxy tail of the EGFR acts to inhibit signalling in the absence of stimulatory ligands. PKC and ERKs interact with EGFR at the juxtamembrane domain and attenuate signalling by negative feedback i.e. serine phosphorylation of EGFR which prevents transduction of signals into the cell. ERK activation also promotes the disassociation of Grb2 and SOS, forming a negative feedback pathway (259).

There are a number of factors that are capable of modulating signalling via the Ras/Raf/Mek/Erk cascade. For example, MEK partner 1 (MP1) enhances the efficiency of Erk phosphorylation by MEK (260) and Sur-8 increases the binding between Ras and Raf (261). Alternatively, SPRED (262;263), RIN1 (264) and RKIP (265) have all been demonstrated to act as negative regulators of the Ras/Raf/Mek/Erk cascade. The p66 isoform of Shc can act as a negative regulator of ERK stimulation (266). Recently Erbin, a protein capable of binding to ErbB2,

via a PDK domain (267), has also been shown to associate with active Ras and impair the activation of Erk (268).

1.9.13. EGFR and hyperproliferative disorders

The role EGFR ligands can play in hyperproliferative disorders is best exemplified by psoriasis. Psoriasis is characterized by epidermal hyperplasia, altered epidermal maturation, and local accumulation of acute and chronic inflammatory cells. The EGFR is overexpressed in psoriatic epithelium (269). Psoriatic lesions exhibit prominent amphiregulin cytoplasmic staining in basal and spinous keratinocytes (270) and in psoriatic epidermis (271). A later study by Cook and colleagues showed that overexpression of amphiregulin in basal keratinocytes correlated with a psoriasis-like phenotype (272).

1.9.14. EGF and Asthma

EGFR, ErbB2 and ErbB3 receptors are expressed in the epithelium of the upper and lower airways as well as in primary cultures of human bronchial epithelial cells and human bronchial epithelial derived cell lines such as H292 and16HBE 14o- (204;273). In asthma it has been observed that the expression of EGFR is increased on the remaining epithelial cells around the site of damage, and on the apical side of the exposed basal cells (273). EGFR expression levels are increased in asthma and correlate to disease severity (208). This increase in EGFR expression is a typical response to tissue injury, however in asthma there is no evidence that this is coupled with an increased proliferative response (25). Furthermore, EGFR expression is insensitive to the effects of corticosteroids (208;274).

Increased expression of EGFR ligands has been reported in asthmatic airways. Amishima *et al* (275) reported that EGF was expressed at higher levels in the bronchial epithelium and glands of asthmatic subjects compared to normals. It has also been demonstrated that EGF immunoreactivity is significantly increased in the submucosa of asthmatics (167). Additionally, TGFα release was enhanced in

asthmatic epithelial cells in response to proinflammatory cytokines, in particular TNFα and combinations of IL-4, IL-13, and allergen (276).

EGF and TGFα have been implicated in induction of MUC5AC expression, a mucin released from goblet cells, via activation of the EGFR/Ras/Raf/ERK-signaling pathway (277). Takeyama *et al*, (278) demonstrated a positive correlation between EGFR immunoreactivity and the area of MUC5AC-positive staining. Increased release of EGFR ligands into the luminal space, such as EGF, coupled with increased EGFR expression may account for the increase in mucus secretion by asthmatic epithelial cells.

The inflammatory cell profile in severe asthma is characterised by high numbers of circulating neutrophils. Hamilton and colleagues (279) demonstrated that damage to the epithelium has the potential to contribute to neutrophilic inflammation through enhanced production of CXCL-8 (IL-8) via EGFR- dependent mechanisms, which again are insensitive to corticosteroids.

1.9.15. Modulation of EGFR expression in asthma

β2 agonists can stimulate cAMP release, causing activation of PKA and subsequent serine phosphorylation of the EGFR (280). Phosphorylation of serine residues on the EGFR deactivates the receptor, thus preventing signalling downstream from the receptor. Additionally, Kimura and Ogihara, (281) showed that increasing concentrations of the β2 agonist metaproterenol, markedly reduced the proliferative effects of TGFα in adult rat hepatocytes. These observations provide an example of where a pre-existing therapy could indeed be counter-productive and prevent epithelial repair in the airways of asthmatics.

Studies have revealed that corticosteroids do not alter either EGFR expression (208) or activation (274). A study by Kibe *et al* (282) reported that corticosteroid treatment inhibited eotaxin expression and eosinophil accumulation, but had no affect on airway hyperresponsiveness, MUC5AC overexpression, or goblet cell hyperplasia induced by IL-13 in a murine model. Corticosteroids have also been

shown to have no effect on the expression of $p21^{waf}$ expression, a cyclin dependent kinase inhibitor.

1.9.16. EGF/EGFR as a novel therapeutic target in chronic asthma

EGFR can regulate mucin production in the airways (283). A treatment whereby secretion is reduced would be beneficial to the asthma sufferer, although complete inhibition of mucus secretion may indeed be counter-productive. Controlled mucus secretion plays a protective role in the innate immune system and helps to eliminate air-borne allergens via ciliary action. Inhibitors of EGFR would potentially also suppress EGFR mediated signals needed to initiate repair of the damaged epithelium, evident in asthmatic airways. Therefore, targeted downstream interventions may be appropriate.

1.10. TGFβ and EGF

TGFβ can interact with a large number of different growth factors and cytokines in the lungs, each affiliation mediating a different outcome. Attempting to unscramble the cellular response to only two growth factors among the thousands of potential couplings that exist in the plethora of growth factors in the lung is a daunting task. This thesis attempts to untangle the intricate interweaving responses to TGFβ2 and EGF chosen because of their much published and often opposing actions in proliferation and differentiation. Furthermore, the EGF and TGFβ families are historically linked as, TGFα and TGFβ were originally co-purified as 'transforming growth factor' on the basis of anchorage independent growth assays (284), lending another intriguing twist to the theme.

Indeed, the TGFβ and EGF signalling pathways are not discrete pathways as they share many down stream signalling molecules. TGFβ and EGF can both mediate distinct cellular events via activation of ERK1 and ERK2 and as a result, TGFβ can potentially act synergistically, antagonistically or independently to EGF. There are a few examples of cross-talk between the two pathways at the level of receptor-ligand interactions. A study by Thompson *et al* (285) using normal rat kidney

fibroblasts indicated that TGFβ was capable of increasing expression of EGFR. TGFβ can also regulate expression of EGFR ligands. For example, a study by Bennett *et al* (286) showed that TGFβ treatment reduces the level of expression of amphiregulin in the human lung adenocarcinoma cell line A549. Conversely, Danielpour *et al* (287) showed that stimulation by EGF in the same cell type could induce the secretion of TGFβ1.

Independently, TGFβ and EGF are known to mediate a number of different cellular events, including growth, migration and branching morphogenesis of the lung. There are a few reports in the literature describing the combined actions of the two growth factors. For example, Mishima *et al* (288) demonstrated that TGFβ could antagonise EGF induced attachment of cells onto a fibronectin matrix, migration over corneal stroma, and proliferation of corneal epithelial cells. TGFβ was also found to inhibit the EGF induced filapodia extension associated with migrating human bronchial epithelial cells (289). Both TGFβ and EGF can control lung branching in the developing foetus. TGFβ causes growth arrest of the developing airways (290-293) whereas EGF has been reported to cause significant elongation of the bronchial tubes (294). In combination, TGFβ promotes cell arrest allowing EGF to induce branching in the outgrowing terminal buds.

These examples show how the action of TGFβ can influence EGF signalling pathways and *vice versa* in a number of ways. Regulation of the pathways can occur at a number of different levels and the signalling outcome is dictated by the relative balance between the TGFβ and EGF signalling pathways. No signalling mediated event can occur in isolation in the cell but the TGFβ-EGF axis is distinct in its degree of overlap, especially when the antagonistic nature of the two growth factors is taken into consideration. The balance between EGF driven proliferation and TGFβ mediated differentiation of fibroblasts is therefore of potential importance in the pathogenesis of airway remodelling in asthmatics.

1.11. Aims

Previously, TGFβ induced differentiation of human lung fibroblasts and the EGF induced proliferation have largely been studied in isolation of other growth factors and cytokines. The aim of this study was to examine differences in response to TGFβ and EGF in primary human lung fibroblasts obtained from the outgrowth of normal and asthma bronchial biopsies using a previously characterised method (68). The study comprises an in-depth examination of how EGF can modify TGFβ2 induced differentiation at both the ligand and receptor level. The effect of TGFβ2 on EGF induced proliferation will also be assessed.

The majority of the literature regarding TGFβ receptor regulation by TGFβ has focused on TGFβ1. TGFβ2 was the main isoform of TGFβ to be released from damaged epithelium in co-culture studies (67). TGFβ2 was therefore chosen in the present study due to its relevance to epithelial-mesenchymal signalling. EGF was chosen as it is the most studied EGFR ligand and because of its reported antagonistic actions against TGFβ.

It was postulated that asthmatic fibroblasts have an altered response to TGFβ2 and EGF. The rationale behind the hypothesis was that other fibrotic diseases such as FPPH and HHT have an altered response to TGFβ due to genetic defects. Asthma is a complex genetic disease, one of the outcomes of which is an increased expression of TGFβ. This study was designed to determine whether asthmatic fibroblasts grown *in vitro* respond differently to TGFβ and EGF stimulation. It has been reported that TGFβ levels are increased in asthma, potentially contributing to the fibrosis described in asthmatic airways. It is hypothesised that the majority of asthmatic patients have some degree of airway remodelling due, not only to an increase of TGFβ in the airways but also an enhanced responsiveness to the growth factor.

Hypothesis 1: Asthmatic bronchial fibroblasts are more responsive to the effects of TGFβ2 induced differentiation and proliferation than normal fibroblasts.

Additionally, the impact of EGF on the TGFβ induced effects will be studied. The aim was to determine whether TGFβ2 and EGF always act antagonistically to one another, whether they can act in a synergistic manner, or indeed be mutually independent.

Hypothesis 2: EGF antagonises the effects of TGFβ2 induced differentiation and proliferation in bronchial fibroblasts.

Finally the effect of the two growth factors on amphiregulin and HB-EGF expression will be examined. These growth factors have potent paracrine activity, HB-EGF being a potent smooth muscle mitogen, and amphiregulin being a mitogen for epithelial cells. Differences in response to TGFβ and EGF elicited by the normal and asthmatic fibroblasts will be recorded and their relevance to disease discussed.

CHAPTER 2

Materials and Methods

CHAPTER 2

MATERIALS AND METHODS

2.1. Subject characterisation

All subjects participating in the study were clinically assessed by history and examination prior to bronchoscopy. Those with asthma were classified according to GINA guidelines. Written consent was obtained from the subjects and ethically approved by the Joint Ethics Committee of Southampton University and General Hospital. All subjects were thoroughly characterised before entering the study.

The subjects recruited (normals, n=23, asthmatics, n=19) were of a similar age (normals, 29.7±9.72, asthmatics, 31.26±10.89) (table 2.1 and 2.2). The normal group consisted of 12 females and 11 males, the asthmatic group consisted of 8 females and 11 males. Only non-atopic normals and atopic asthmatics were accepted into the study, as determined by results to a skin prick test to 8 common aeroallergens. If the wheal exceeded 4mm, 15 minutes after addition of allergen the subject was considered to be atopic.

Patients performed lung function tests to determine the degree of airway obstruction and hyperresponsiveness. Airway hyperresponsiveness was measured by methacholine challenge, a 20% fall in forced expiratory volume in one second (FEV1) in response to methacholine signified hyperresponsiveness. Normal subjects had no evidence of bronchial hyperresponsiveness. The average provocation concentration (PC20) causing a 20% decline in FEV1 for asthmatic subjects was 3.53±2.9mg/ml. Subjects with asthma had a reduced lung function (81.29% predicted FEV1 ±10.18%) compared to controls ($p<0.05$). All asthmatics were prescribed β2 agonists, and 10 subjects were taking inhaled steroids throughout the course of the study. Those on inhaled corticosteroids were all using less than 1,500μg/d of beclomethasone. To distinguish between the differing severities of asthma, the asthmatic subjects were divided into mild (receiving only

β2 agonists) and moderate/severe asthmatics (recieving β2 agonists and corticosteroids).

Code	Age at Bronchoscopy	Gender	PC20 (mg/ml)	FEV1 (% predicted)	Inhaled Steroid	Short acting β2 agonist
1	38	M	>8	120	N	N
2	35	F	>8	100	N	N
3	30	F	>8	105	N	N
4	53	F	>8	116	N	N
5	46	M	>8	90.23	N	N
6	31	M	>8	102	N	N
7	25	F	>8	97	N	N
8	45	F	>8	121	N	N
9	31	M	>8	90.6	N	N
10	32	F	>8	81	N	N
11	24	F	>8	113	N	N
12	19	M	>8	96	N	N
13	19	M	>8	114.1	N	N
14	21	M	>32	104	N	N
15	20	M	>32	118	N	N
16	21	M	>32	93	N	N
17	20	F	>32	97	N	N
18	21	F	>32	97	N	N
19	21	M	>32	95	N	N
20	27	M	>8	100	N	N
21	40	F	>8	112	N	N
22	28	F	>8	119	N	N
23	36	F	>8	118	N	N
Average	29.70			104.30		
Standard Deviation	9.72			11.48		

Table 2.1: Subject charactersitics for normal subjects

Y indicates that the subject was receiving medication, N indicates that the subject was not receiving medication.

Code	Age at Bronchoscopy	Gender	PC20 (mg/ml)	FEV1 (% Predicted)	Inhaled Steroid	Short acting β2 agonist
1	24	M	4.42	96.7	N	Y
2	20	F	4.28	78	Y	Y
3	34	M	0.7	79	N	Y
4	20	F	5.7	83	N	Y
5	20	F	1.49	75	N	Y
6	42	M	4.49	96	Y	Y
7	26	M	1.17	78	Y	Y
8	21	F	0.5	70	Y	Y
9	36	M	1.2	69	Y	Y
10	21	M	0.62	76	Y	Y
11	46	M	0.04	67	Y	Y
12	57	M	5.62	89.2	Y	Y
13	29	F	7.97	97.3	Y	Y
14	42	F	3.28	94	Y	Y
15	35	M	1.16	89	N	Y
16	21	M	1.06	68	N	Y
17	35	F	8.5	79	Y	Y
18	23	F	8		Y	Y
19	42	M	6.83	79	N	Y
Average	31.26		3.53	81.29		
Standard Deviation	10.89		2.90	10.18		

Table 2.2: Subject characteristics for asthmatic subjects
Y indicates that the subject was receiving medication, N indicates that the subject was not receiving medication.

Bronchoscopy was undertaken by an experienced respiratory physician in accordance with current guidelines. Prior to bronchoscopy, subjects were premedicated with nebulised salbutamol (2.5mg) and intravenous atropine (0.6mg) and midazolam (0-8mg intravenously) to achieve moderate sedation. Lignocaine (4.5mg/kg, maximum dose 300mg) was used for local anaesthesia and continuous

oxygen was given via a nasal cannulae. Biopsy samples were taken from the second and third generation bronchi using alligator forceps.

2.2. Primary fibroblasts

All media and supplements were ordered from Invitrogen (Paisley U.K.) unless otherwise specified. Primary fibroblasts were obtained by outgrowth from bronchial biopsies. Biopsies from each subject were placed in a petri dish with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v heat inactivated foetal bovine serum (FBS), 50IU/ml penicillin, 50µg/ml streptomycin, 1x non-essential amino acids, 1mM sodium pyruvate and 2mM glutamine. The tissue was chopped into pieces and scored onto a petri dish using two sterile scalpel blades to aid attachment of the fibroblasts. The tissues were incubated in a humidified Heraeus incubator at 37°C, 5% CO_2 for approximately three weeks during which time fibroblasts migrated from the tissue and proliferated on the base of the culture dish. On passage, the fibroblast cultures were typically split 1:5. Cultures were used for assays up to passages 10, but were typically assayed at passage 5-7. Primary fibroblasts were routinely checked for smooth muscle contamination. None of the cultures used in this study stained positively for either smooth muscle heavy chain myosin or desmin, both smooth muscle marker proteins. The staining for smooth muscle markers was performed by Dr Fabio Bucchieri, Dr Ken Matsumoto and James Wicks.

2.2.1. Trypsinisation of confluent cell monolayers

Trypsin-EDTA concentrate (x10) was diluted to a 1x solution with Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. Prior to use, all media and trypsin were pre-warmed to room temperature. The cell monolayer was washed twice with HBSS to remove traces of Ca^{2+}, Mg^{2+} and serum. Approximately 1-2ml 1x Trypsin-EDTA was added to a T75cm^2 flask, enough to cover the bottom surface. Primary fibroblasts were removed by incubation at room temperature for approximately 1 minute, followed by sharp tapping of the flask. The action of the trypsin was halted by the addition of growth media supplemented with 10% FBS.

The cells were then centrifuged at 150g for 5 minutes to remove the trypsin and the cell pellet was re-suspended in the required volume of growth medium.

2.2.2. Counting cells

If necessary, cell clumps were disrupted by aspiration through a 25gauge needle before plating out in order to obtain a single cell suspension. Cells were counted using the Trypan blue exclusion method. The dye can enter cells whose membranes are not intact i.e. non-viable cells, and stain them blue. This method gives both a viable cell count (Trypan blue excluded cells) and a total cell count (including blue cells). A small aliquot of cell suspension (100µl) was centrifuged at 150g for 5 minutes. The media was removed and the cells re-suspended in 100µl of HBSS, to remove serum proteins present in the growth media that may prevent trypan blue uptake into the cells. 20µl of cell suspension was added to 30µl HBSS and 50µl 0.4% (w/v) Trypan blue. The cells were counted using an improved Neubauer haemocytometer (depth 0.1mm, 400mm^2). The mean of the cells in the central 1mm^2 square and four surrounding squares was calculated in both the top and bottom grid. The count was equivalent to 0.1mm^3. The number of cells per ml was calculated by multiplying the mean count by 5 (dilution factor) x 10^4.

2.2.3. Cryogenic storage

Frozen aliquots of primary fibroblasts were routinely prepared to maintain stocks. Trypsinised cells were placed into medium to neutralise the action of the trypsin. The cells were centrifuged at 150g and the medium was discarded. The cells were re-suspended in chilled (4°C) growth medium supplemented with 10% FBS and 10% DMSO, the latter acting as a cryoprotectant. Approximately 1ml cell solution was placed into a cryotube (Invitrogen, Paisley, U.K.), frozen overnight at –80°C and then placed into liquid nitrogen vapour at –150°C for long-term storage. A confluent T75cm^2 flask of primary fibroblasts was routinely distributed into four cryotubes (approx. 0.75 x 10^6 cells/tube). To regenerate the cells, the cryotubes were rapidly thawed and placed into 10ml of warmed media in a T75cm^2 flask. The flask was placed in the incubator for 6 hours until the cells had adhered. The

medium was then replaced with fresh growth medium to remove all traces of DMSO.

2.2.4. Treatment of cells

Cells were grown to the desired confluency and then serum starved for 24 hours in UltraCulture (Cambrex Bio Science Wokingham Ltd, Berkshire U.K.) supplemented with 50IU/ml penicillin, 50μg/ml streptomycin and 2mM glutamine to make them quiescent. This ensured that all the cells were quiescent, so that when stimulated their responses would be similar. After 24 hours the culture medium was replaced by the appropriate stimulus diluted in UltraCulture.

The type and concentration of growth factor and time of incubation varied according to the experiment. TGFβ2 and EGF were purchased from Peprotech (London, U.K.). HB-EGF, amphiregulin and TGFα were purchased from R&D systems (Minneapolis, U.S.A.). Working stocks of growth factor were routinely aliquotted and stored at -80°C at a concentration of 1μg/ml or higher.

2.3. Methylene Blue Proliferation assay

Fibroblasts were routinely photographed before and after treatment to record any morphological changes occurring in the cells. The cells were stained with methylene blue in order to clearly visualise modifications to cell size, shape and orientation. Elution of the dye provided a measure of cell biomass (295). Proliferation assays were performed on 24 well plates. The fibroblasts were seeded at 8 x 10^3/well, serum starved 24 hours after plating, and then treated for the appropriate amount of time (up to 5 days) with the appropriate stimulus diluted in serum free medium, as detailed in the results section. After treatment the medium was removed and the cells fixed overnight with 500μl formal saline (4% (v/v) formaldehyde in 0.9% (v/v) saline solution) at room temperature. The formal saline was then removed and 300μl methylene blue (1% (w/v) methylene blue in 10mM borate buffer, pH 8.5) was added to each well for 30 minutes. Excess dye was removed from the plates by careful washing with tap water. The plates were

then air-dried and if required, photographs were taken using a digital camera (Nikon Coolpix 995).

Methylene blue dye was eluted with 200µl 1:1 Ethanol: 0.1M HCl. 20µl eluted dye was transferred to a 96 well plate and diluted with 80µl 1:1 Ethanol: 0.1M HCl. The samples were read at 630nm on a microplate spectrophotometer (Labsystems Multiskan Ascent). Cell biomass was calculated from a standard curve (see figure 2.1). A standard curve was constructed with dilutions of fibroblasts from $1x10^5$ (determined by a trypan blue count of viable cells) down to 195 cells/well. The fibroblasts were fixed after 6 hours to ensure the cells had adhered but before the cells could proliferate. Cells were fixed and stained as described above.

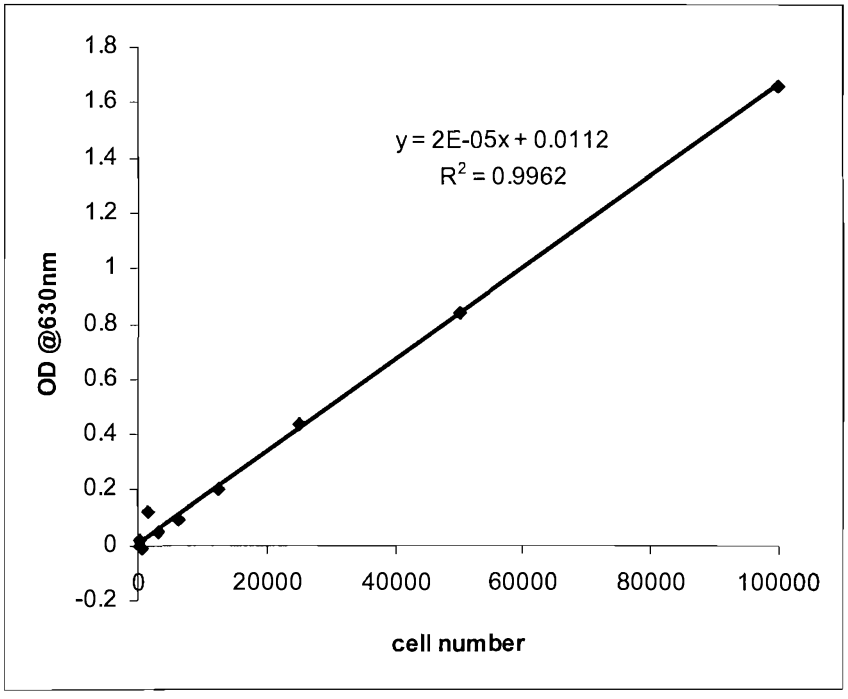


Figure 2.1: Methylene Blue standard curve for primary fibroblasts

Primary fibroblasts were seeded at decreasing concentrations in a 24 well Nunc tissue culture dish. The cells were fixed after 6 hours, stained with methylene blue and then eluted with 1:1 0.1M HCl:Ethanol. The optical density at 630nm was plotted against the number of fibroblasts initially seeded in the well to provide a reference standard curve for other methylene blue elution assays.

To check the accuracy of a methylene blue assay, it was compared to a direct cell count using the Trypan blue exclusion method. Fibroblasts were plated at two concentrations (n=6) and grown in growth medium for two days. Half the cells were then fixed with formal saline and subsequently stained, while the other half were trypsined and counted directly. The results were very similar as shown in table 2.3.

Initial cell density	Average number of cells counted by Methylene Blue method	Average number of cells counted by Trypan Blue method
5×10^4	115148	92300
1×10^5	175015	172300

Table 2.3: Comparison of Methylene Blue Elution assay to direct cell counting

2.4. RNA extraction and analysis

All reagents and tubes used for RNA work were sterile and DNase and RNase free. Promega Barrier tips (Southampton, U.K.) were used on the pipettes throughout the procedure. The distilled water was RNase and DNase free MilliQ quality distilled water (Millipore U.K. Ltd). RNA work was carried out in a defined area in the laboratory to prevent DNA contamination.

2.4.1. RNA extraction

The primary fibroblasts were serum starved for 24 hours and then treated with the appropriate stimulus. RNA was extracted from the cells using Trizol reagent (Invitrogen), a mono-phasic solution of phenol and guanidine isothiocyanate that allows single step extraction of RNA. The fibroblast monolayer was detached from a T25 flask by gentle agitation after the addition of 1ml Trizol Reagent. The Trizol solution was pipetted up and down a number of times before transferring to a 1.5ml microfuge tube. The samples were left at room temperature for five minutes to allow cell components to dissolve and the dissociation of soluble protein factors from the RNA. The samples were stored at -80°C until the RNA was extracted.

The Trizol samples were thawed and 200µl of chloroform added for each 1ml of Trizol. Each tube was shaken vigorously by hand for 15 seconds and then incubated at room temperature for 10 minutes. The tubes were centrifuged at 12,000g for 15 minutes at 4°C. The top aqueous layer containing the RNA was transferred into a sterile microfuge tube containing 500µl isopropanol. The tubes were mixed by hand and incubated overnight at –20°C. The samples were centrifuged at 12,000g for 30 minutes at 4°C. The supernatant was removed leaving a small white pellet of RNA. The pellet was washed with 1ml 75% ethanol, mixed and then centrifuged at 7,500g for 5 minutes at 4°C. The ethanol was removed and the tube was pulsed again to remove final traces of liquid. The pellet was air dried for approximately 5 minutes until the pellet turned transparent.

2.4.2. DNase treatment

To remove contaminating genomic DNA, the total RNA was treated with DNase. The only DNA present after the reverse transcriptase reaction should be that corresponding to the cDNA reverse transcribed from the RNA. 1µl RQ1 DNaseI (Promega), 2µl 10x DNase buffer (Ambion, Austin, USA) and 17µl dH_2O was added to each tube containing a pellet of RNA. The reaction was incubated at 37°C for 1 hour, addition of 5µl neutralisation buffer (Ambion, Austin, USA) halted the action of the enzyme. The tubes were mixed by hand, incubated at room temperature for 2 minutes and then pulse spun. The samples were stored at -80°C until required.

2.4.3. Agarose gel

The gel apparatus and combs were carefully washed and air-dried prior to use and the sides of the gel tray constructed using masking tape. 0.75g agarose was added to 100ml 1x Tris-Borate-EDTA (TBE) buffer and then heated for 4 minutes on a medium heat in the microwave to dissolve the agarose. 15µl Ethidium bromide (500µg/ml stock) was added carefully to the cooled agarose gel to allow visualisation of the bands, due to its ability of interchelating into RNA. The gel was then poured into the tray and a comb inserted. Once the gel was set, the masking tape was removed and the gel placed into the horizontal gel apparatus (Biorad), with enough TBE buffer to cover the gel by approximately 3mm.

The gel was loaded with 2µl sample RNA diluted in 8µl 1x RNA loading buffer (30% glycerol, 0.5% Bromophenol Blue, 5mM EDTA and 20mM Tris, pH7.5). The gel was run at 80mA (constant) for approximately 30 minutes. The bands were visualised under UV light using the GeneSnap programme on the Gene Genius Bioimaging system (Syngene). The presence of two clear bands (see figure 2.2), an 18S and 28S band, with no smearing confirmed the integrity of the RNA.

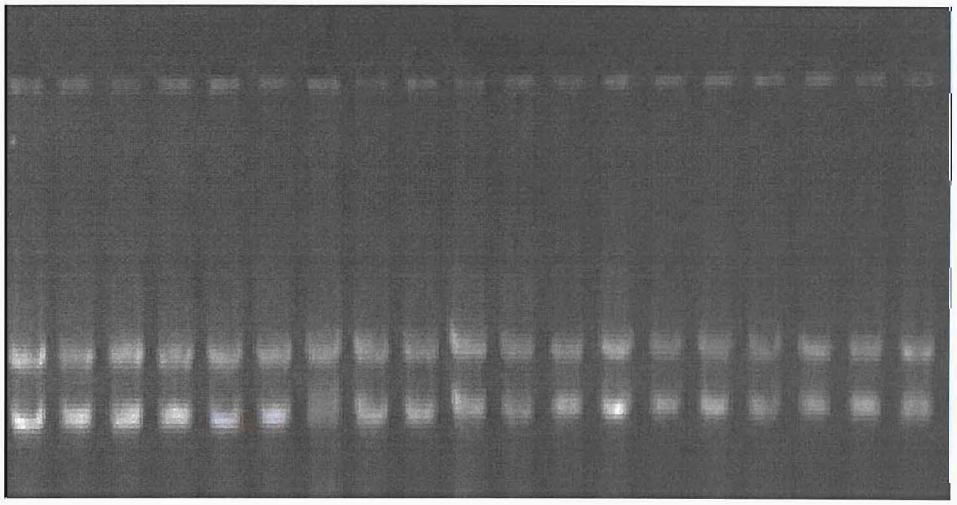


Figure 2.2: RNA samples run on a 0.75% agarose gel
Under UV light, two clear bands are visible for each sample. This indicates that the RNA is of good quality.

2.4.4. RNA quantification -Ribogreen™

Initially RNA quantification was performed using the RiboGreen™ quantification technique. The RiboGreen® reagent is non-fluorescent when free in solution. Upon binding RNA, the fluorescence of the RiboGreen® reagent increases more than 1000-fold. The RiboGreen™ method quantifies the concentration of multiple RNA samples by reference to a standard curve (1ng/ml – 1μg/ml). This method is faster than determining the absorbance of individual samples at 260nm (A_{260}) and avoids the disadvantages of the A_{260} method such as interference by contaminants (e.g. residual phenol and protein) commonly found in preparations.

A 1x Tris-EDTA (TE) buffer was prepared by diluting a 10x TE stock (100mM Tris pH8, 5mM EDTA). RNA for the standard curve was diluted 1:500 to a concentration of 100ng/ml and standards of 50, 40, 30, 20 and 10ng/ml were prepared. The RNA standard used was purchased from Invitrogen (Paisley U.K.) and came as the RNA control in the SuperScript kit. The RNA samples were diluted to a final concentration of 1:2000, 1:4000 and 1:8000 in **1x TE** buffer. 100μl sample/standard was added to the 96 well plate. Ribogreen reagent (Molecular probes, Eugene U.S.A.) was diluted 1:2000 and 100μl was added to

each well. The plate was incubated in the dark for 5 minutes and then read by the Cytofluor II microplate Fluorometer (Bioresearch, U.S.A.) at an excitation wavelength of 480nm and an emission wavelength of 520nm, gain 90, after shaking for 1 minute. RNA values were calculated in μg/ml by reference to the standard curve (see figure 2.3).

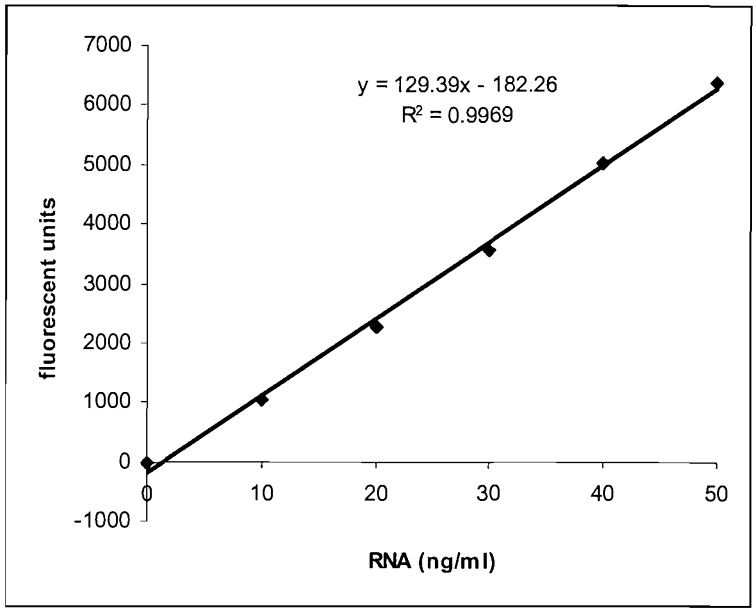


Figure 2.3: Representative RiboGreen standard curve

2.4.5. RNA quantification – agarose gel

In latter experiments it was deemed that RNA quantification and quality control could be performed simultaneously by running both the samples and a standard curve on an agarose gel, therefore preventing the need for performing a RiboGreen™ assay to quantify RNA. Standard fibroblast RNA was extracted, quantified and diluted to 0.2μg/μl. The standard curve was prepared by adding 1μl (0.2μg), 2μl (0.4μg), 3μl (0.6μg) and 4μl (0.8μg) of standard RNA to RNA loading buffer to give a final volume of 10μl in individual wells. The RNA was quantified using the GeneTools programme by assigning a track for each individual sample and reading the pixel intensity of bands for both the 18S and 28S bands together. A standard curve (see figure 2.4) was drawn in Excel from the standard pixel intensities and sample RNA values were calculated from the gradient and y-intercept.

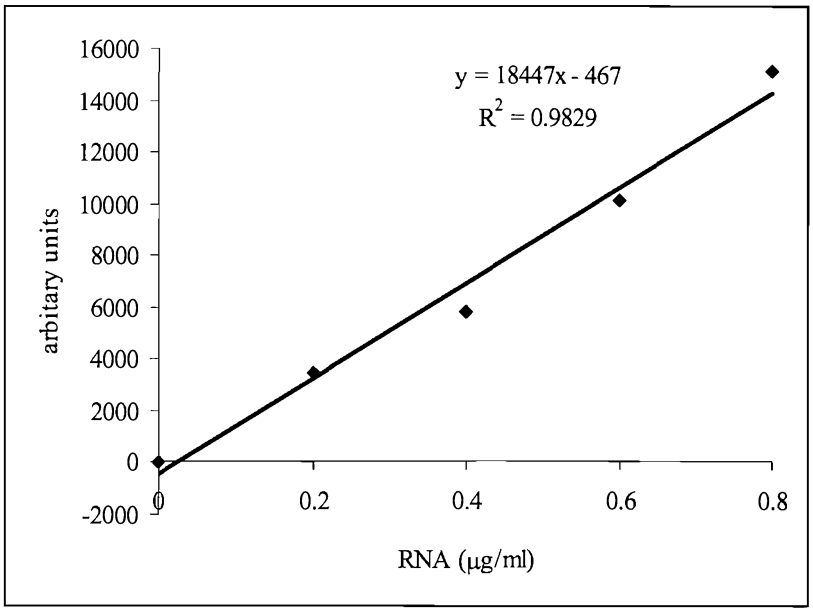


Figure 2.4: Representative RNA standard curve obtained from an agarose gel.

2.4.6. Reverse transcriptase (RT) reaction

The RNA extracted using the Trizol protocol was reverse transcribed to create cDNA. The cDNA was the template used for the Taqman PCR reactions. The reaction was carried out in sterile thin walled strips of 200µl microtubes. 1µg of RNA was incubated with 1µl random hexamer primers (3µg/ml) (MWG Biotech, Milton Keynes, U.K.) and 1µl dNTP (10mM) (Invitrogen). The final volume was made up to 10µl with distilled water. A negative RT control and negative template control were included where either no RNA template or no RT enzyme was added. The samples were incubated for 5 minutes at 85°C using the Eppendorf Mastercycler gradient (Brinkmann, Westbury, U.S.A.) to denature the RNA, ensuring it was single stranded.

The tubes were removed from the machine and placed in a dry ice-ethanol bath for five minutes. A RT super mix was prepared for all the tubes (the volume for 1 tube comprised 0.5µl M-MLV RT enzyme (Promega, Southampton, U.K.), 4µl 5x RT buffer (Promega, Southampton, U.K.) and 5.5µl dH_2O). 10µl RT super mix was added and then incubated for 1 hour at 42°C in a water bath. The RT samples were then diluted 1:10 in DNase free water and stored at –20°C.

2.4.7. Real time quantative PCR

Taqman probes are oligonucleotides (about 20-25 bases) containing a fluorogenic reporter dye FAM (6-carboxyfluorescein) and a quencher dye TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine). The probe is complementary to a target sequence of cDNA and binds to the region of interest, between the forward and reverse primers, during the annealing stage of PCR (see figure 2.5). During the cycle, the Taq polymerase will bind and extend the cDNA. The Taq polymerase enzyme has 3' exonuclease activity and when in contact with the probe will cleave the FAM from the probe. This causes an increase in fluorescent intensity of the reporter dye due to its physical separation from the quencher. The time taken for the fluorescence to reach the threshold (CT value) is indicative of the amount of cDNA added to the reaction mix. Therefore the lower the CT value, the more cDNA encoding the target gene present.

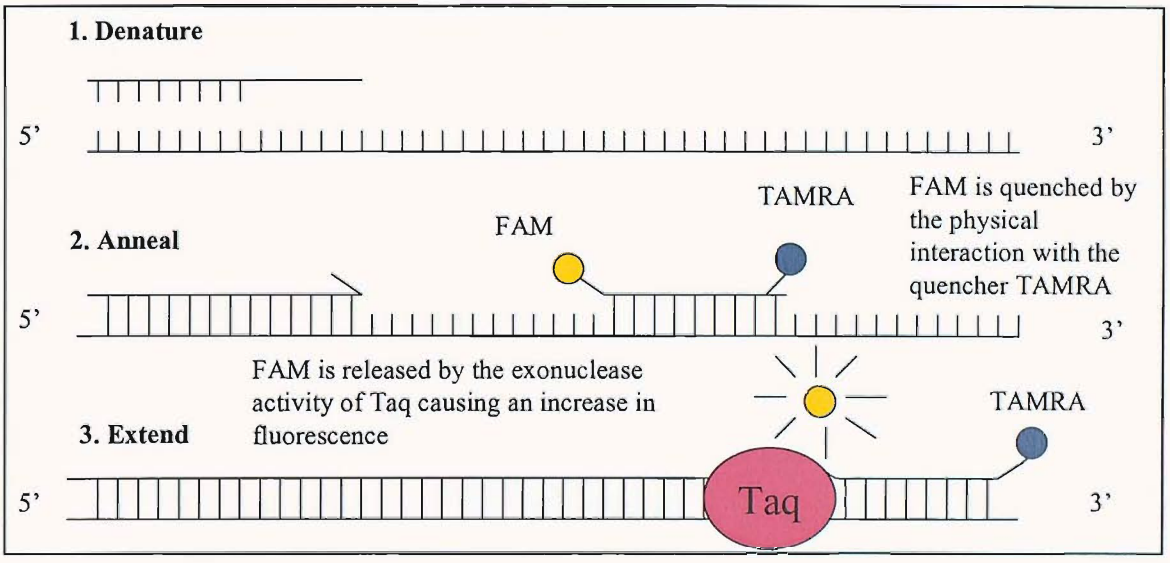


Figure 2.5: Schematic representation of probe activity during a Taqman PCR reaction

2.4.8. Primers and probes

The forward and reverse primers and probes were ordered from MWG biotech or Oswell and optimised according to the Perkin-Elmer protocol. Primers and probes

were designed by Dr R. Powell using either Primer Express (Perkin-Elmer Biosystems, Warrington, UK) or beacon Designer 2.1 (see table 2.4). The primers were designed to span two exons on the gene of interest on the cDNA. The primers and probes therefore cannot recognise any genomic DNA, only cDNA transcribed from the mRNA.

Target	Primers	Probe
TGFβRI	F 5' GCCACAACCGCACT GTCA3' R 5' TGAACAAGCAATGGTAAA CCTGAG 3'	FAM 5'CCATCGAGTGCC AAATGAAGAGGACCT 3' TAMRA
TGFβRII	F 5' CTGTGGCCGCTGCACAT 3' R 5' TTGTTGTCAGTGACTATCA TGTCGTTA 3'	FAM 5' CCTGTGGACGCG TATCGCCAGC 3' TAMRA
TGFβRIII	F 5' CCCCCAGAAGTTGCCT AAGTG 3' R 5' CATCATGGCCCAGATT ATCGA 3'	FAM 5' CTGACGAAGCCT GCACCTCGCTG 3' TAMRA
α smooth muscle actin	F 5' GACAGCTACGTGGGTG ACGAA 3' R 5' TTTTCCATGTCGTCCCA GTTG 3'	FAM 5' TGACCCTGAAGT ACCCGATAGAACATGGC T 3' TAMRA
EGFR (ErbB1)	F 5' GCGTCTCTTGCCGGAAT GT 3' R 5' TCCCTTGGCTCACCCTC C 3'	FAM 5' CCACGCATTCCCTGCCTC GG 3' TAMRA
TGFβ1	F 5' TGGACATCAACGGGTTC ACTA 3' R 5' AAGCAGGAAAGGCCG GTT 3'	FAM 5' CGAGGTGACCTGGCCACCA TTCATT 3' TAMRA
TGFβ2	F 5' GACCAACCGGCGGA AGA 3' R 5' CAGCAATTATCCTGCAC ATTTCTAA 3'	FAM 5' CAATAGGCCGCATCCAAA GCACGT 3' TAMRA

TGFβ3	F 5' CAAATTCAAAGG CGTGGACA 3' R 3' TTAGATGAGGGTTGT GGTGATCCT 3'	FAM 5' TCCACGGCCATGGTCATCC TCAT 3' TAMRA
CTGF	F 5' GCGGCTTACCGACTGG AA '3 R 5' GGACCAGGCAGTTG GCTGTA '3	FAM 5'CACGTTTGGCCCAGACCC AACTATGAT 3' TAMRA
Amphiregulin	F 5' GTGGTGCTGTCGCTCTTGA TAC 3' R 5'GCTTCCCAGAGTAGGT GTCATTG 3'	FAM 5' TCCAATCCAGCAGCATAAT GGCCTGA 3' TAMRA
HB-EGF	F 5' GATCTGGACCTTTTGAG AGTCACTT 3' R 5' TCCCGTGCTCCTCCTTG TT 3'	FAM 5' AGCCACAAGCACGGGCCA CACCA 3' TAMRA
ADAM 12	F 5' AGCTATGTCCTAGAACC AATGAAAAGTG 3' R 5' CCCCGGACGCTTTTCAG 3'	FAM 5'ACCAACAGATACAA ACTCTTCCCAGCGAAGAT 3' TAMRA
ADAM 17	F 5' GAAGTCCCAGGAGGC GAT T 3' R 5' TCCTTACACTTGCCAAGA TCCA 3'	FAM 5' CACAGGTAATAGCAGTGA GTGCCCGCCT 3'TAMRA
TIMP3	F 5' GACATGCTCTCCAATTTC GGTTA 3' R 5' GTCTGTGGCATTGATG ATGCTT 3'	FAM 5' CCTGGCTACCAGTCCAAAC ACTACGCCT 3' TAMRA

Table 2.4: Sequences of Primers and probes used

2.4.9. Melt curves

A melt curve was performed on every primer set to determine whether the primers were specific to the target sequence. A melt curve plots the change in fluorescence

against temperature after addition of SYBR green to the PCR mix. SYBR green is a dye that fluoresces when bound to double stranded DNA. The change in fluorescence is therefore highest at the temperature where the double stranded PCR products melt. The presence of additional lower melting point peaks usually indicates primer dimers. Primer dimer peaks are often wider than the target peak since different combinations of primer/primer interactions can result in a variety of short products with slightly different melting points. Primer dimer formation is favoured at high concentrations of primer and but is also encouraged by poor primer design. Primer dimer formation should be avoided as it decreases the accuracy between duplicates, uses up limited PCR reagents and reduces the priming efficiency of the target template.

SYBR Green reactions:

6.5µl 2x qPCR Master mix

0.06µl SYBR Green (Eurogentec, Seraing, Belgium)

0.12µl Fluorescein (Bio-Rad Laboratories, Hercules, California)

1µl Primer mix (15pmol Forward and Reverse primers)

Template 5µl (1:10 of RT)

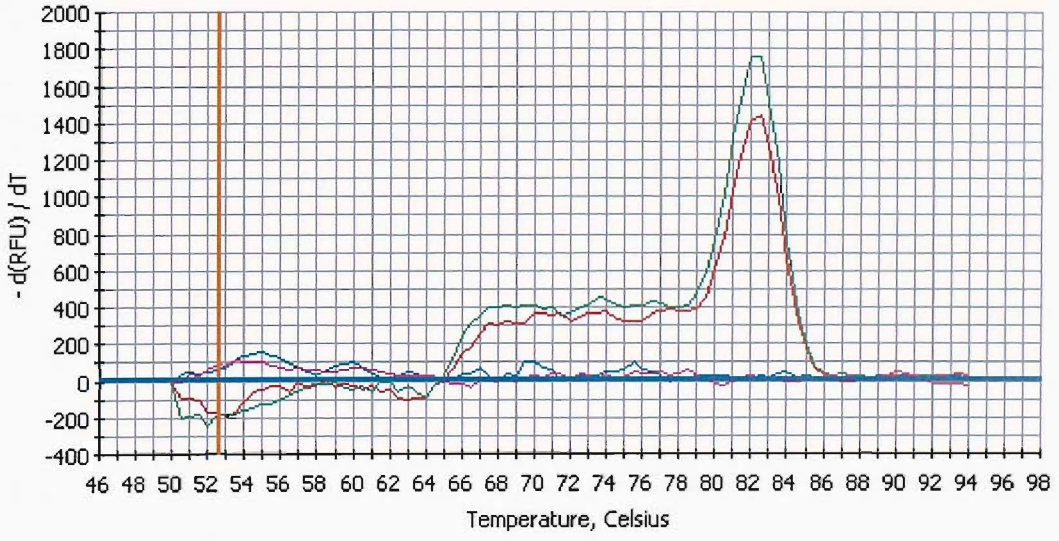


Figure 2.6: Representative melt curve for TGFβ2

The green and red lines represent the melt curve from one sample in duplicate. The blue and pink lines are the negative control.

2.4.10. Taqman PCR reaction

Taqman PCR reactions were carried out using two different protocols. Initially Taqman reactions were carried out on the ABI Prism 7700 sequence detection Taqman machine (Applied Biosystems, Cheshire U.K.) using a final reaction volume of 25µl. Genes were measured by a multiplex reaction using the probe of interest combined with a probe for 18S ribosomal RNA (rRNA). As two probes were used, the emission wavelengths had to be far enough apart to allow their individual detection. The FAM label on the target probe and the VIC label on the 18S rRNA probe allowed for this difference. Quantitation was achieved by running a standard curve on each plate, created using the samples being run on that plate. Due to the purchase of a new Bio-Rad Icycler Taqman machine (Bio-Rad Laboratories Ltd, Hertfordshire, U.K.), the protocol was modified to increase both the cost efficiency and replication efficiency.

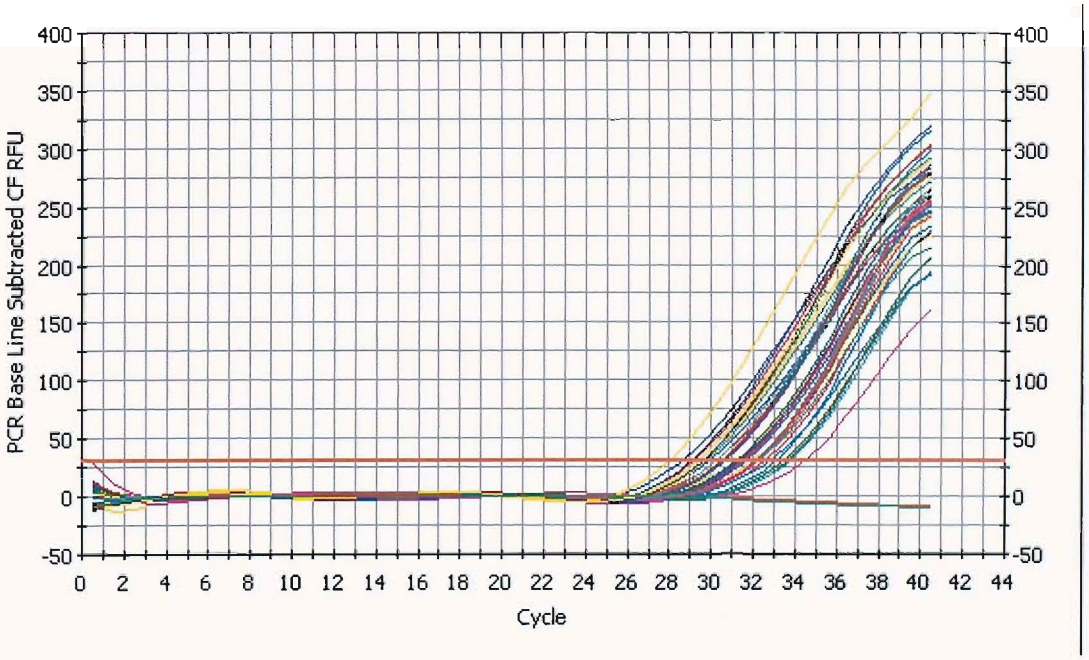


Figure 2.7: Typical trace from Icycler showing CT values for TGFβ RII

The orange horizontal line indicates the point at which threshold is reached. The point where each sample crosses the threshold is termed its CT value. The plot also shows two RT negative controls which have not amplified (orange and green lines).

2.4.11. ABI Prism 7700 protocol

The PCR reaction was carried out in sterile thin walled strips of 200µl microtubes. 5µl of diluted cDNA template was added to 20µl reaction mix (19µl Taqman Master mix and 1µl target primer/probe mix).
The Taqman Master mix was prepared as follows (volumes indicated are per tube):
2.5µl 10x real time PCR buffer (no $MgCl_2$) (Eurogentec, Seraing, Belgium)
5µl $MgCl_2$ (6.6mM) (Eurogentec, Seraing, Belgium)
2µl dNTP (0.26mM) (Eurogentec, Seraing, Belgium)
0.125µl Hot Goldstar Taq enzyme (0.625units) (Eurogentec, Seraing, Belgium)
9µl dH_2O
0.375µl 20x ribosomal 18S primers and probe mix (Applied Biosystems, Foster City, U.S.A.).

Primer and probe mix was made as follows:
22.5µl of forward primer (100µM) was added to 22.5µl reverse primer (100µM) with a volume of probe to give a final concentration of 6.25pmol/µl. The primer/probe mix was made up to 100µl with dH_2O. 1µl of this stock gave a final concentration of 250nM probe and 900nM primer.

Samples were run in triplicate. A standard curve was created by pooling equal volumes of template from each of the tubes being measured and double diluting the template in dH_2O to achieve a six point standard curve in duplicate. A No Template control (5µl dH_2O) and negative RT controls were also included on each plate.

The Taqman reaction was performed on an ABI Prism 7700 sequence detection system. The Taq polymerase was activated on heating to 95°C for 10 minutes followed by a 50°C incubation for 2 minutes. The amplification consisted of 40 cycles of denaturation at 95°C for 15 seconds followed by an annealing/extension phase for 1 minute at 60°C.

The ABI Prism 7700 Sequence software plotted an emission intensity verses time (cycle number) graph. The computer programme set a threshold for both the FAM and VIC graphs from which CT values were automatically generated. Standard curves (fig. 2.8) were constructed for both the FAM and VIC probes using MS Excel. The top standard (undiluted pooled sample) was given a nominal value of 1 and then logged.

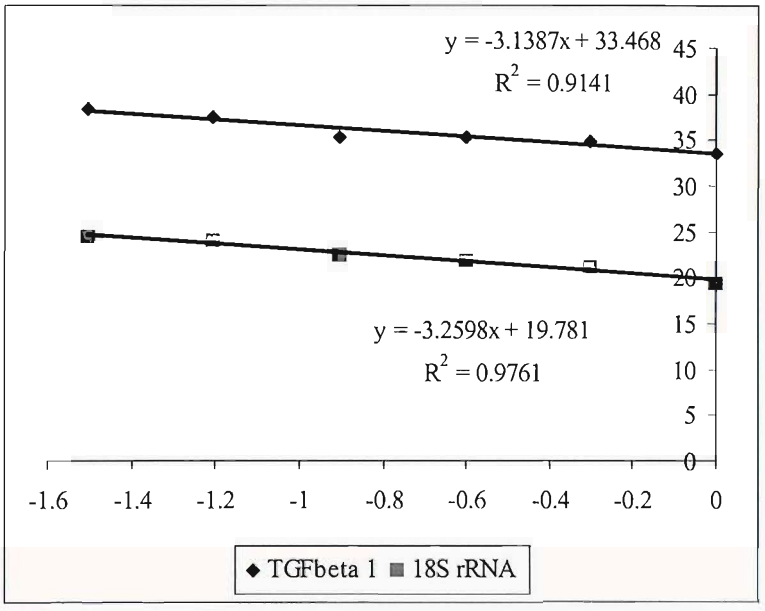


Figure 2.8: Representative Taqman PCR Standard Curve for TGFβI and 18s rRNA

The amount of cDNA present in each sample was determined from the VIC CT value of the sample, calculated from the ABI Prism 7700 Sequence software programme, using the VIC equation (y-c)/m from the plot of the standard curve. The amount of target cDNA (i.e. the FAM signal) was also calculated using the FAM gradient. FAM and VIC values were then both antilogged. Normalised values were determined by dividing the FAM value by the VIC value. The duplicates or triplicates were then averaged to obtain the amount of target gene relative to the endogenous control.

2.4.12. Biorad Icycler – Modifications to ABI Prism 7700 protocol

Optimisation experiments on the new Icycler (Bio-Rad, Cheshire, U.K.) showed that lower concentrations of primer and probe could be used, i.e. 15pmol primer rather than 22.5pmol and 3.1pmol probe rather than 6.3pmol. The replicates were improved on the new machine, allowing samples to be run in duplicate.

The final volume of the reaction mix was reduced from 25µl to 12.5µl, with no reduction in sensitivity or accuracy. The reaction mix was therefore changed to 2x 6.5µl Taqman master mix, 1µl primer and probe mix and 5µl sample. The reactions were run in ABgene Thermo-Fast 96 Non-skirted plates (Epsom, U.K.) instead of the 8 well strips and were covered by Bio-Rad Icycler iQ optical tape. Taqman PCR reactions were not multiplexed on the Icycler, although 18S rRNA remained the internal control and was measured in a sample set prior to measuring the gene of interest. The PCR cycling conditions were the same as those used for the ABI Prism 7700 apart from the 2 minute incubation at 50°C at the start of the 40 cycles which was removed. Quantitation, and real-time detection of the PCR products were followed on the IcyclerIQ real time detection system.

2.4.13. Real-time Quantitative PCR analysis

Relative expression levels for genes measured on the Icycler were calculated using the ΔΔCT calculation as previously described (user bulletin 2 ABI 7700 Sequence Detection System, http://docs.appliedbiosystems.com/search.taf). It was previously demonstrated by Dr Rob Powell that the assays used in these calculations produced a very similar gradient on a standard curve indicating that they have similar amplification efficiencies. The principle underlying the calculation is based on the assumption that a decrease in the delta CT by 1 unit equals a doubling of target gene.

Both target and 18S rRNA gene expression was measured in duplicate. The target gene CT value was subtracted from the averaged 18S rRNA CT value obtained for that sample. The lowest "target gene-18S rRNA value" for a control was used to

normalise all other samples in the data set to it. This was achieved by subtracting that number from all the other sample "target gene-18S rRNA values". A ΔΔCT value was obtained for each sample by applying the equation $2^{(-x)}$. The duplicates were then averaged and samples could be directly compared to one another to demonstrate alterations in gene expression upon treatment.

2.5. ELISA protocols

The enzyme linked immunosorbant assay (ELISA) protocol is a sensitive technique for measuring either antigen or antibody in a sample, e.g. conditioned media. The ELISAs used in the current study are indirect sandwich ELISAs. An antibody raised against a specific antigen e.g. amphiregulin is coated overnight onto a polystyrene 96 well microtitre plate. The microtitre plate was washed and blocked with buffer containing BSA, which prevents non-specific binding. Samples and standards were added to the microtitre plate and incubated for 1-2 hours to allow attachment of the antigen to the bound antibody. A second detection antibody, raised against a different epitope of the antigen, was then added to the microtitre plate. The biotinylated detection antibody allows subsequent binding of a streptavidin-horse radish peroxidase (SA-HRP) conjugate. Adding a substrate (i.e. H_20_2 with TMB as chromagen) leads to colour production in proportion to the amount of antigen present in the samples. The reaction is stopped by the addition of acid and the colour intensity of the individual wells is determined by a spectrophotometer reading at 450nm. The amount of antigen present in a given sample can then be calculated from the standard curve.

2.5.1. ELISA buffers and reagents

The buffers and reagents specified below were used for the amphiregulin and HB-EGF ELISAs. All reagents used in the buffer preparation were purchased from Sigma-Aldrich (Poole, U.K.).

Coupling buffer

Na_2CO_3 (1.59g/l), $NaHCO_3$ (2.93g/l) and NaN_3 (0.2g/l) was dissolved in distilled water. The pH was corrected to 9.6 and the buffer stored at room temperature.

10x blocking buffer

NaCl (80g/l), $Na_2HPO_4.2H_2O$ (14.2g/l), KH_2PO_4 (2g/l), KCl (2g/l) and BSA Fraction V (50g/l) was dissolved in dH_2O and the pH corrected to pH7.4. The buffer was stored at $-20^{\circ}C$ and diluted to a working concentration of 1x with distilled water on the day of use.

Assay buffer

1x blocking buffer/ Tween 20 (0.1%)

10x PBS

Na_2HPO_4 (12.8g/l), NaCl (85g/l) and $NaH_2PO_4.2H_2O$ (1.56g/l) was dissolved in distilled water and the pH corrected to 7.4. The buffer was stored at room temperature and diluted to 1x before use.

Wash buffer

1x PBS /Tween 20 (0.05%)

Chromagen diluent

13.6g sodium acetate.$3H_2O$ was dissolved in distilled water. The pH was corrected to 6.0 with 0.1M citric acid and volume made up to 1 litre.

TMB stock solution

30mg tetra-methyl benzidine (TMB) was dissolved in 5ml dimethyl sulphoxide (DMSO). The stock solution was stored protected from light at room temperature and was stable for up to 1 month

Chromagen solution

12ml chromagen diluent, 200µl Tetramethylbenzidine (TMB) stock solution and 1.2µl 30% H_2O_2 was freshly prepared prior to use.

2.5.2. Amphiregulin ELISA

Nunc Maxisorp plates (Nalgene-Nunc, Herefordshire, U.K.) were incubated overnight at 4°C with 100µl capture antibody (R&D, MAB262) diluted in coupling buffer to 2µg/ml. The capture antibody was then removed and the plates washed four times with wash buffer. The plates were blocked for 1 hour at room temperature with 1x blocking buffer. An amphiregulin standard (262-AR purchased from R&D systems, Minneapolis, U.S.A.) curve (2ng/ml – 0.032ng/ml) was prepared using serial dilutions and a blank consisting of assay buffer alone was

added to each plate. Cell culture media from treated fibroblasts (100µl) was added undiluted to the ELISA plate. An Ultraculture blank was added to the plate, no amphiregulin was detected in the media prior to addition to the cells. Both standard and samples were incubated for 2 hours at room temperature.

The plate was washed four times with PBS/Tween 20 (0.05%). Biotinylated detection antibody (BAF262 purchased from R&D systems, Minneapolis, U.S.A.) was diluted to 150ng/ml and incubated on the plate (100µl/well) for 2 hours at room temperature. The plate was washed a further four times. SA-HRP conjugate (Amersham Biosciences UK Ltd, Buckinghamshire, U.K.) diluted 1:1000 in assay buffer was added to the plate (100µl/well) and incubated for 30 minutes at room temperature. The plate was washed and 100µl chromagen solution was added and incubated in the dark. The reaction was stopped after 30 minutes by the addition of 50µl $2H_2SO_4$. The plate was read on a Labsystems Multiskan Ascent (Thermo, Basingstoke, U.K.) spectrophotometer at 450nm and sample concentrations were calculated by reference to the standard curve (figure 2.9) after a blank subtraction.

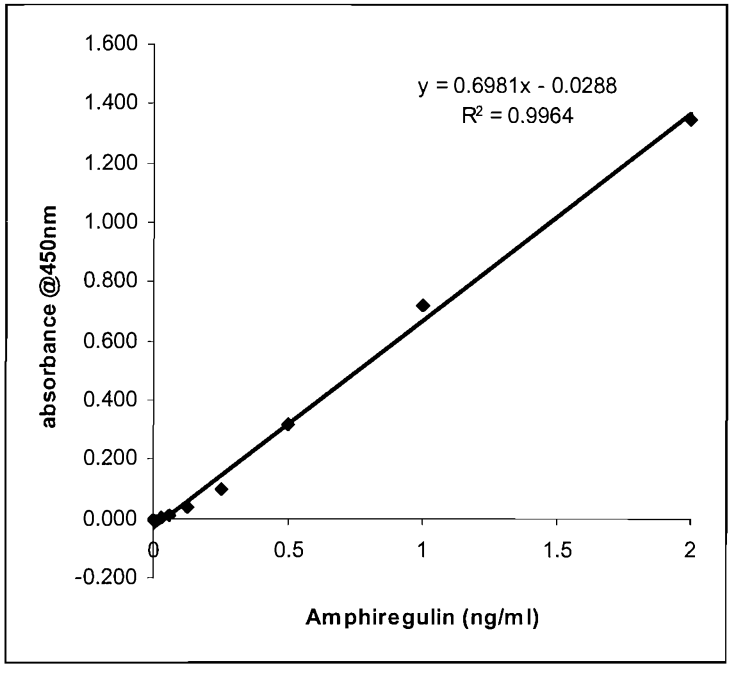


Figure 2.9: Representative Standard curve for amphiregulin ELISA

2.5.3. Design of HB-EGF ELISA

There were no commercially available ELISAs on the market to measure HB-EGF. As a result, an HB-EGF ELISA was developed using a matched pair of antibodies against HB-EGF (MAB 259, BAF 259) from R&D Systems (Minneapolis, U.S.A). MAB 259 is a monoclonal antibody raised against recombinant sf21-derived HB-EGF. The company suggested that the antibody could detect as low as 2.5ng/ml using a direct ELISA approach. BAF259 is a biotinylated goat polyclonal antibody, also raised against recombinant sf21-derived HB-EGF.

A sandwich ELISA was developed, with MAB 259 used as the capture antibody and BAF 259 as the detection antibody. The signal was amplified using streptavidin-HRP, with TMB used as a colorimetric endpoint. Initial studies showed that the sensitivity of the assay was improved using increasing concentrations of capture and detection antibody (fig. 2.10). Capture antibody was shown to give the best signal at 1μg/ml (fig. 2.10) and detection antibody at 800ng/ml (fig. 2.11). Streptavidin-HRP conjugate (Amersham Biosciences) was shown to slightly improve the signal in comparison to streptavidin-HRP complex and therefore was used henceforth (fig. 2.11).

The specificity of the ELISA was determined by assaying amphiregulin and EGF. Neither growth factor gave a positive signal (results not shown), demonstrating that the antibodies showed no cross reactivity with other EGFR family ligands. Using the optimised ELISA protocol, the sensitivity of the ELISA was 4pg/ml (fig. 2.12). This was 600 times more sensitive than that suggested by R&D systems.

2.5.4. HB-EGF ELISA

HB-EGF was detected in conditioned media after prior treatment with 10ng/ml TGFβ2 (Peprotech, London, U.K.) for 24 hours followed by a 6 hour incubation with 10nM PMA (Promega, Southampton, U.K.), 100μg/ml hexadimethrine bromide (Sigma-Aldrich, Poole, U.K.) and αEGFR antibody diluted to 10μg/ml (In house).

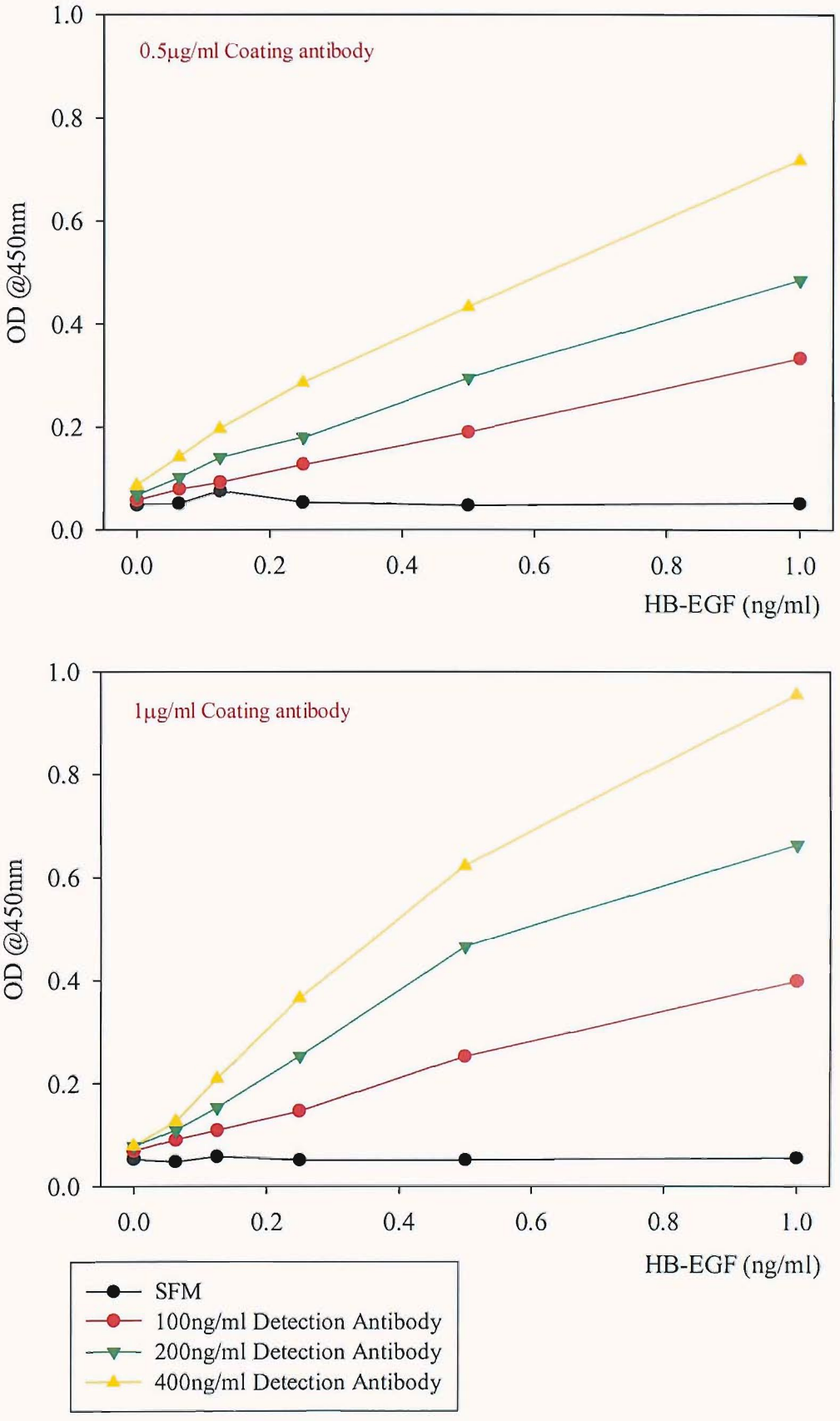
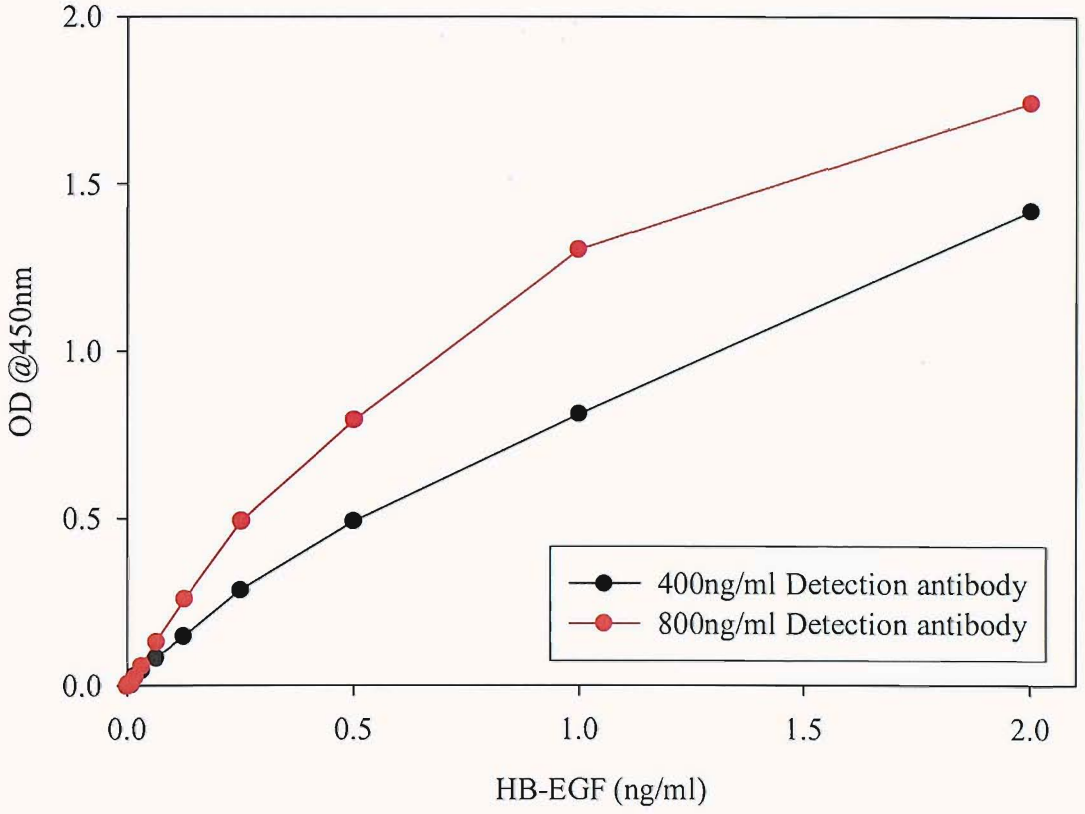


Figure 2.10: Optimisation of antibody concentration for HB-EGF ELISA

Coating (MAB 259) and detection (BAF 259) antibody were used at a range of concentrations to determine the optimum signal for HB-EGF detection in an ELISA format.



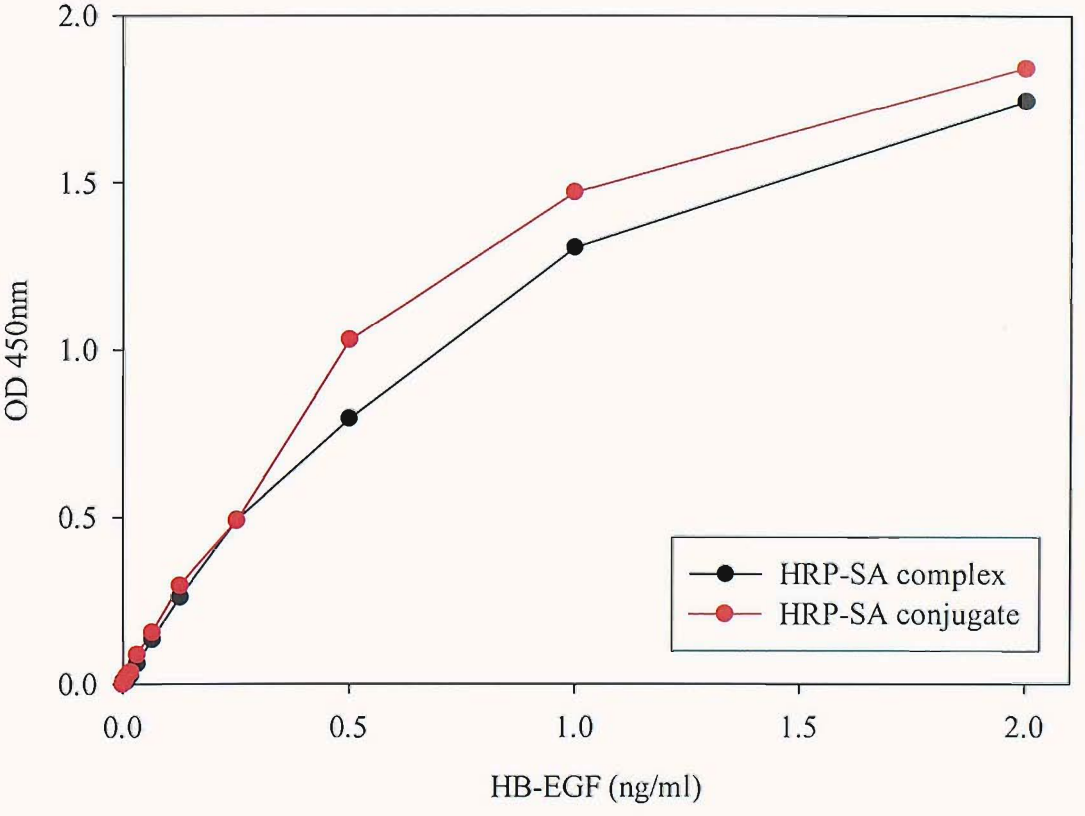


Figure 2.11: Optimisation of HB-EGF ELISA

In determining the optimal conditions for detection of HB-EGF, detection antibody concentration was further titrated and two different signal amplification solutions were tested. Further studies were performed using 800ng/ml of detection antibody and HRP-SA conjugate (Amersham Biosciences) at a 1:1000 dilution.

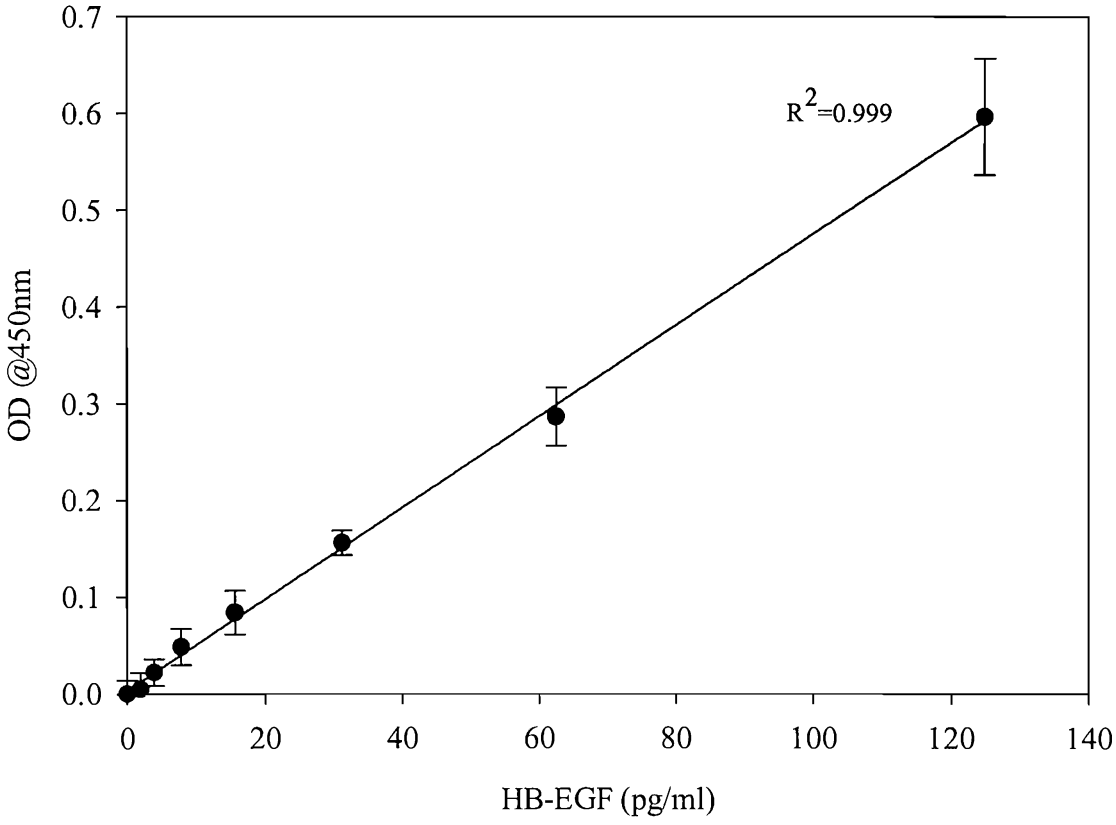


Figure 2.12: Standard Curve for Optimised HB-EGF ELISA

The error bars represent the standard deviation of the intra-assay variation (n=6) with the an R^2 value of 0.999. The standard used to consruct the standard curve was recombinant HB-EGF purchased from R&D systems (cat. no. 259-HE).

Anti human HB-EGF antibody (MAB259 purchased from R&D systems, Minneapolis, U.S.A) was diluted to 1μg/ml in coupling buffer. 100μl was added per well of a 96 well Nunc Maxisorp plate (Nalgene-Nunc, Herefordshire, U.K.) and incubated overnight at 4°C without shaking. The coating buffer was flicked out of the plate and the plate was washed four times with PBS/Tween 20 (0.05%). 300μl/well of 1x blocking buffer was placed into the wells and incubated for 1 hour at room temperature. The plate was washed four times with PBS/Tween 20 (0.05%).

The standard curve was prepared which ranged from 125pg/ml to 2pg/ml HB-EGF. 100μl standard/undiluted sample was added to the 96 well plate and incubated for 2 hours. The plate was washed four times. The detection antibody (BAF259 purchased from R&D systems, Minneapolis, U.S.A) was diluted to 800ng/ml and incubated for 2 hours at room temperature with shaking. The plate was washed a further four times. 100 μl of streptavidin-HRP conjugate (Amersham Biosciences UK Ltd, Buckinghamshire, U.K.) diluted 1/1000 in assay buffer was added to each well and incubated for 30 minutes at room temperature. The plate was washed four times with PBS/Tween 20 (0.05%). 100 μl/well of chromagen solution was added and incubated for 30 minutes with occasional shaking in the dark. To stop the reaction, 50 μl of 2M H_2SO_4 was added to each well. The plate was read on a Labsystems Multiskan Ascent spectrophotometer at 450nm and sample concentrations were calculated by reference to the standard curve.

2.5.5. TGFβ1 ELISA

The TGFβ ELISA allowed quantitation of total (active and latent TGFβ) and active TGFβ1 (non-acid treated) in conditioned medium. The ELISA kit (Emax ImmunoAssay System TGFβ1, cat. no. G7591) was ordered from Promega (Southampton, U.K.) and the protocol was followed according to manufacturer's instructions. The ELISA is specific for the measurement of TGFβ1, there is typically less than or equal to 3% cross reactivity with TGFβ2 or 3 at 10ng/ml.

Latent TGFβ in samples was activated by acidification to give a measure of total TGFβ present. 3µl 1M HCl was added to 150µl media and incubated for 15 minutes at room temperature. The sample was then neutralised by the addition of 3µl 1M NaOH. The samples were diluted 1:4 in sample buffer and stored at –20°C. Active TGFβ samples were not acidified.

TGFβ monoclonal antibody was diluted 1:1000 in carbonate coating buffer (0.025M sodium bicarbonate, 0.025M sodium carbonate, pH9.7). 100µl was added per well of a 96 well falcon plate (BD Bioscience, New Jersey, U.S.A.) and incubated overnight at 4°C without shaking. The coating buffer was flicked out of the plate and 270µl/well blocking buffer (diluted 1:5 in distilled water) was placed into the wells followed by an incubation at 37°C for 35 minutes. The plate was washed five times with TBST wash buffer (20mM Tris-HCl pH7.6, 150mM NaCl and 0.05% (v/v) Tween®20). The TGFβ1 standard was diluted 1:1000 with sample buffer. 200µl was added to two wells of the ELISA plate and then doubling serial dilutions were made down the plate. A blank comprising 100µl sample buffer was added to each plate. Samples (total TGFβ1 samples previously diluted 1:4, active TGFβ1 samples added neat) were added in duplicate or triplicate to the plate. The plate was incubated with shaking for 1 hour and 30 minutes at room temperature. The plate was then washed 5 times as before. The anti-TGFβ1 polyclonal secondary antibody was diluted 1:1000 in sample buffer. 100µl of antibody was added/well. The plate was incubated for 2 hours with shaking at room temperature. The plate was washed five times as before. The TGFβ-HRP conjugate was diluted 1:100 in sample buffer and incubated with shaking for 2 hours at room temperature. The plate was washed five times as before.

During the above TGFβ HRP conjugate incubation, the TMB One Solution was equilibrated to room temperature. 100µl was added/well and the plate incubated for 15 minutes at room temperature. The reaction was stopped by the addition of 100µl 1N HCl. The absorbance of the samples was determined using the Labsystems Multiskan Ascent at a wavelength of 450nm and the concentration of TGFβ in samples determined by reference to the standard curve (fig. 2.13).

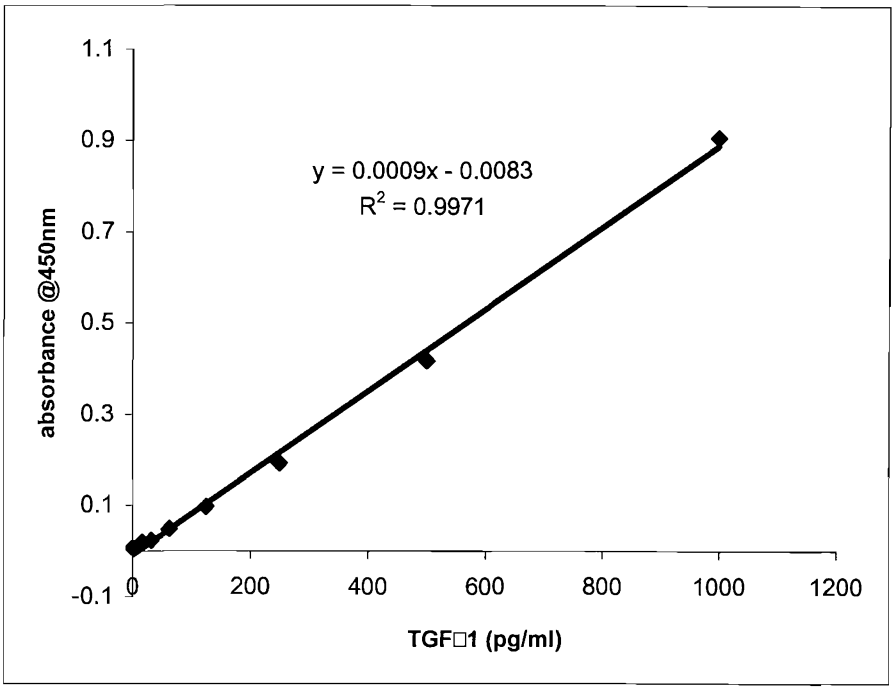


Figure 2.13: Representative standard curve for TGFβ1 ELISA

2.6. Immunofluorescent Techniques

Antibodies conjugated directly or indirectly to a fluorochrome can be utilised to detect antigens expressed either on the cell surface of, or within permeabilised fibroblasts. Upon ultraviolet light absorption, the fluorochrome emits its own light at a longer wavelength (fluorescence) and thus allows localization of antibody-antigen complexes. Immunofluorescent staining of fixed fibroblasts allows the visualisation of the antigen, showing its cellular location and physical appearance. Semi quantitation can be performed on the samples although it is a subjective approach. Flow cytometry is a more accurate measure of changes in the actual amount of protein present in the cell.

2.6.1. Immunofluorescent staining of fixed fibroblasts

All reagents used in the buffer preparation were purchased from Sigma-Aldrich (Poole, U.K.) unless otherwise specified.

10x PBS

Na_2HPO_4 (12.8g/l), NaCl (85g/l) and $NaH_2PO_4.2H_2O$ (1.56g/l) was dissolved in distilled water and the pH corrected to 7.4. The buffer was stored at room temperature and diluted to 1x before use.

Blocking buffer

10ml DMEM (Invitrogen, Paisley, U.K.) supplemented with 10% FBS (Invitrogen, Paisley, U.K.) and 1% BSA.

Mowiol

20g mowiol was dissolved in 80ml 1x PBS and heated at 60°C until dissolved. 40ml of glycerol was added to the mowiol, which was then mixed for several hours. Aliquots were stored at –20°C until required.

Mowiol/DABCO

On the day of staining, 0.25g DABCO was added to 10ml Mowiol.

Fibroblasts were grown on 8 well chamber slides (LabTech II, Fisher Scientific UK Ltd, Leicestershire, U.K.) and fixed with cold methanol for 15 minutes at –20°C. Slides were stored dry at –20°C until required.

Immunofluorescent staining was performed at room temperature in the dark. The fixed fibroblasts were washed with PBS and then blocked with DMEM (10% FBS, 1% BSA) for 1 hour. The slides were washed with PBS (4x2 minutes). The fibroblasts were then incubated with 4.4μg/ml αSMA antibody (A2547, Sigma) for 2 hours; a mouse IgG_{2a} isotype control at the same concentration was used to test for non-specific staining. After 2 hours the fibroblasts were washed with PBS (4x2 minutes). Rabbit α mouse FITC (F0261, DAKO, Cambridgeshire, U.K.) labelled secondary antibody was diluted 1:100 to 10μg/ml and incubated for 1 hour. The slides were washed again 4 times with PBS. The nuclei were then counterstained with 7-aminoactinomycin D (7-AAD, Sigma-Aldrich, Poole, U.K.) for 5 minutes at a concentration of 12.5μg/ml in PBS. The fibroblasts were washed (PBS, 4 times) and the slides mounted with Mowiol/DABCO (Sigma-Aldrich, Poole, U.K.) under a cover slip. The mounted slides were examined under a Leica DMRBE fluorescent microscope (Milton Keynes, U.K.). Images were captured with a Hamamatsu camera (Hamamatsu photonics UK limited, Hertfordshire, U.K.) using the Wasabi analysis programme (Hamamatsu photonics UK limited, Hertfordshire, U.K.).

2.6.2. Flow Cytometry – αSMA expression

Flow cytometers use the principle of hydrodynamic focusing for presenting cells to a laser in a single cell suspension. As each cell intercepts the laser it scatters light. Light that is scattered in the forward direction is proportional to the size of the cell. Light can also enter the cell and be reflected and refracted by the nucleus and other contents of the cell, thus the side scatter is proportional to the granularity of the cell. The light source can also excite any fluorochromes (e.g. FITC) attached to the cells to a higher energy state. The energy is released as a photon of light with specific spectral properties unique to different fluorochromes. Scattered and emitted light from cells and particles are then converted to electrical pulses by optical detectors, most commonly a photomultiplier tube. The electrical pulses are

then processed by a series of linear and log amplifiers. Logarithmic amplification is most often used to measure fluorescence in cells as this type of amplification expands the scale for weak signals and compresses the scale for "strong" or specific fluorescence signals. The signals are processed by an Analog to Digital Converter (ADC) which in turn allows for events to be plotted on a graphical scale.

Fibroblasts were seeded at 5 x 10^4 cells per well in a 24 well Nunc plate. The cells were serum starved after 24 hours in culture for a further 24 hours. Fibroblasts were then treated in duplicate with 0.4nM TGFβ2, 0.04nM TGFβ2 or SFM alone for 24 hours. The staining and flow cytometric analysis of αSMA was performed with the help and expertise of Dr Fabio Bucchieri. Cells were washed with HBSS and then detached with trypsin. The cell solution was neutralised with DMEM containing 10% FBS. The neutralised cell suspension was centrifuged for 5 minutes at 150g at 4°C in FACS tubes. The cells were washed twice with 1ml PBS and then fixed with ice cold methanol for 15 minutes. Following fixation the cells were washed twice with PBS to remove the methanol and then incubated with either a monoclonal αSMA antibody conjugated with FITC (F3777 purchased from Sigma-Aldrich, Poole, U.K.) diluted 1:100 in PBS (1% BSA, 0.1% Tween 20) or isotype control (IgG2a) for 30 minutes. The stained fibroblasts were washed twice and then re-suspended in 300μl PBS. A FACScan (Beckton Dickinson UK Ltd, Oxfordshire, U.K.) was used to perform flow cytometric analysis on the stained fibroblasts using the settings shown in table 2.5. Flow cytometry data was analysed using WinMDI 2.8.

Forward Scatter (FSC)	E-1	Amp 4.90	Lin
Side Scatter (SSC)	240	1	Lin
FL1	490		Log

Table 2.5: FACScan settings used to measure αSMA expression

For each sample, 10,000 events were collected. The cells were gated to remove the influence of cell debris (the events recorded in the bottom left hand side of the scatter plots in figure 2.14) as well as minimalising the effect of autofluorescence

(figure 2.15). αSMA antibody specificity was demonstrated by comparing the events collected from antibody stained cells to those collected with an isotype control (figure 2.16). The antibody stained cells showed a shift to the right, indicating an increase in fluorescence.

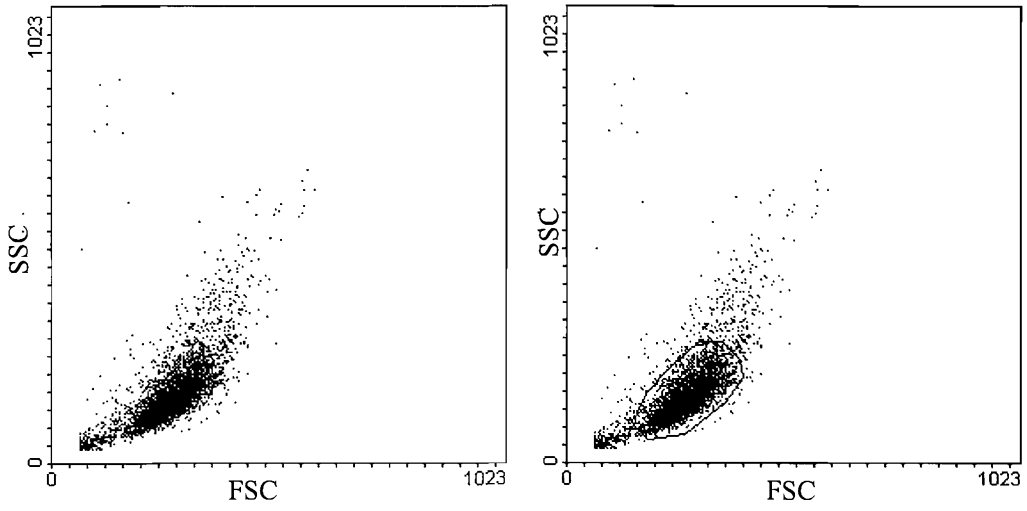


Figure 2.14: Representative scatter plot showing fibroblasts gated according to size

The y axis (side scatter) represents the size of the cells, the higher the value, the larger the cell. The x axis (forward scatter) represents the granularity of the cells. The right hand plot shows the cells that have been gated for analysis, i.e. the cells inside of the black polygon.

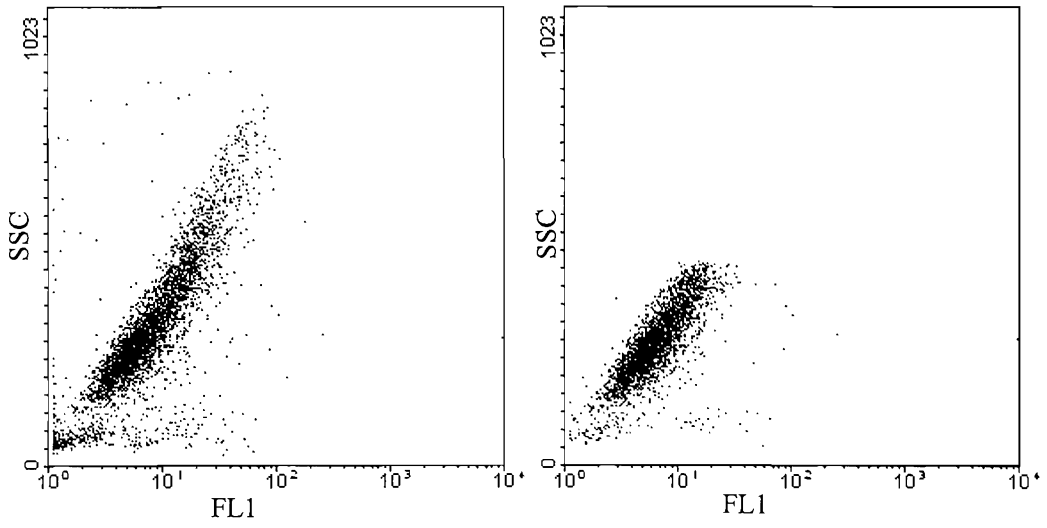
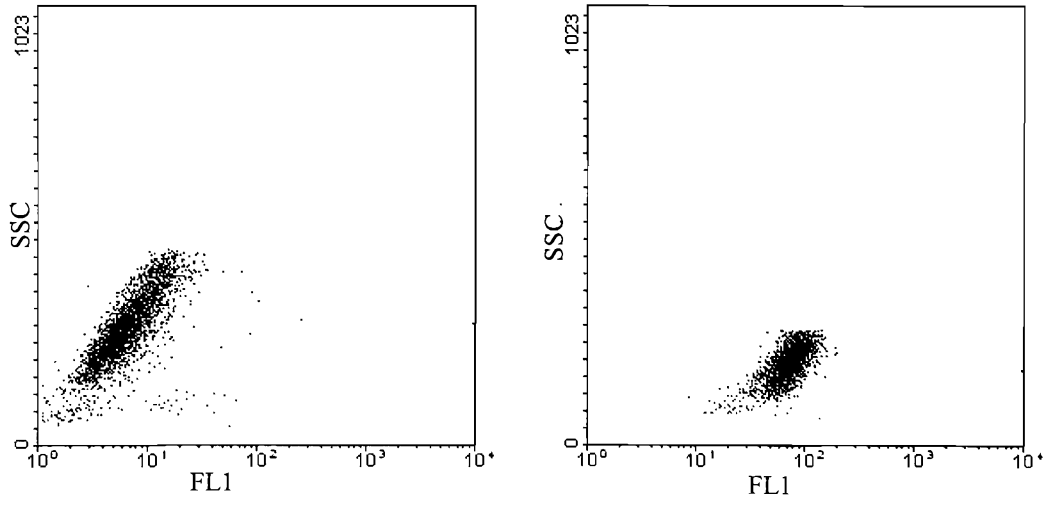


Figure 2.15: Autofluorescence is removed by gating isotype control fibroblasts according to size.

Fibroblasts were stained with the isotype control (IgG2a) for αSMA antibody. The fluorescence intensity for FITC (FL1, x axis) was plotted against the granularity of the cells (side scatter). The effect of gating (right plot) removes any autofluorescence produced by the cells (events in the top right hand corner of the left plot).



Isotype control for αSMA antibody

αSMA antibody

Figure 2.16: Representative scatter plot showing αSMA specific staining. Plotting the fluorescence intensity for FITC (FL1, x axis) against the granularity of the cells (side scatter) enables differences in αSMA expression to be revealed. The scatter plot shows that TGFβ2 treated fibroblasts stained with the antibody show a shift to the right compared to the isotype control.

2.6.3. Flow Cytometry –TGFβ receptors

Fibroblasts were seeded at 5 x 10^4 cells per well in a 24 well Nunc plate. The cells were serum starved after 24 hours in culture for a further 24 hours. Fibroblasts were the treated in triplicate +/- 0.4nM TGFβ2 for 24 hours. Cell were washed with HBSS and detached with trypsin. The cell solution was neutralised with DMEM containing 10% FBS. Staining of the primary fibroblasts was performed as recommended in R&D Systems biotinylated rhTGFβ1 Fluorokine™ kit (Cat. No. NFTG0). Staining was carried out on ice to prevent receptor internalisation.

For each subject studied there were 2 TGFβ2 treated samples, 2 serum free medium (SFM) controls, 1 TGFβ2 treated negative control and 1 SFM negative control. The neutralised cell suspension was centrigued for 5 minutes at 150g at 4°C in FACS tubes. The cells were washed twice with 1ml PBS and then incubated with

10µl biotinylated TGFβ1 (3µg/ml) or negative control supplied with the kit (soyabean trypsin inhibitor (5µg/ml) biotinylated to the same degree as the cytokine).

A specificity control was also supplied with the kit. 20µl of polyclonal chicken anti-human TGFβ1 blocking antibody (2mg/ml) was mixed with TGFβ1-biotin for 15 minutes at room temperature. In a separate tube 1 x 10^5 cells were pretreated with purified mouse IgG for 15 minutes at room temperature to block Fc-mediated reactions.

The negative control, specificity control and samples were all incubated for 1 hour at 4°C. After 1 hour, 10µl of avidin-FITC was added to each tube and then incubated at 4°C in the dark for 30 minutes. The cells were then washed twice with 2ml RDF1 wash buffer (supplied with the kit) and then re-suspended in 200µl of RDF1 wash buffer.

A Becton Dickinson FACScan was used to perform flow cytometric analysis on the stained fibroblasts. Dr Fabio Bucchieri helped with running the samples on the FACScan and in analysing the data. The machine was set as indicated in table 2.6, using the Cell Quest software. Flow cytometry data was analysed using WinMDI 2.8. For each sample, 10,000 events were collected.

Forward Scatter (FSC)	E-1	Amp 5.08	Lin
Side Scatter (SSC)	280	1	Lin
FL1	490		Log

Table 2.6: FACScan settings used to measure TGFβ receptors

2.7. Statistics

The data provided by the primary cells were not normally distributed so non-parametric statistical tests were performed to assign significance. In order to make multiple comparisons within a data set, data were initially subjected to a Friedman

and a Kruskal-Wallis H test using SPSS for windows 11.5 (Surrey, U.K.). A Friedman test was performed on dependent samples i.e. two or more treatments compared to control. Kruskal-Wallis H tests were performed on independent samples i.e. normal samples compared to mild asthmatic and moderate severe asthmatic samples.

If a normal or asthmatic data set gave a p value of less than 0.05 in a Friedman statistical test, the data was reanalysed using a Wilcoxon Signed Ranks Test. Wilcoxon Signed Ranks Test. If a p value of less than 0.05 was obtained using a Kruskal-Wallis H test the data was reanalysed using a Mann Whitney U test. Bonferoni corrections were applied where necessary when multiple comparisons were made within the same data set.

Data were presented graphically using box and whisker plots, showing the median interquartile range, 10^{th} and 90^{th} percentile, using SigmaPlot 5 (SPSS, Surrey, U.K.). To aid interpretation of the data, individual data points were represented on the graph using different symbols. Normal data points were unfilled, mild asthmatic data points were turquoise and moderate severe asthmatic subjects were coloured blue.

CHAPTER 3

TGFβ2 Induced Effects on Bronchial Fibroblasts

CHAPTER 3

TGFβ2 INDUCED EFFECTS ON BRONCHIAL FIBROBLASTS

It is postulated that the increase in myofibroblasts observed in the remodelled airways of asthmatics is not just a consequence of the TGFβ release associated with chronic inflammation, but an increased responsiveness to TGFβ. The following experiments were designed to identify differences between the differentiation and proliferation potential of normal and asthmatic fibroblasts and to relate any changes to TGFβ ligand and receptor expression.

Hypothesis: Asthmatic bronchial fibroblasts are more responsive to TGFβ2 than normal fibroblasts.

3.1. Alpha Smooth Muscle Actin (αSMA expression)

The most documented function of TGFβ is its ability to transform fibroblasts into myofibroblasts. The differentiation of fibroblasts into myofibroblasts has been reported in fibroblasts isolated from a number of different organs including the lung, skin and eye. In the liver, TGFβ induces the differentiation of the fat storing hepatic stellate cells into myofibroblasts (296). TGFβ has also been implicated in the transformation of mesengial cells of the kidney (297) into myofibroblast-like cells.

The most common distinguishing feature of the myofibroblast is the presence of αSMA. The expression of αSMA is directly correlated to wound contraction, appearing at the initiation of and disappearing at the completion of the contraction process (298). In asthma, it is postulated that lung myofibroblasts are active for prolonged periods of time, resulting in deposition of excess extracellular matrix and perpetuation of the inflammatory cycle due to the secretion of proinflammatory cytokines.

Initial studies were designed to determine the sensitivity of human lung fibroblasts to the transforming actions of TGFβ2. It was hypothesised that asthmatic fibroblasts are more responsive to TGFβ2 than normals. In relation to their potential to differentiate, this may be interpreted as an increased responsiveness to TGFβ2 at lower doses or increased expression of αSMA at equivalent doses of growth factor.

3.1.1. RT-PCR analysis of αSMA expression

To determine which time point to study αSMA mRNA expression in bronchial fibroblasts, a kinetic experiment was performed on a representative normal fibroblast culture (fig. 3.1). Fibroblasts were treated for 0, 4, 24, 48 and 66 hours with three concentrations of TGFβ2 and a serum free medium (SFM) control. RNA was isolated from the cells using the Trizol extraction protocol and αSMA mRNA measured by Taqman PCR, each sample was measured in duplicate.

αSMA expression, normalised to 18S rRNA, was altered upon treatment with TGFβ2 and varied according to the length of stimulation. 0.4nM (10ng/ml) TGFβ2 induced the greatest induction of αSMA at 24 hours, although by 66 hours the expression of αSMA had diminished to 50% of that at 24 hours. Both 0.04 (1ng/ml) and 0.006nM (150pg/ml) TGFβ2 induced αSMA expression, but at 50% of the level of that induced by 0.4nM TGFβ2. The SFM control did not induce αSMA at any of the time points tested. From this study it was decided to study αSMA expression at only 0.4nM and 0.04nM as the 0.006nM response varied very litlle from the 0.04nM response. As 24 hours was the optimum time point, further studies on a larger numbers of fibroblast cultures were performed at this time.

To determine disease related differences in the TGFβ2 induced expression of αSMA, αSMA mRNA was measured after stimulating confluent, quiescent T25cm^2 flasks of normal (n=8), mild asthmatic (n=7) and moderate/severe asthmatic (n=8) fibroblasts with 0.04nM and 0.4nM TGFβ2 for 24 hours.

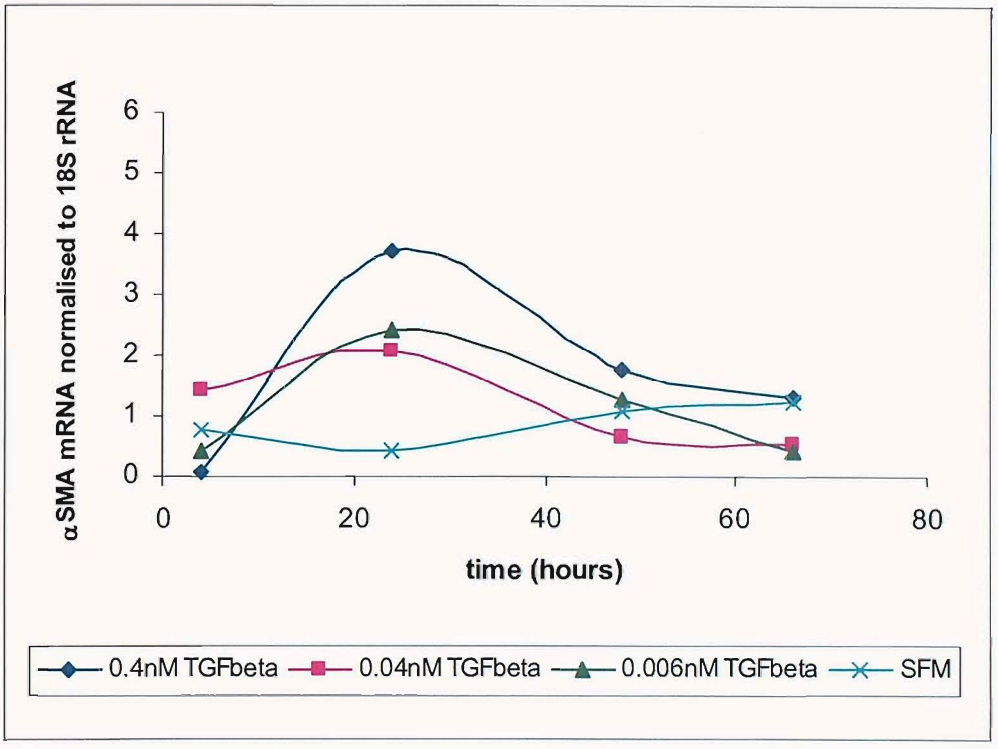


Figure 3.1: αSMA expression over time after TGFβ2 treatment in fibroblasts from a representative normal subject

Fibroblasts were grown on a 6 well plate and grown until 70% confluent. The cells were then serum starved for 24 hours and then treated with TGFβ2 (0.4nM, 0.04nM or 0.006nM) or serum free media for 4, 24, 48 or 66 hours. RNA was isolated from the cells using the Trizol extraction protocol and αSMA mRNA measured by Taqman PCR. Each sample was measured in duplicate.

The results revealed that αSMA expression, normalised to 18S rRNA, was increased in a concentration dependent fashion after stimulation with TGFβ2 for 24 hours (fig. 3.2.), regardless of disease status. αSMA expression, upon treatment with 0.4nM TGFβ2, increased significantly in the normal (p=0.034), mild asthmatic (p=0.036) and moderate severe (p=0.024) fibroblasts. These results obtained from primary lung fibroblasts are consistent with previous findings, in that TGFβ2 induces αSMA expression.

Surprisingly, the αSMA expression level in the normal SFM control was higher in the healthy controls compared to the mild (p=0.015) and moderate severe (p=0.046) asthmatic fibroblasts. This observation is novel and the reverse of that predicted. Although the basal expression levels of αSMA were lower in the asthmatic fibroblasts, it should be noted that upon stimulation with 0.4nM TGFβ2, there were no differences in αSMA between the normal and asthmatic fibroblasts.

3.1.2. Immunofluorescent detection of αSMA protein

In order to determine whether the changes described at the gene level were translated to the protein level, immunofluorescent staining for αSMA protein was performed on TGFβ2 treated fibroblasts. Immunofluorescent staining was initially used as it provides a positive visual readout of protein expression, indicating both cellular location and arrangement within the cell.

In order to maximally induce αSMA, fibroblasts (n=4) were treated for 5 days +/- TGFβ2, replenishing the media every two days. The control fibroblasts grown in serum free medium (SFM) had either a low expression of αSMA, or were αSMA negative (fig 3.3). All four fibroblast cultures treated with TGFβ2 showed intense αSMA staining after 5 days. The staining revealed that the majority of TGFβ treated cells were αSMA positive and that the αSMA was arranged in long filaments parallel to one another within the cell. Although the number of fibroblasts studied was small, there was no apparent disease related difference in

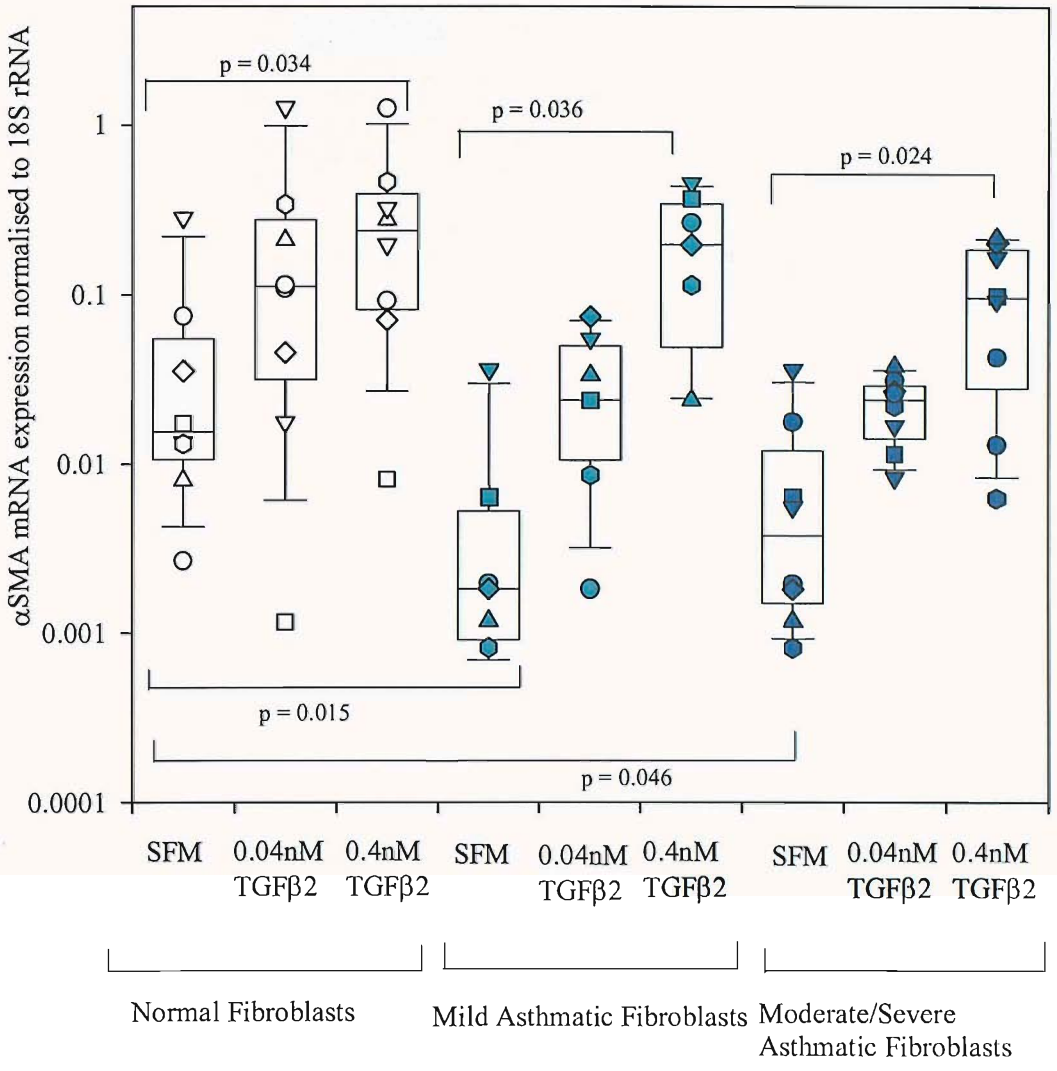


Figure 3.2. TGFβ2 induces αSMA mRNA expression

Normal (n=8), mild (n=7) and moderate/severe asthmatic (n=8) fibroblasts were treated with TGFβ2 for 24 hours. αSMA expression was measured using RT-PCR and normalised using 18S rRNA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, data from mild asthmatic subjects as turquoise symbols and moderate/severes as dark blue.

Comparisons between normal, mild and moderate/severe asthmatic data were analysed for significance using a Kruskal-Wallis H statistical test. There was a significant difference between the groups for the SFM data ($p=0.027$) but not for the 0.04nM TGFβ2 ($p=0.088$) or 0.4nM TGFβ2 ($p=0.292$). Accordingly, a Mann Whitney U test was performed on the basal expression data for αSMA, statistical comparisons where $p<0.05$ were noted on the graph.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within the normal, mild and moderate/severe asthmatic groups. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within a group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

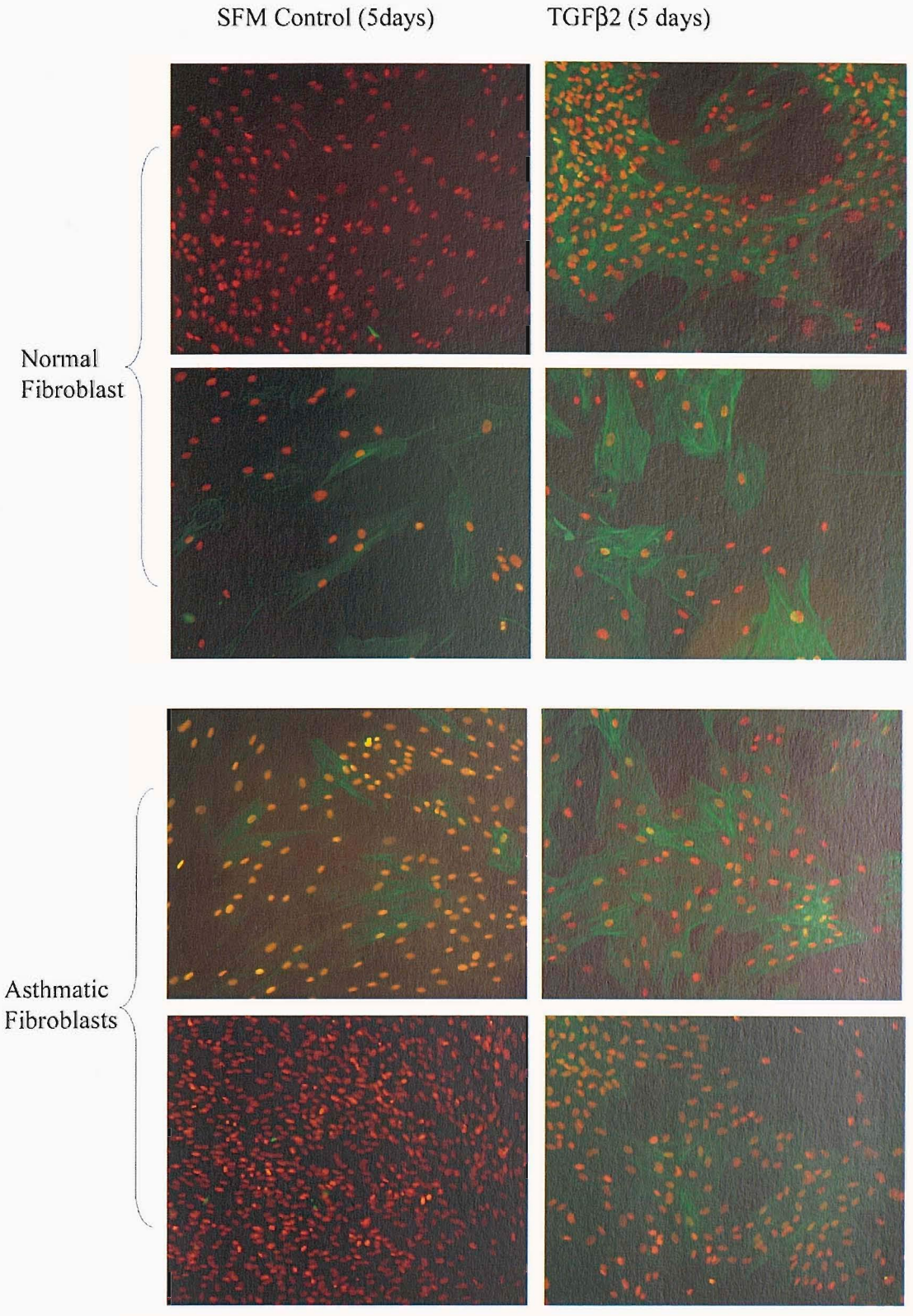


Figure 3.3 TGFβ2 induces αSMA in bronchial fibroblasts

Fibroblasts (n=4) were grown for 5 days in serum free medium (SFM) with or without 0.4nM TGFβ2. αSMA antibody (Sigma A2547, diluted 1:500) was visualised using FITC conjugated secondary antibody. Nuclei were counter-stained with 7-aminoactinomycin D. Digital images were taken with a Hamamatsu camera (x10 magnification) using a Leica DM-RBE microscope and analyzed using the Wasabi analysis programme (Hamamatsu, GMHB).

the expression of αSMA. It was also noted that αSMA was induced regardless of the starting confluency of the culture.

3.1.3. Flow Cytometric measurement of αSMA protein

In order to obtain a quantitative readout of αSMA expression, flow cytometric analysis was performed on the fibroblasts. Although both techniques rely on fluorescently labelled antibodies, flow cytometry is a more sensitive detection technique to study αSMA expression. The fibroblasts were trypsinised, stained and run on the FACScan (Beckton Dickinson). Mean fluorescence intensity (MFI) and percentage of αSMA positive cells were calculated as a result of treatment. Confluent normal (n=7), mild (n=4) and moderate severe asthmatic (n=9) fibroblasts were treated with 0.04nM and 0.4nM TGFβ2 for 24 hours prior to analysis. Although the asthmatic data have been plotted together, mild asthmatic subjected are represented as turquoise symbols and moderate/severe asthmatic fibroblasts as blue symbols to distinguish between the two sets of data.

Upon treatment, TGFβ2 significantly increased the number of αSMA positive cells in a dose dependent manner in both normal and asthmatic fibroblasts (fig 3.4). The increase in αSMA protein expression reached significance after stimulation with the higher dose of TGFβ2 in the normal ($p=0.036$) and asthmatic ($p=0.002$) fibroblasts. The lower dose (0.04nM) of TGFβ2 also caused a significant increase ($p=0.006$) in the percentage of αSMA positive asthmatic fibroblasts. Although only four mild asthmatic fibroblasts were analysed, the mean % of αSMA positive cells was lower than the moderate severe group, both basally (mild asthmatics 11.6% (median) compared to 46.05% for the moderate severe asthmatics) and upon 0.4nM TGFβ2 treatment (mild asthmatics 36.34% compared to 59.65% for the moderate severe asthmatics).

MFI data revealed that the highest dose of TGFβ2 in the normal fibroblasts, and both doses of TGFβ2 in the asthmatic fibroblasts, increased the amount of αSMA per cell (fig 3.5). After stimulation with 0.4nM TGFβ2 there was a 1.88 fold

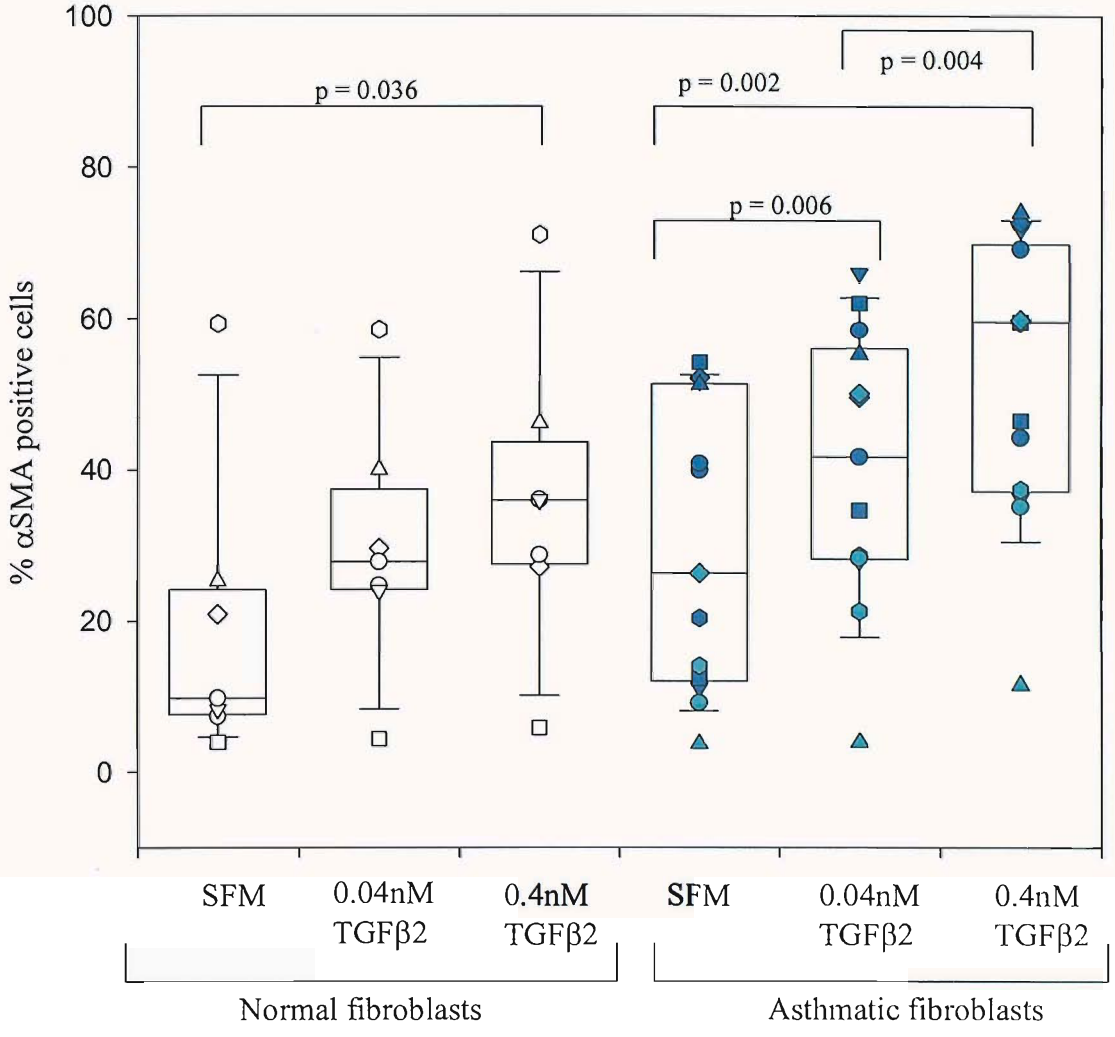


Figure 3.4 TGFβ2 increases the number of αSMA positive fibroblasts

Normal (n=7), mild (n=4) and moderate severe asthmatic (n=9) fibroblasts were treated with TGFβ2 for 24 hours. The number of αSMA positive cells was measured by flow cytometry using an αSMA-FITC labelled antibody.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal (p=0.006) and asthmatic (p=0.000) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

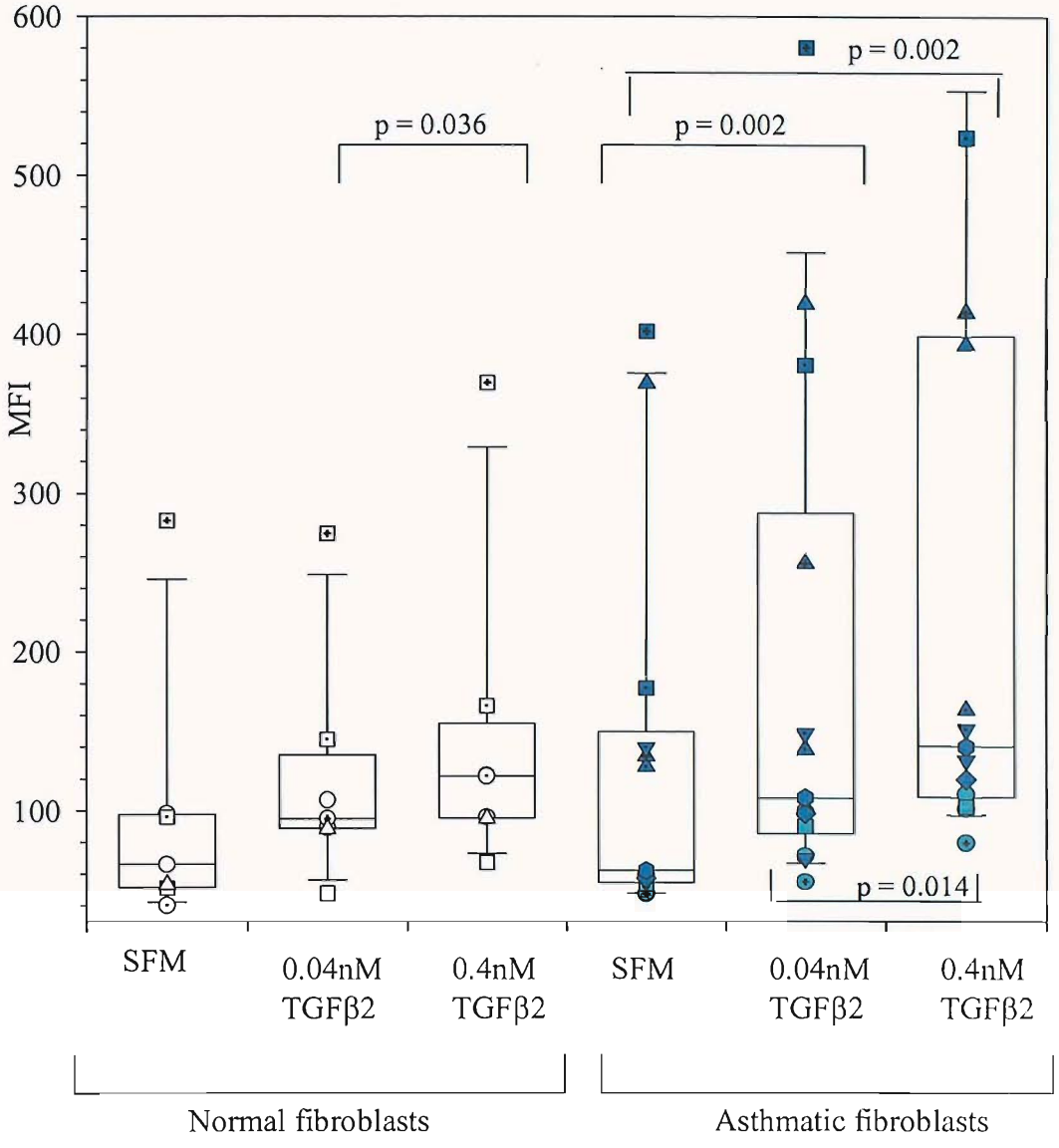


Figure 3.5 TGFβ2 increased αSMA protein expression in fibroblasts

Normal (n=7), mild (n=4) and moderate severe asthmatic (n=9) were treated for 24 hours with TGFβ2. αSMA expression was measured by flow cytometry using an αSMA-FITC labelled antibody.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.021$) and asthmatic ($p=0.000$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

induction in MFI in the normal fibroblasts, a 2.03 fold induction in the mild asthmatic fibroblasts and a 2.02 fold induction in the moderate severe fibroblasts.

Stephenson *et al* (299), demonstrated that an increase in αSMA in kidney mesangial cells was correlated to an increase in cell size. In order to differentiate between a real increase in αSMA and a hypertrophic mediated increase, the size of the fibroblasts before and after treatment with TGFβ2 was measured. Using flow cytometry measurements, it was found that there was no significant difference in cell size in either the normal ($p=0.174$) or the asthmatic group ($p=0.180$), according to a Friedman test, indicating that the increase in αSMA expression was not due to hypertrophy.

To summarise, there was a trend for a dose dependent TGFβ2 induced increase in αSMA expression at the protein and mRNA level in the bronchial fibroblasts, regardless of disease status. To determine whether the differences suggested above between the normal and asthmatic fibroblasts are the result of differences in the TGFβ receptor profile, the effects of TGFβ2 on both receptor and ligand expression was studied.

3.2. TGFβ receptor expression

An increased number of TGFβ receptors may facilitate the transduction of signal in fibroblasts. Indeed, an elevated TGFβ receptor profile in asthmatic fibroblasts may enhance their sensitivity to the growth factor and contribute to their fibrogenic phenotype. Therefore it is postulated from the hypothesis that asthmatic fibroblasts express more TGFβ receptors compared to normal fibroblasts, either basally and/or upon treatment with TGFβ2.

Growth factor receptors are generally down regulated in response to ligand binding by internalisation. Zwaagstra *et al*, (300) reported that TGFβ RI, RII and RIII are rapidly down regulated in response to TGFβ1. It was suggested that down regulation occured via a two-phase mechanism involving receptor aggregation at the cell surface followed by internalisation.

To determine the optimum time point to measure TGFβ receptor mRNA expression, a time course was performed on one normal fibroblast culture. The initial experiment revealed complex variation in receptor expression due to changes relating to both time and concentration of TGFβ2 (results not shown). In reference to TGFβ RI, kinetic analysis of receptor expression revealed no substantial increase in the receptor at any of the time points tested. Analysis of TGFβ RII revealed a TGFβ dependent suppression and a time dependent increase at 24 hours, TGFβ RIII changed very little over time. Subsequent studies were carried out at the 24-hour time point.

3.2.1. Basal TGFβ receptor mRNA expression

The data presented in figure 3.6. shows that all three receptor subtypes are expressed in bronchial fibroblasts in culture. There was a higher basal expression level of TGFβ RI ($p=0.01$) in normal compared with asthmatic fibroblasts. There was a trend for reduced expression of TGFβ RII ($p=0.071$) and betaglycan ($p=0.063$) in asthmatic fibroblasts, although the observations did not reach significance. In summary, there appeared to be a lower basal expression of TGFβ receptors in asthmatic fibroblasts, compared to normal fibroblasts at the mRNA level. This finding does not suggest that there is an enhanced expression profile of TGFβ receptors.

3.2.2. Altered TGFβ receptor mRNA expression in response to TGFβ2

It was postulated that TGFβ2 would down regulate receptor mRNA expression. TGFβ RI levels (fig 3.7) in normal fibroblasts were decreased by an average of 2.71 fold after stimulation with 0.04nM TGFβ2 ($p=0.024$). However, the high dose of TGFβ2 caused a 0.63 fold reduction compared to control, an observation which did not reach significance. The asthmatic fibroblasts responded differently to the TGFβ2, with 0.04nM TGFβ2 causing a 1.51 fold increase in expression (not significant) and the 0.4nM TGFβ2 concentration causing an average 4.56 fold

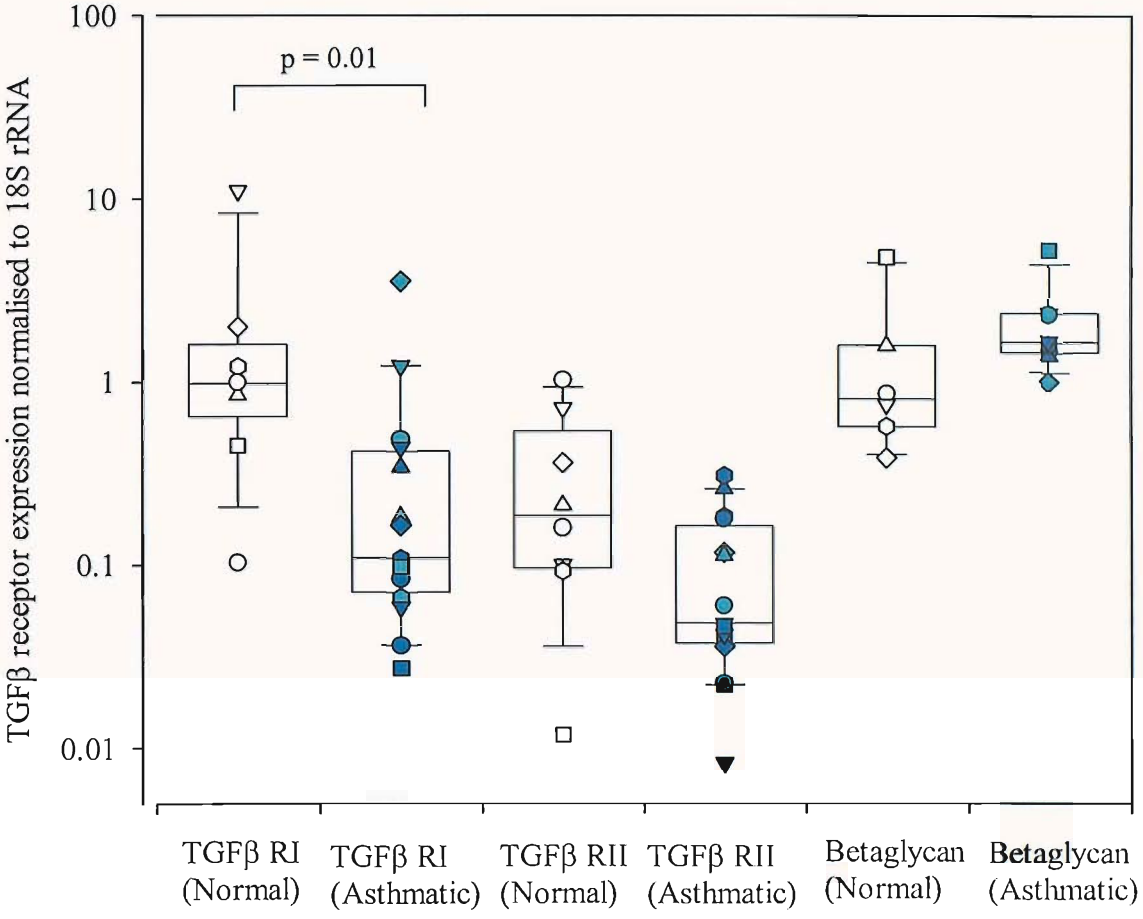


Figure 3.6. Asthmatic fibroblasts express lower levels of TGFβ RI than normals

Normal (n=6-8) and mild (n=5-7) and moderate severe asthmatic (n=3-8) were serum starved for 48 hours and TGFβ2 receptor expression analysed using RT-PCR.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols. Mann Whitney U tests were used to determine inter-group differences. Statistical comparisons where $p<0.05$ were noted on the graph.

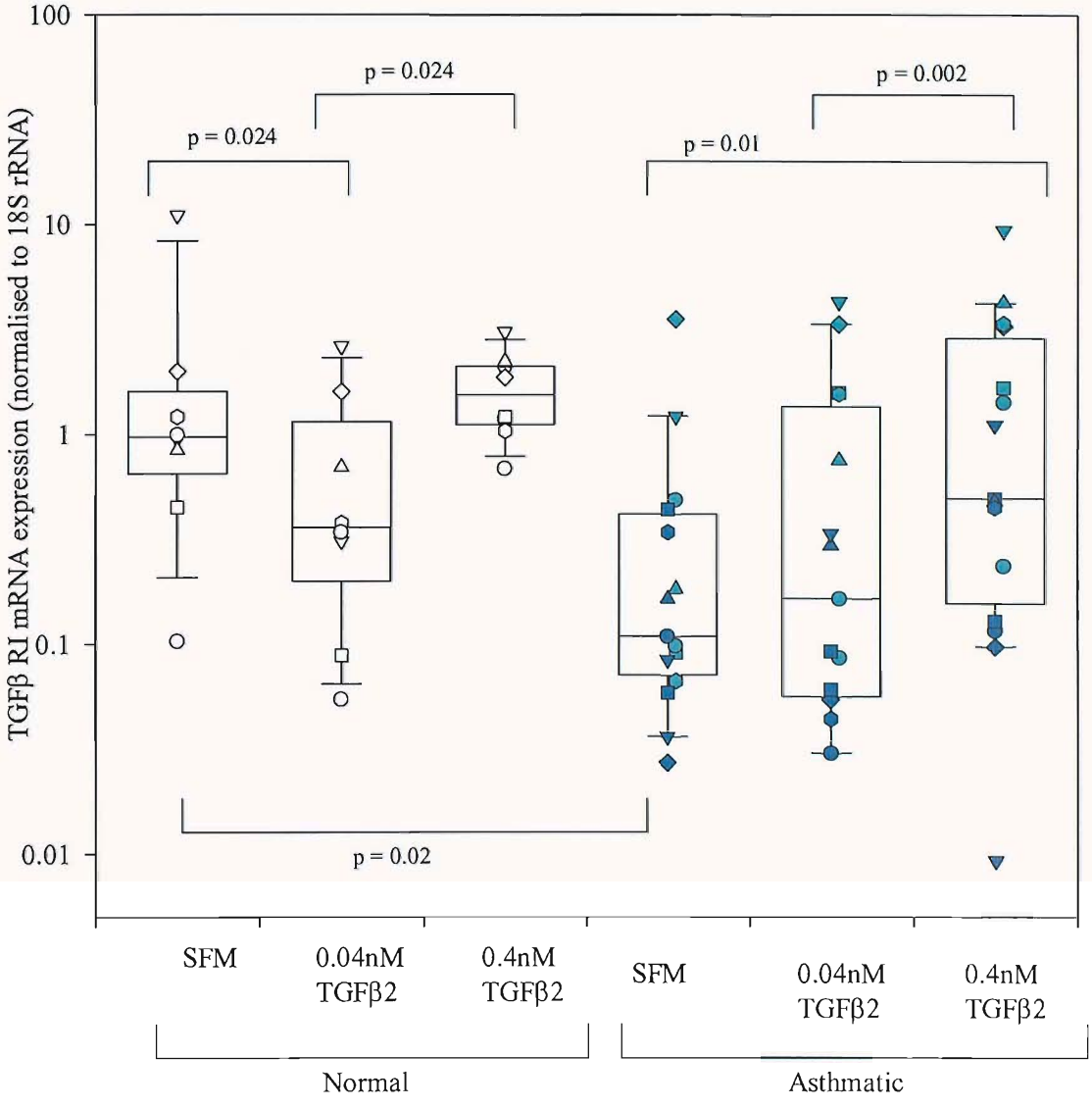


Figure 3.7. TGFβ2 induced TGFβ RI mRNA expression in asthmatic fibroblasts

Normal (n=8) and mild (n=7) and moderate severe asthmatic (n=8) fibroblasts were treated with TGFβ2 for 24 hours. TGFβ RI expression was measured using RT-PCR. Expression levels were normalised against 18s rRNA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2 (statistical comparisons where $p<0.05$were noted on the graph). To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.002$) and asthmatic ($p=0.001$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

increase in mRNA levels (p=0.01). The difference in response may be due to a lower basal level of TGFβ RI in the asthmatic fibroblasts (p=0.02), which therefore permitted a greater increase upon treatment.

There was a trend for suppressed TGFβ RII expression after TGFβ2 treatment in bronchial fibroblasts (fig 3.8), as suggested by the initial kinetic analysis. Although in the normal fibroblasts this did not achieve significance, in the asthmatic fibroblasts TGFβ RII expression was significantly reduced by an average 1.3 fold after treatment with 0.4nM TGFβ2 (p=0.04). Betaglycan expression appeared to be suppressed upon treatment with 0.4nM TGFβ2 in normal and asthmatic fibroblasts (fig 3.9), but not significantly.

To summarise, as suggested by the literature, TGFβ2 down regulated the expression of TGFβ RII and betaglycan at the gene level. TGFβ RI was regulated differently to the other receptors, with TGFβ2 increasing the expression of TGFβ RI in the asthmatic fibroblasts by approximately 4.5 fold. Indeed, the only significant differences in the data between the normal and asthmatic cultures were seen in the expression of TGFβ RI. TGFβ RI mRNA from asthmatic fibroblasts was lower basally but positively responded to TGFβ2 resulting in increased expression after 24 hours, suggesting an increased responsiveness to TGFβ2.

3.2.3. Detection of TGFβ receptors

It was important to determine whether the data collected at the gene level translated into differences at the protein level. In order to measure changes in protein expression a number of TGFβ receptor antibodies were purchased. It was planned to measure TGFβ receptor expression by immunofluorescence techniques and Western blotting. Unfortunately, the TGFβ RI and RII antibodies purchased detected a number of different molecular weight proteins by western blotting. In immunofluorescence experiments, the antibodies gave diffuse cellular staining of fixed fibroblasts, treated with or without TGFβ2 (results not shown).

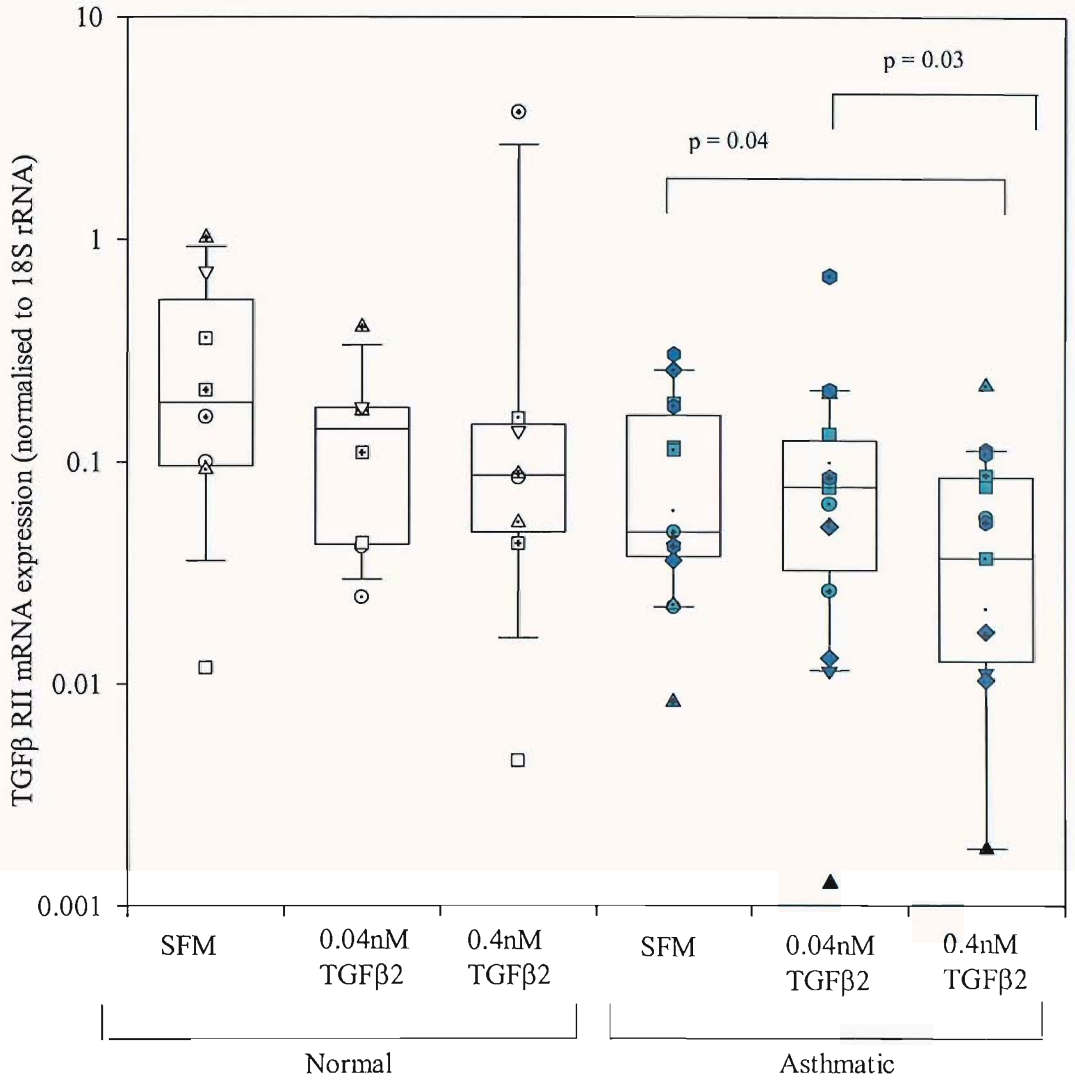


Figure 3.8. TGFβ2 suppressed TGFβ RII mRNA expression in bronchial fibroblasts

Normal (n=8) and mild (n=7) and moderate severe asthmatic (n=8) fibroblasts were treated with TGFβ2 for 24 hours. TGFβ RII expression was measured by RT-PCR and expression normalised against 18S rRNA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.044$) and asthmatic ($p=0.038$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

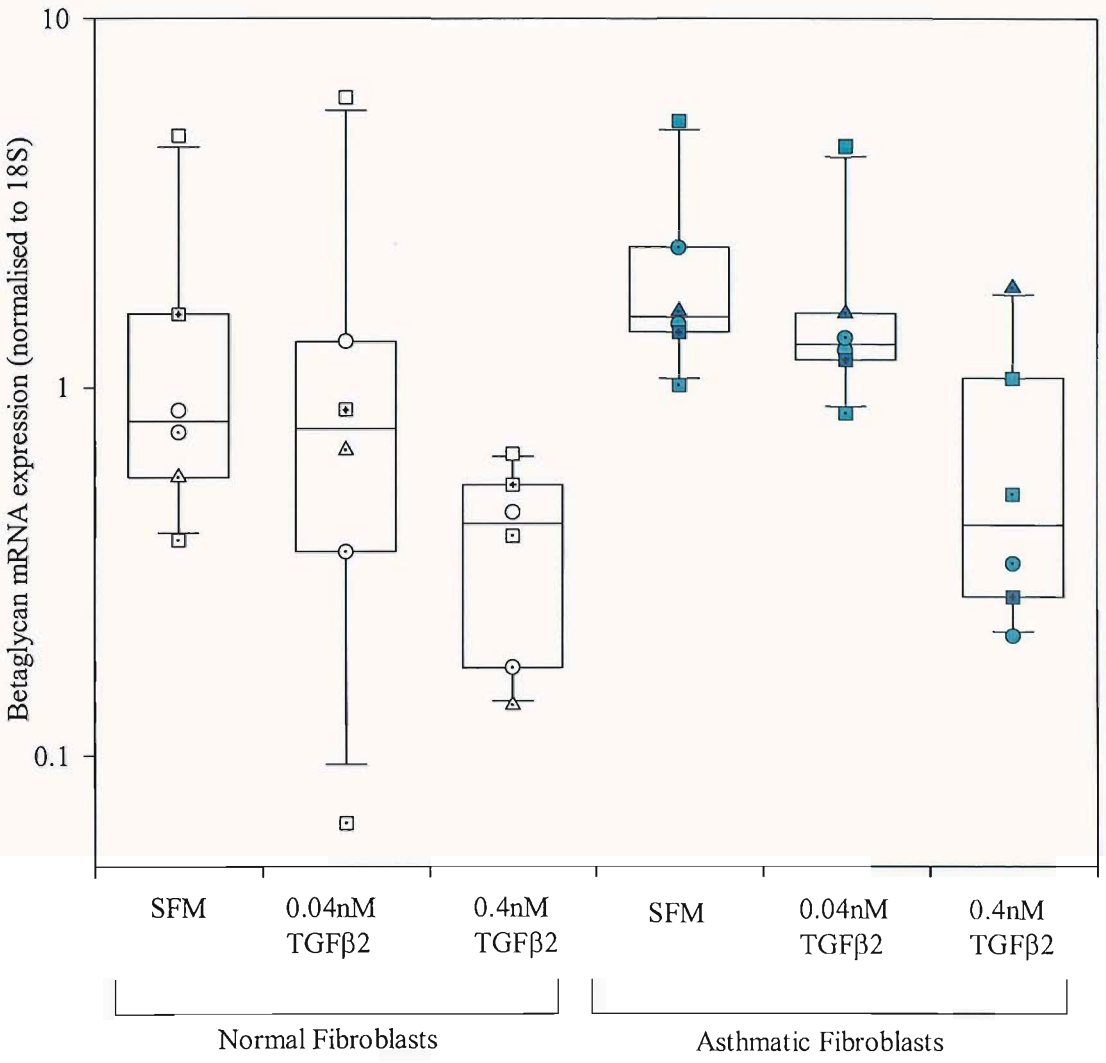


Figure 3.9. TGFβ2 suppresses betaglycan mRNA expression

Normal (n=6) and mild (n=4) and moderate severe asthmatic (n=2) were treated with TGFβ2 for 24 hours. Betaglycan expression was measured using RT-PCR and normalised against 18S rRNA using the standard curve method (ABI Prism 7700 sequence detection Taqman machine).

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there was a significant difference within the asthmatic ($p=0.03$) group but not the normal group ($p=0.135$). The intra-group comparisons did not reach significance in the Wilcoxon Signed Ranks test once Bonferoni corrections were applied to correct for multiple testing.

To overcome the difficulties of studying TGFβ receptor expression at the protein level, the binding of TGFβ to its receptors was measured by flow cytometry. The assay is a functional readout of receptor expression on the cell surface of live cells. From the mRNA data it was predicted that 0.4nM TGFβ would down regulate TGFβ RII and betaglycan expression, thus causing a reduction in the number of binding events.

3.2.4. Measurement of TGFβ receptor expression

A preliminary experiment was performed to determine the optimum method of detaching primary bronchial fibroblasts from culture dishes, using trypsin, cell dissociation solution and EDTA. The protocol suggested using EDTA to prevent receptor cleavage by trypsin. EDTA and cell dissociation solution however were both ineffective at removing the fibroblasts, taking approximately 20 minutes to detach and causing the fibroblasts to clump. The trypsin detached all of the cells quickly without needing to disperse the cells with a needle. Trypsin did not appear to cause TGFβ receptor cleavage; indeed the staining intensity was greatest with trypsin compared to EDTA or cell dissociation solution as revealed by preparing slides of the cells after staining and viewing them down a fluorescent microscope (results not shown).

To measure TGFβ1 binding in bronchial fibroblasts, TGFβ2 treated normal (n=7) and moderate severe asthmatic (n=6) trypsinised fibroblasts, and their corresponding SFM control cultures, were incubated with biotinylated TGFβ1. The fibroblasts were indirectly labelled using streptavidin-FITC. Mean fluorescence intensity (MFI) and percentage of TGFβ1 positive cells was calculated using flow cytometry. Approximately 50% of the fibroblasts analysed were TGFβ receptor positive (fig. 3.10). Treatment with TGFβ2 did not significantly alter the number of TGFβ receptor positive cells in either the normal or asthmatic fibroblasts. The MFI also did not alter significantly in response to TGFβ2 (fig. 3.11) but the asthmatic fibroblasts appeared to have a lower MFI than the normal fibroblasts, suggesting that there were fewer TGFβ1 binding sites per TGFβ receptor positive cell.

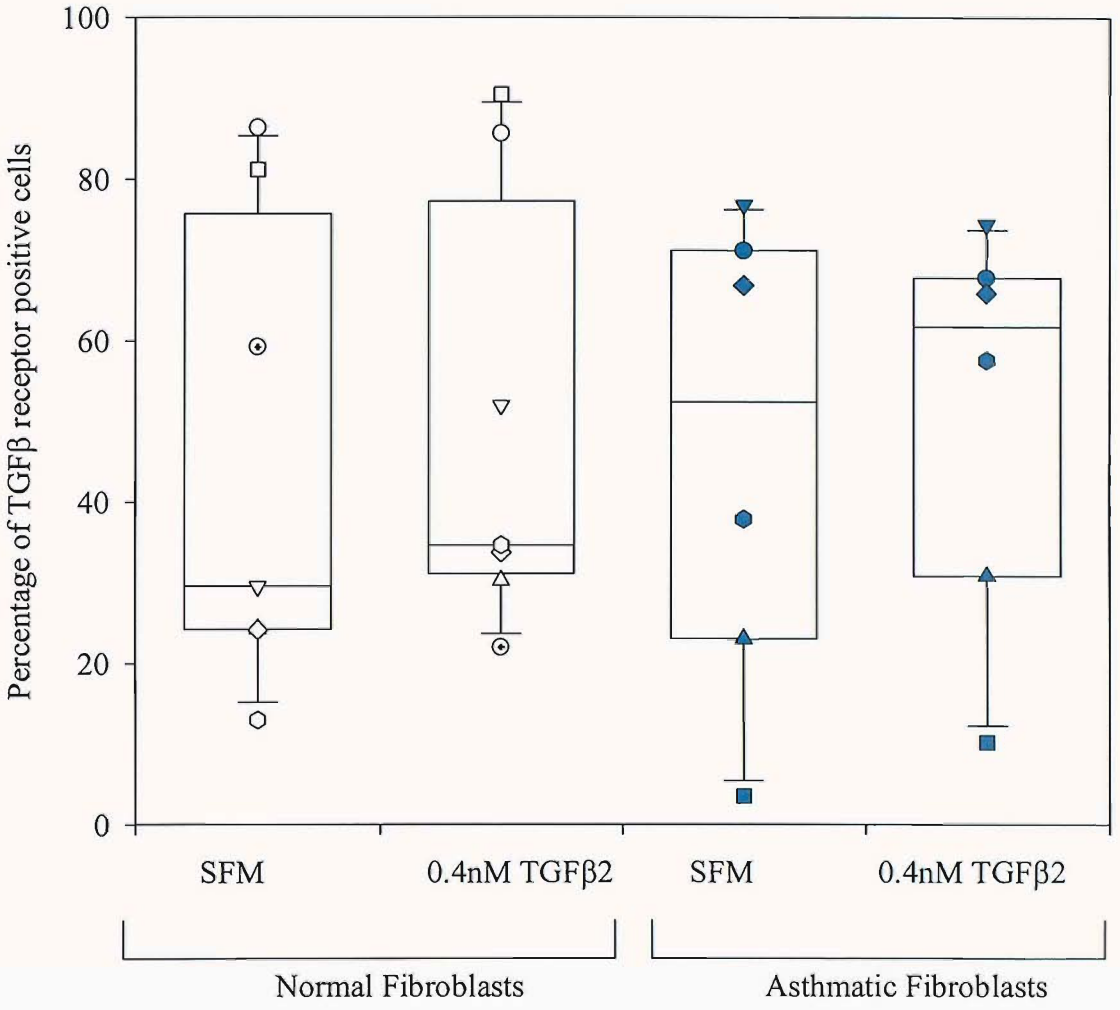


Figure 3.10. TGFβ2 did not alter cell surface TGFβ receptor expression

Normal (n=7) and moderate-severe asthmatic (n=6) fibroblasts were treated with TGFβ2 for 24 hours. TGFβ receptor expression was measured in live fibroblasts using flow cytometry.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, data from asthmatic subjects as blue symbols.

TGFβ receptor expression did not significantly change in response to treatment (Wilcoxon Signed Ranks test) and no differences in expression were noted between the asthmatic and normal population of fibroblasts (Mann Whitney U test).

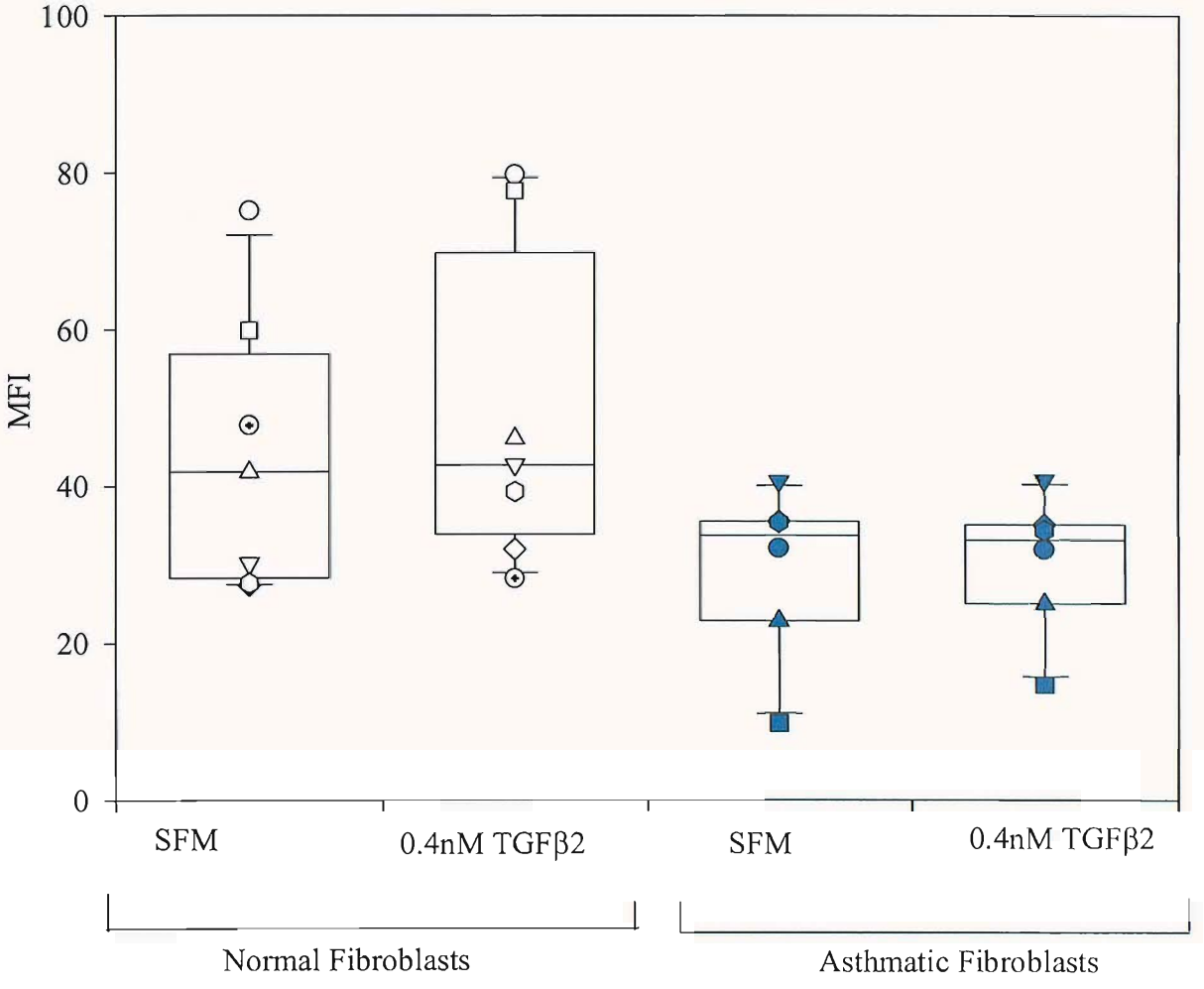


Figure 3.11. Pretreatment with TGFβ2 did not alter binding of TGFβ1 to its receptors

Normal (n=7) and asthmatic (n=6) fibroblasts were treated with TGFβ2 for 24 hours. TGFβ receptor number was measured by flow cytometry using biotinylated TGFβ1 and steptavidin-FITC conjugate.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, data from asthmatic subjects as blue symbols.

TGFβ receptor expression did not significantly change in response to treatment (Wilcoxon Signed Ranks test) and no differences in expression were noted between the asthmatic and normal population of fibroblasts (Mann Whitney U test).

To summarise, this study suggests that the ability of a cell to specifically bind TGFβ1 is unaffected by stimulation with 0.4nM TGFβ2 for 24 hours, although I have previously shown that this condition can induce αSMA expression (fig 3.2), an event associated with fibroblast differentiation.

3.3. TGFβ ligand expression

Although there were no changes in the receptor expression after stimulation with TGFβ2, it is possible that asthmatic fibroblasts have a different secretory phenotype to normal fibroblasts, thus enhancing their fibrogenic capacity. The literature surrounding the regulation of TGFβ at the mRNA level shows TGFβ regulation to be dependent on TGFβ isoform, cell type and the length of stimulation. For example, Bascom *et al* (301) reported that TGFβ1 was upregulated by TGFβ1, unlike TGFβ2 and 3, which were not significantly induced by TGFβ1. In contrast, TGFβ2 increased the expression of TGFβ1, 2 and 3. Conversely, McCartney-Francis *et al* (302) demonstrated that TGFβ1 is capable of autoinduction at the transcript level whereas TGFβ2 mRNA was unchanged after treatment with TGFβ2 in human peripheral blood monocytes. Although there are a number of inconsistencies in the literature, a common feature appears to be the ability of TGFβ1 to upregulate its own expression.

The current study was specifically designed to examine the expression and release of TGFβ ligands in response to stimulation with TGFβ2 for 24 hours. Based on previous reports in the literature it was hypothesised that TGFβ ligand expression is increased in response to TGFβ2 and that there is a disease dependent increase in expression.

3.3.1. RT-PCR analysis of TGFβ1 mRNA

There was a concentration dependent response to TGFβ2 in the normal and asthmatic group of fibroblasts (fig. 3.12) in respect to TGFβ1 induction. According to a Friedmans test, significant differences within the normal ($p=0.016$) and

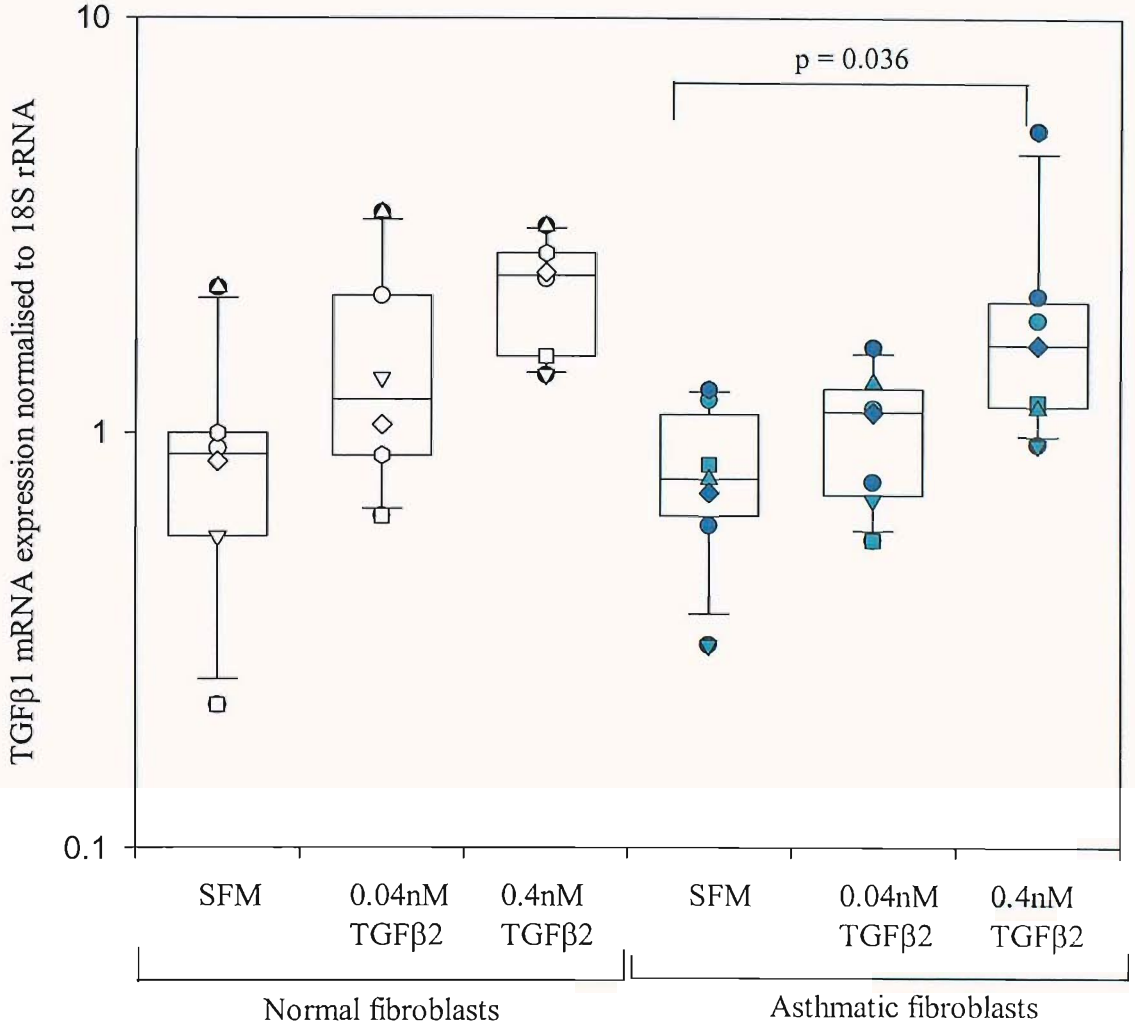


Figure 3.12. TGFβ2 induces TGFβ1 mRNA expression

Normal (n=6) and mild (n=4) and moderate severe asthmatic (n=3) fibroblasts were treated for 24 hours with TGFβ2. TGFβ1 expression was measured using RT-PCR and normalised against 18S rRNA using the standard curve method (ABI Prism 7700 sequence detection Taqman machine).

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.016$) and asthmatic ($p=0.012$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

asthmatic (p=0.012) data were revealed. However, the Wilcoxon Signed Ranks test showed only the 2.5 fold increase in TGFβ1 mRNA, upon treatment with 0.4nM TGFβ2 (p=0.036) in the asthmatic fibroblasts, significant.

A Friedman test revealed that there was a significant difference between the treatment groups of the normal fibroblasts treated with TGFβ2 (p=0.016) in relation to TGFβ2 mRNA expression. There was a non-significant trend for increased TGFβ2 mRNA expression in response to increasing TGFβ2 concentration (fig. 3.13) in the asthmatic fibroblasts. TGFβ2 did not alter TGFβ3 expression in the normal or the asthmatic fibroblasts (fig.3.14). These data suggest that TGFβ synthesis and release were not significantly increased at the mRNA level in asthmatic fibroblasts compared to normals.

3.3.2. Measurement of TGFβ protein

As TGFβ1 mRNA was induced by 0.4nM TGFβ2 in both the normal and asthmatic fibroblasts, TGFβ1 ELISAs were performed on conditioned media from TGFβ2 treated fibroblasts. It was predicted from the mRNA data that treating fibroblasts with TGFβ2 would increase the total amount of TGFβ1. TGFβ is secreted as an inactive complex with latency associated peptide (LAP) and latency TGFβ binding protein 1. TGFβ can be activated *in vivo* by a number of molecules including thrombospondin (TSP1) (81) and furin (303) although the precise sequence of events has not been elucidated. *In vitro*, latent TGFβ can be activated by acidification. Active and latent TGFβ were both measured using the Promega TGFβ1 ELISA kit. Total TGFβ measurements were achieved using samples that were previously acidified and then neutralised. Active TGFβ was measured in samples that had not been acidified.

Active TGFβ1 was detected at low levels in conditioned media from bronchial fibroblasts (fig. 3.15). Results from the current study show that active TGFβ1 levels decreased upon treatment with TGFβ2 in fibroblasts but the observations did not reach significance. Conversely, there was a trend whereby total TGFβ1

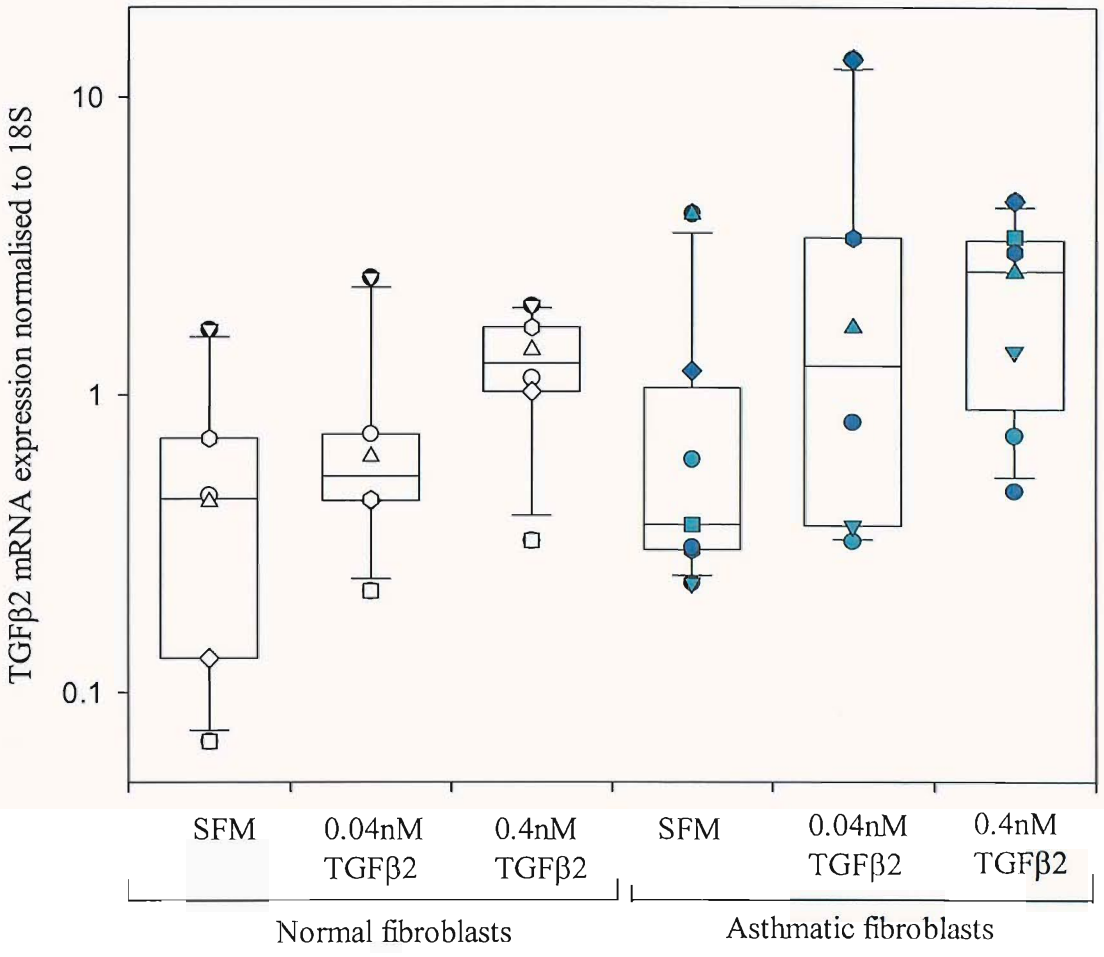


Figure 3.13. TGFβ2 induces TGFβ2 mRNA expression in normal fibroblasts

Normal (n=6) and mild (n=4) and moderate severe asthmatic (n=3) fibroblasts were treated with TGFβ2 for 24 hours. TGFβ2 mRNA expression was measured using RT-PCR and normalised against 18S rRNA using the standard curve method (ABI Prism 7700 sequence detection Taqman machine).

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there was a significant difference within the normal ($p=0.016$) group but not the asthmatic group ($p=0.311$). The intra-group comparisons did not reach significance in the Wilcoxon Signed Ranks test once Bonferoni corrections were applied to correct for multiple testing.

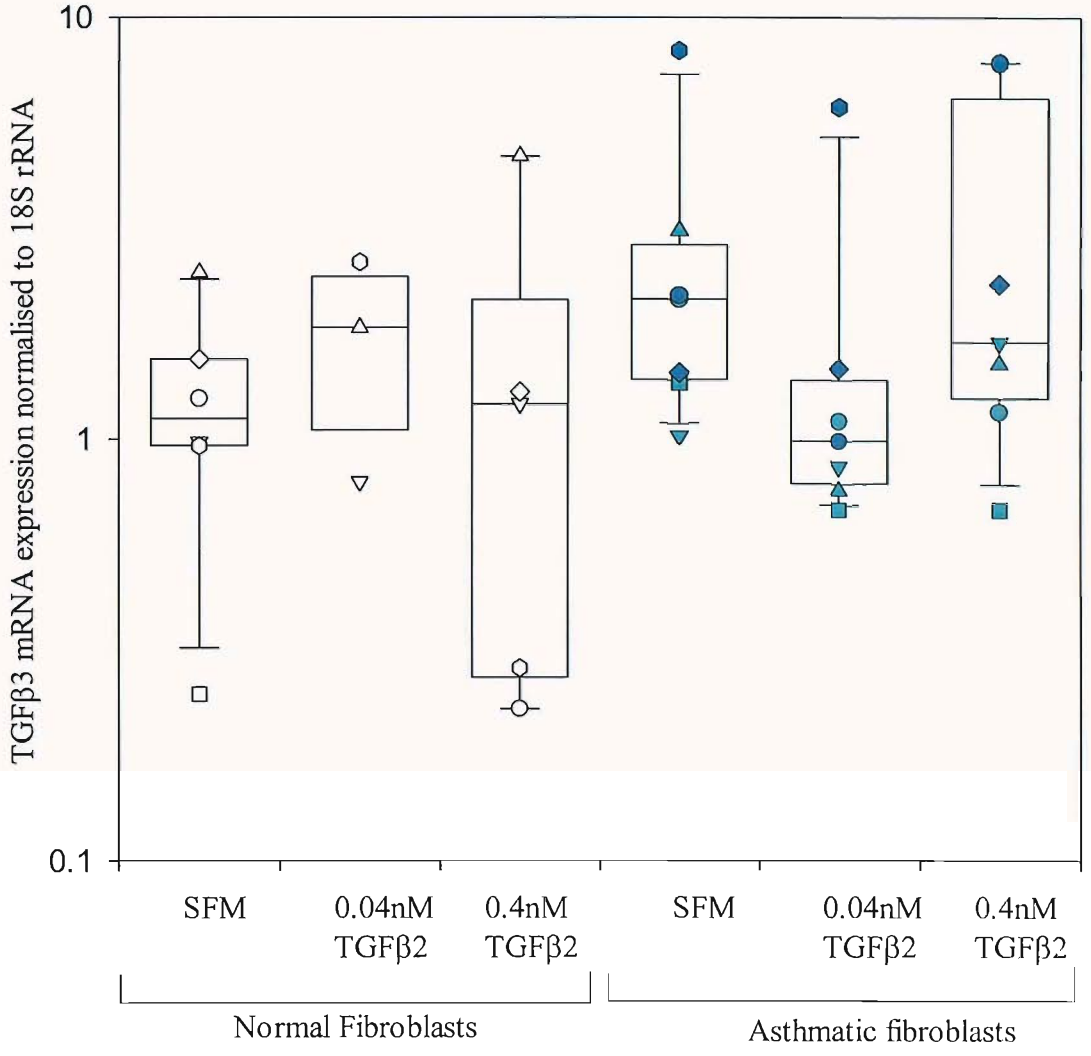


Figure 3.14. TGFβ2 did not alter TGFβ3 mRNA expression

Normal (n=3-6) and mild (n=4) and moderate severe asthmatic (n=3) fibroblasts were treated with TGFβ2 for 24 hours. TGFβ3 mRNA expression was measured by RT-PCR and expression normalised against 18S rRNA using the standard curve method (ABI Prism 7700 sequence detection Taqman machine).

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there was no significant difference within either the normal (p=0.717) or the asthmatic group (p=0.066).

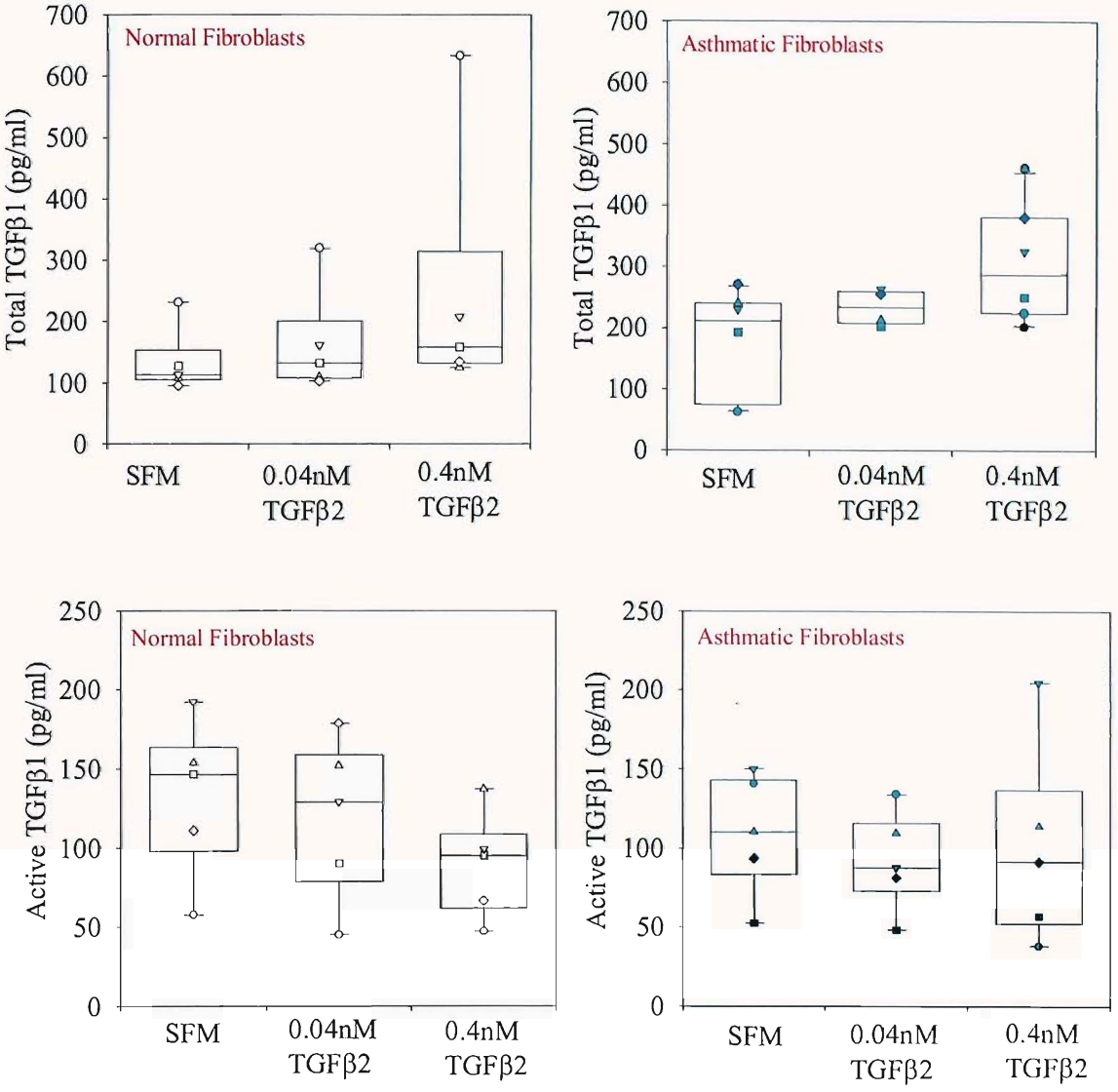


Figure 3.15. Release of TGFβ1 in response to TGFβ2

Normal (n=5) and asthmatic (n=4-6) fibroblasts were treated for 24 hours with TGFβ2. TGFβ1 (active and total) release into the cell culture media was measured by TGFβ1 ELISA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there was no significant difference within the normal ($p=0.074$) or the asthmatic group ($p=0.091$) for active TGFβ2. There was a significant intra-group difference in the normal fibroblast group ($p=0.007$) for total TGFβ2. The intra-group comparisons did not reach significance in the Wilcoxon Signed Ranks test once Bonferoni corrections were applied to correct for multiple testing.

production was increased in response to TGFβ2 (normal fibroblasts, p=0.007), the observations did not reach significance in the asthmatic group, perhaps because of the small number of samples examined.

To summarise, the data suggests that addition of TGFβ2 to bronchial fibroblasts may cause a small decrease in the amount of active TGFβ1 present. A reduction in the active TGFβ1 level could be indicative of either binding events or degradation of the molecule. There was no difference between the normal or asthmatic fibroblasts in their ability to induce the release of TGFβ1 in response to TGFβ2. These results do not suggest that TGFβ release is enhanced in asthmatic fibroblasts compared to normals.

3.4. Proliferation

The data as yet has shown that although asthmatic fibroblasts displayed a difference in the regulation of TGFβ RI this did not correlate into a significant difference in αSMA expression. To determine whether asthmatic fibroblasts do indeed demonstrate an altered response to TGFβ2, another functional readout of TGFβ signalling was selected. The effect of TGFβ on proliferation was chosen, as it was physiologically relevant to asthma, with increased numbers of fibroblasts and myofibroblasts being reported in asthmatic airways (28). A difference in the proliferative phenotype of asthmatic fibroblasts would add considerable weight to the hypothesis that there is an increased responsiveness to TGFβ2 in asthmatic fibroblasts.

TGFβ has been reported to have different effects on the proliferation of fibroblasts. Dube *et al*, (42) reported that TGFβ1 had no significant effect on bronchial fibroblast proliferation. Kay *et al*, (138) reported that corneal stromal fibroblasts produce FGF-2 in response to TGFβ and this is suggested to be a direct stimulator for TGFβ mediated cell proliferation. It was further reported by Thannickal *et al*, (139) that TGFβ1 (2ng/ml) upregulated FGFR-1 and –2 in a time dependent manner, thus contributing to the proliferative response. McAnulty *et al*, (140), also

demonstrated that TGFβ (0.2pM) could stimulate fibroblast proliferation. At concentrations of 16pM and above, the TGFβ effect on proliferation was found to be inhibitory. It was suggested that PGE2 was produced in response to the TGFβ and this was mediating the anti-proliferative effects. PGE2 has been shown to decrease the expression of TGFβ RI and RII and also has the effect of decreasing the binding of TGFβ to TGFβ RI and RII by 50% (304). Indomethacin, an inhibitor of PGE2 overcame the inhibitory effects observed in the 1997 McAnulty study (140) and restored the proliferative effects seen previously using low concentrations of ligand.

In the present study the proliferative effect of TGFβ2 on bronchial fibroblasts was measured by a methylene blue elution assay. To limit the effect of autocrine or paracrine growth factor production, media was replenished with TGFβ2 every two days for a total of 5 days. Changes in cell biomass were determined by staining the fixed fibroblasts with methylene blue. For internal consistency, all measurements were calculated as fold induction relative to day 0.

3.4.1. Kinetics of TGFβ2 induced proliferative effects

Figure 3.16 shows the effect of TGFβ2 on the proliferation of a representative asthmatic fibroblast culture, over 5 days. TGFβ2 at both 0.4nM and 0.04nM appeared to have a weak inhibitory effect compared to the basal proliferative potential of the cells. 0.004nM TGFβ2 did not appear to have an inhibitory effect over the five days studied. The greatest proliferative difference between the treatments was seen at 5 days. Subsequent studies to compare growth related differences in fibroblast cultures isolated from normal or asthmatic subjects were performed at this later time point. Therefore the experiment was repeated with normal (n=11) and asthmatic (n=17) fibroblasts to determine whether TGFβ2 significantly inhibited proliferation and whether the effect was the same regardless of disease status.

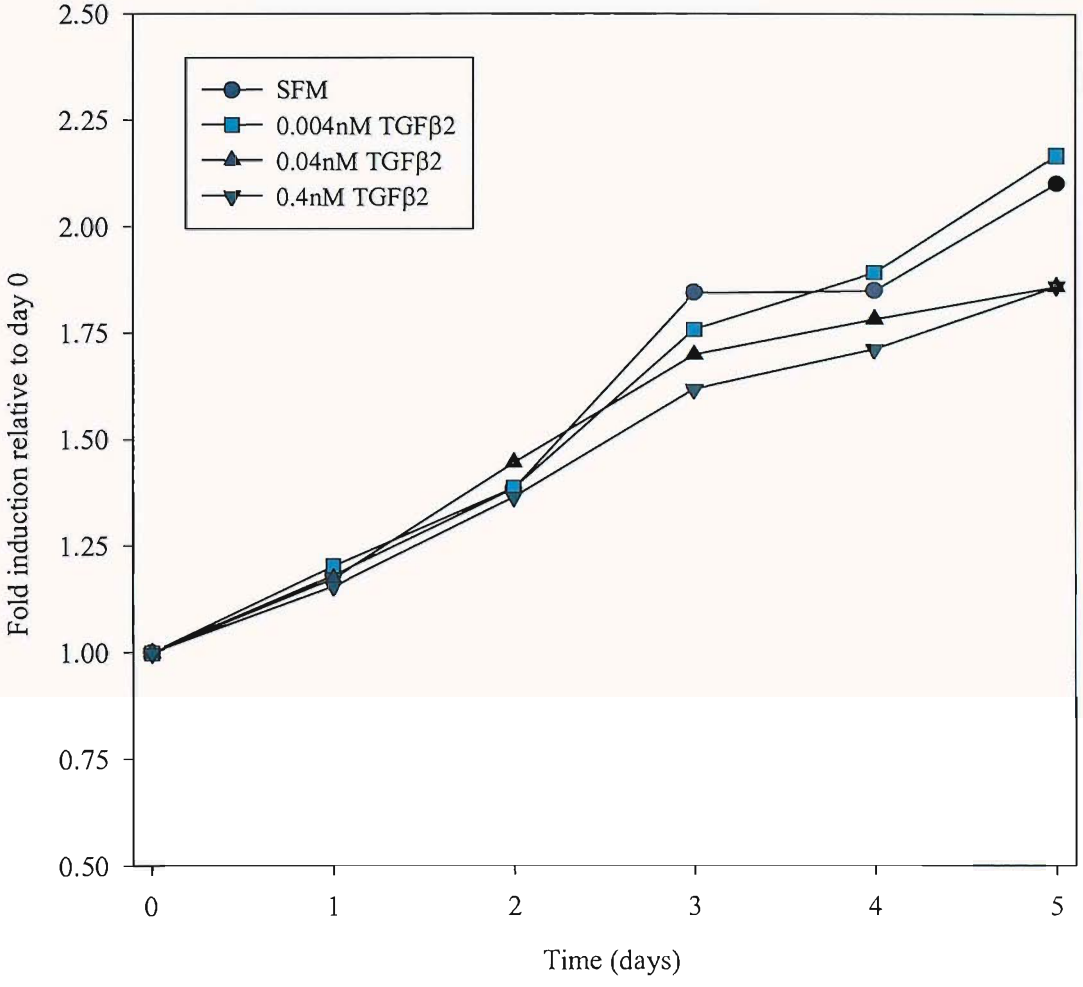


Figure 3.16. Time course of TGFβ2 induced proliferation

The graph shows proliferation data from one representative asthmatic fibroblast culture where cells were grown for 5 days in media supplemented with TGFβ2. Media was replenished every 2 days and the final increase in cell biomass was measured using a methylene blue elution assay. Data points were performed in triplicate and the average of the three plotted.

3.4.2. TGFβ induced growth effects

Fibroblasts proliferated in SFM between day 0 and 5, although the degree of proliferation showed considerable subject dependent variation, independent of disease status. Over the 5 days, the fold increase relative to day 0 ranged from a 1.5- to 10-fold, averaging to approximately a 4-fold induction (fig. 3.17) in the cultures studied.

The concentration dependent effects of TGFβ2 on the growth of primary fibroblasts were examined using 0.004nM, 0.04nM and 0.4nM TGFβ2. After 5 days in culture, 0.4nM TGFβ2 significantly inhibited the growth of both normal ($p=0.026$) and asthmatic fibroblasts ($p=0.003$), compared to the SFM control. Lower concentrations of TGFβ2 had no significant effect on the proliferation of the fibroblasts, as assessed at day 5 compared to SFM. There was no difference between the normal and asthmatic cultures at any of the concentrations of TGFβ2 tested. This result indicates that there is not an increased responsiveness to TGFβ2 in asthmatic fibroblasts in terms of proliferation.

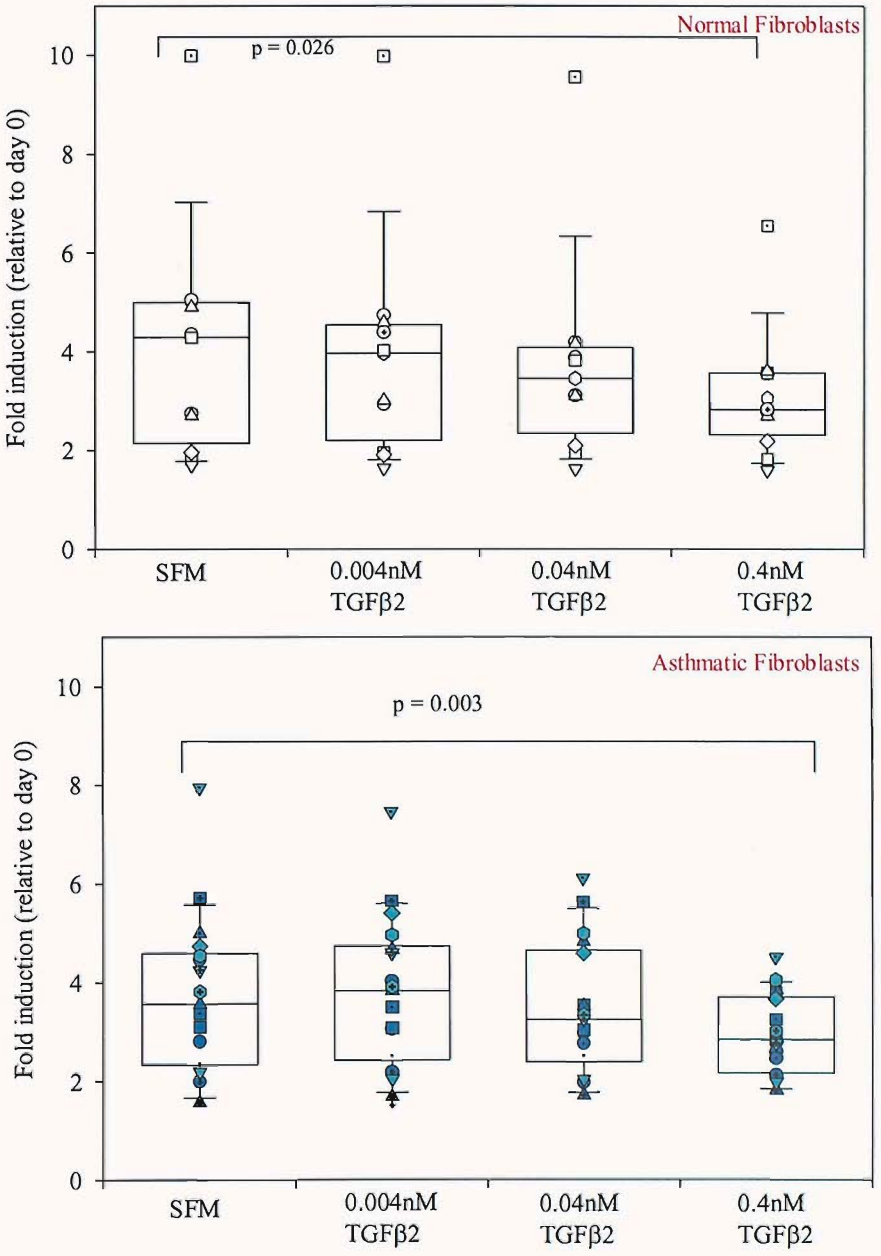


Figure 3.17. TGFβ2 suppresses the proliferation of bronchial fibroblasts

Normal (n=11) and mild (n=7) and moderate severe asthmatic (n=10) fibroblasts were treated with TGFβ2 for 5 days. Media was replenished on days 2 and 4. Increase in cell biomass was measured by methylene blue elution assay. Data are presented as fold induction relative to day 0 in box plots showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2 (statistical comparisons where $p<0.05$were noted on the graph). To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.006$) and asthmatic ($p=0.001$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

3.5. Discussion

Asthmatic airways undergo a process of remodelling, resulting in a more rapid decline in lung function over time than that experienced by the population as a whole. Fibroblasts and their differentiated form, the myofibroblast, play key roles in remodelling.

Myofibroblasts have a classically defined role in the process of wound healing, by participating in the deposition of scar tissue as well as releasing growth factors and cytokines to help orchestrate the ongoing inflammation. They are normally a transient cell type although in fibrotic diseases such as asthma they are present in the submucosa for prolonged periods of time. As a result the myofibroblasts release excess quantities of matrix proteins, chemoattractants such as eotaxin, smooth muscle mitogens such as HB-EGF and angiogenic growth factors such as ET-1. The myofibroblast thus contributes to subepitheial fibrosis and thickening of the *lamina reticularis*, although the ability of myofibroblasts to contribute to the bronchoconstriction associated with the early asthmatic response is unlikely.

The aim of this chapter was to determine whether asthmatic fibroblasts respond differently to TGFβ2, a fibrogenic growth factor that is released in enhanced amounts in asthmatic airways. In designing drugs to combat remodelling, it is important to determine whether asthmatic fibroblasts are phenotypically different to normal fibroblasts or whether they are just responding to the local environment.

3.5.1. Differentiation

Taqman PCR is a very sensitive detection technique to measure changes in mRNA expression. However, as it measures the total number of copies of αSMA mRNA in an RT sample, it is impossible to distinguish between an increase in the number of αSMA positive cells and an increase in the amount of αSMA per cell. Flow cytometry overcomes this problem by being capable of measuring both of these parameters. As flow cytometry relies on the use of antibodies, it measures changes in protein. The combined use of RT-PCR and flow cytometry, with observations

confirmed by immunofluorescent staining of fixed fibroblasts ensured that a thorough examination of αSMA expression was made.

Induction of αSMA expression is indicative of the myofibroblast phenotype. TGFβ2 induced αSMA mRNA in a concentration dependent fashion in both normal and asthmatic fibroblasts after 24 hour stimulation. The RT-PCR data also suggested that asthmatic fibroblasts express a lower basal level of αSMA mRNA than normal fibroblasts. This is a very novel finding and the reverse of what was expected. There were no differences between the TGFβ2 induced increase in αSMA expression between the normal and asthmatic fibroblasts.

The protein data revealed that levels of filamentous αSMA did not differ between the two groups, basally and upon TGFβ2 stimulation. TGFβ2 induced not only the number of αSMA positive cells but also the amount of αSMA per cell. It appeared that the mild asthmatic fibroblasts expressed on average, fewer αSMA positive cells and less αSMA per positive cell, although this did not reach significance due to the small number of subjects studied.

3.5.2. TGFβ receptors

There are no reports in the literature regarding the regulation of TGFβ receptor expression in non-transformed primary lung fibroblasts. The current study revealed that neither 0.4nM nor 0.04nM TGFβ2 increased TGFβ RI expression in normal bronchial fibroblasts, although TGFβ2 induced expression of TGFβ RI mRNA in asthmatic fibroblasts. The difference in basal expression levels and regulation of TGFβ RI is interesting, especially if TGFβ RI expression is the rate-limiting step in the transduction of signal. The lower expression level may be the cause of the lower level of αSMA mRNA in the asthmatic fibroblasts. It was unfortunate that all of the TGFβ RI antibodies tested were non-specific. To overcome the problem of measuring TGFβ receptor protein, TGFβ1 binding was measured by flow cytometry. Although this approach determined the level of specific TGFβ1 binding, the staining did not distinguish between different receptor subtypes.

Unfortunately, the level of non-specific binding of TGFβ1 to the cells was not assessed, an additional compounding factor. The total binding of TGFβ1 reflects only the binding to saturable high-affinity sites - a component may involve non-specific ligation.

If the TGFβ RI antibodies had been shown to be specific, it would have been interesting to further this work by performing dual staining for TGFβ RI and αSMA by flow cytometry. This would have demonstrated whether low basal TGFβ RI levels were correlated with low αSMA levels in asthmatic fibroblasts.

Reports in the literature appear mixed in reference to the regulation of TGFβ RI, but a study by Ward *et al* in 1998 (305) reported elevated TGFβ RI mRNA levels 5-fold (305) in vascular smooth muscle cells in response to TGFβ1. Neither finding in any way contradicts the other but it does show the complexities of studying receptor expression. It also highlights how caution should be applied in extrapolating results from one cell type to predict the effects in another.

There was a non-significant trend for TGFβ2 down regulation of TGFβ RII and betaglycan in the current study. This finding is interesting in light of a recent publication by Lopez-Casillas *et al*, (306) who showed that betaglycan promoter activity is inhibited by all three isoforms of TGFβ in myoblasts.

Results from this chapter showed that TGFβ receptor protein levels, measured indirectly by specific binding of TGFβ1, did not appear to change after treatment with TGFβ2 and hence fibroblast differentiation. The differences between mRNA and protein data may be indicative of differences in regulation, post translation. However, the most likely explanation is the kinetic delay between alterations in message level and the translation into protein.

3.5.3. Ligand

The current study showed that TGFβ2 is capable of inducing TGFβ1 in bronchial fibroblasts. Differences in the expression and release of TGFβ ligands between the

normal and asthmatic fibroblasts were mainly inconclusive due to the small number of subjects studied. However, the results obtained suggested that there are no differences in the release of latent and active TGFβ1. In light of previous findings in asthmatic biopsies where TGFβ levels are significantly increased, these results are surprising. It is possible that the levels of TGFβ from mesenchymal cells are increased in asthma in response to TGFβ release from inflammatory cells, not due to a genetic difference between normal and asthmatic fibroblasts. Another explanation is that TGFβ is more readily degraded in normal fibroblasts, but has a longer half-life in asthmatic lungs due to the increased deposition of ECM proteins. Johnson *et al* (36) have revealed that asthmatic fibroblasts synthesise different ECM proteins to normal fibroblasts. Differences in matrix bound latent TGFβ1 were not accounted for in the ELISA. To prevent this prejudice in further studies, TGFβ1 could be measured by immunofluorescent staining in fixed fibroblasts. Alternatively, the fibroblasts could be removed from the culture plastic by detergent and the remaining ECM analysed for TGFβ1 levels.

TGFβ3 expression remained the same or decreased in response to treatment. There are a number of reports in the literature showing TGFβ3 to have opposing effects to TGFβ1 and –2. The difference in regulation of the growth factor suggests that the TGFβ3 may have a different role to TGFβ1 and TGFβ2 in the lung. A study by Coker *at al* (307) reported on differences between TGFβ1 and TGFβ3 expression in the normal and fibrotic lung. The study showed that TGFβ3 was not detected in mesenchymal cells and TGFβ3 transcript levels from the fibrotic lung tissue were not altered from control lung. This study however has positively identified TGFβ3 mRNA transcripts in fibroblasts.

3.5.4. Proliferation

TGFβ is known to inhibit the proliferation of cells of epithelial origin.
For example, TGFβ2 induces the expression of the CDKI, $p21^{waf}$, at the mRNA and protein level in both human bronchial epithelial cells (25) and type II alveolar epithelial cells (308), to cause growth arrest. Evidence in the literature suggests

that the proliferative effect of TGFβ on mesenchymal cells appears to be dose dependent, with low doses being stimulatory and high doses being inhibitory.

The current study has not shown TGFβ2 to be a mitogen, indeed the results conclude that 0.4nM TGFβ2 acts to prevent the proliferation of fibroblasts. It has also been shown that 0.4nM TGFβ2 induced the highest degree of fibroblast differentiation. In order for a cell to differentiate, the cell must first stop dividing. It is suggested therefore that the lack of proliferative activity in response to TGFβ2 is necessary to promote the transformation of the fibroblast into a myofibroblast. It would have been interesting to determine whether myofibroblasts can indeed proliferate further or whether they are a terminal phenotype. Dual staining of αSMA, to indicate differentiation, with a cell cycle progression marker such as cyclin D, would go some way to answer this.

3.5.5. Conclusions

The TGFβ induced effects on TGFβ receptor and ligand expression in primary bronchial fibroblasts identified in this study are novel. Few studies are so focused on the effects of one growth factor or have the facilities to utilise such an extensive bank of primary material. The results described in this chapter are important in comprehending the regulation of TGFβ receptors and to promote a better understanding of signalling in the asthmatic airways.

To summarise, the regulation of the TGFβ receptors were complex at the mRNA level, but did not alter the ability of TGFβ1 to bind to the cells at the time point studied. The up-regulation of TGFβ ligand in response to TGFβ2 may permit an enhanced fibrotic response to the initial release of TGFβ, which can then further promote the phenotypic change from fibroblast to myofibroblast. The overall conclusion to the work presented here is that there is no major difference in the phenotype of normal and asthmatic fibroblasts in their response to TGFβ2, suggesting that the increased number of myofibroblasts and matrix accumulation seen in asthmatic airways is predominantly a response by the fibroblasts to the increased deposition of TGFβ.

3.5.6. Novel Findings

- TGFβ2 increased αSMA mRNA and protein in normal and asthmatic fibrblasts, no difference between the two groups

- Basal αSMA expression was lower in the asthmatic fibroblasts at the mRNA level but not the protein level.

- TGFβ2 increased TGFβRI mRNA in asthmatic but not normal fibroblasts

- TGFβ2 decreased TGFβRII mRNA in asthmatic but not normal fibroblasts

- Although changes in TGFβ receptor mRNA were observed in the asthmatic fibroblasts in response to TGFβ2, stimulation with TGFβ2 did not alter the ability of TGFβ1 to bind to the fibroblasts.

- TGFβ2 induces TGFβ1 and TGFβ2 mRNA, protein data was inconclusive

- TGFβ2 at 0.4nM weakly suppressed the basal proliferation of normal and asthmatic fibroblasts

CHAPTER 4

Co-ordinate Effects of TGFβ2 and EGF on Bronchial Fibroblasts

CHAPTER 4

CO-ORDINATE EFFECTS OF TGFβ2 AND EGF ON BRONCHIAL FIBROBLASTS

Results from the previous chapter showed that bronchial fibroblasts can respond to and synthesise TGFβ. The ability of bronchial fibroblasts to respond to EGF in terms of differentiation has not been well documented, although EGF is a recognized mitogen for mesenchymal cells. The aim of the chapter was to examine in detail the co-ordinate effects of EGF and TGFβ2 on bronchial fibroblasts. As there are a number of reports describing the antagonistic nature of TGFβ2 and EGF, it was postulated that EGF acts to antagonise the effects of TGFβ on bronchial fibroblasts. It was predicted therefore that EGF would prevent the differentiation of fibroblasts and induce them to proliferate.

Immunolocalisation studies have revealed that EGF is primarily expressed in bronchial glands (273;309), although there is weak expression in the bronchial epithelium and smooth muscle (275). Interestingly, stronger EGF immunoreactivity has been demonstrated in the bronchial epithelium, glands, smooth muscle (275) and submucosa (167) of asthmatic subjects compared to normals.

Hypothesis: EGF antagonises the effects of TGFβ2 in bronchial fibroblasts.

4.1. Effect of EGF on TGFβ2 induced αSMA mRNA Expression

There are no published data regarding the direct effects of EGF on the regulation of αSMA in lung fibroblasts. A study by Kirkland *et al*, (310) showed that HB-EGF, another ligand for the EGFR, did not induce αSMA expression. Furthermore, HB-EGF was shown to strongly inhibit the TGFβ1 induced increase in αSMA in the fibroblast cell line MRC-5. As the ligands for the EGFR commonly perform similar functions in cells, it was postulated that EGF could prevent fibroblasts from differentiating into fibroblasts in the presence of TGFβ2.

In order to study the effect of EGF on the TGFβ2 induced increase in αSMA gene expression, 8 normal and 15 asthmatic fibroblast cultures were treated for 24 hours with TGFβ2 and EGF, alone or in combination. RNA was extracted from the fibroblast cultures, and gene expression was measured semi-quantitatively using RT-PCR and normalised to 18S rRNA.

The results from chapter 3 (fig. 3.1) demonstrated that 0.4nM TGFβ2 caused a concentration dependent increase in αSMA after 24 hours. Upon treatment with EGF, αSMA expression in the normal fibroblasts appeared to be suppressed below basal levels (fig. 4.1). EGF alone suppressed αSMA expression in 11 of the 15 asthmatic subjects tested, although this observation did not reach significance ($p=0.069$).

EGF appeared to slightly suppress the TGFβ2 induced increase in αSMA, although again not significantly. The expression pattern was very similar in the asthmatic fibroblasts. Treatment with EGF did suppress the TGFβ2-induced increase in αSMA expression in the asthmatic fibroblasts ($p=0.01$). Although EGF suppressed αSMA mRNA expression, the combination of TGFβ2 and EGF however, still significantly induced αSMA mRNA expression above that observed for the SFM control ($p=0.046$).

To determine whether EGF could suppress the TGFβ2 induced mRNA expression of αSMA over a longer period than 24 hours; a 5-day time course was performed on a representative normal (fig. 4.2A) and asthmatic (fig. 4.2B) fibroblast culture. The fibroblast cultures were re-fed every 2 days, consistent with the protocol used for the proliferation assays, thus diminishing the contribution of autocrine growth factors being released into the media. TGFβ2 induction of αSMA was time dependent, reaching a maximum 60-fold increase at day 3 in the normal fibroblast culture. αSMA mRNA expression then decreased almost back down to SFM levels by day 5. The combination of TGFβ2 and EGF induced a moderate 20-fold increase by day 2. After replenishing the media with growth factor, there was a rapid increase in αSMA expression by day 3 (95-fold induction relative to day 0),

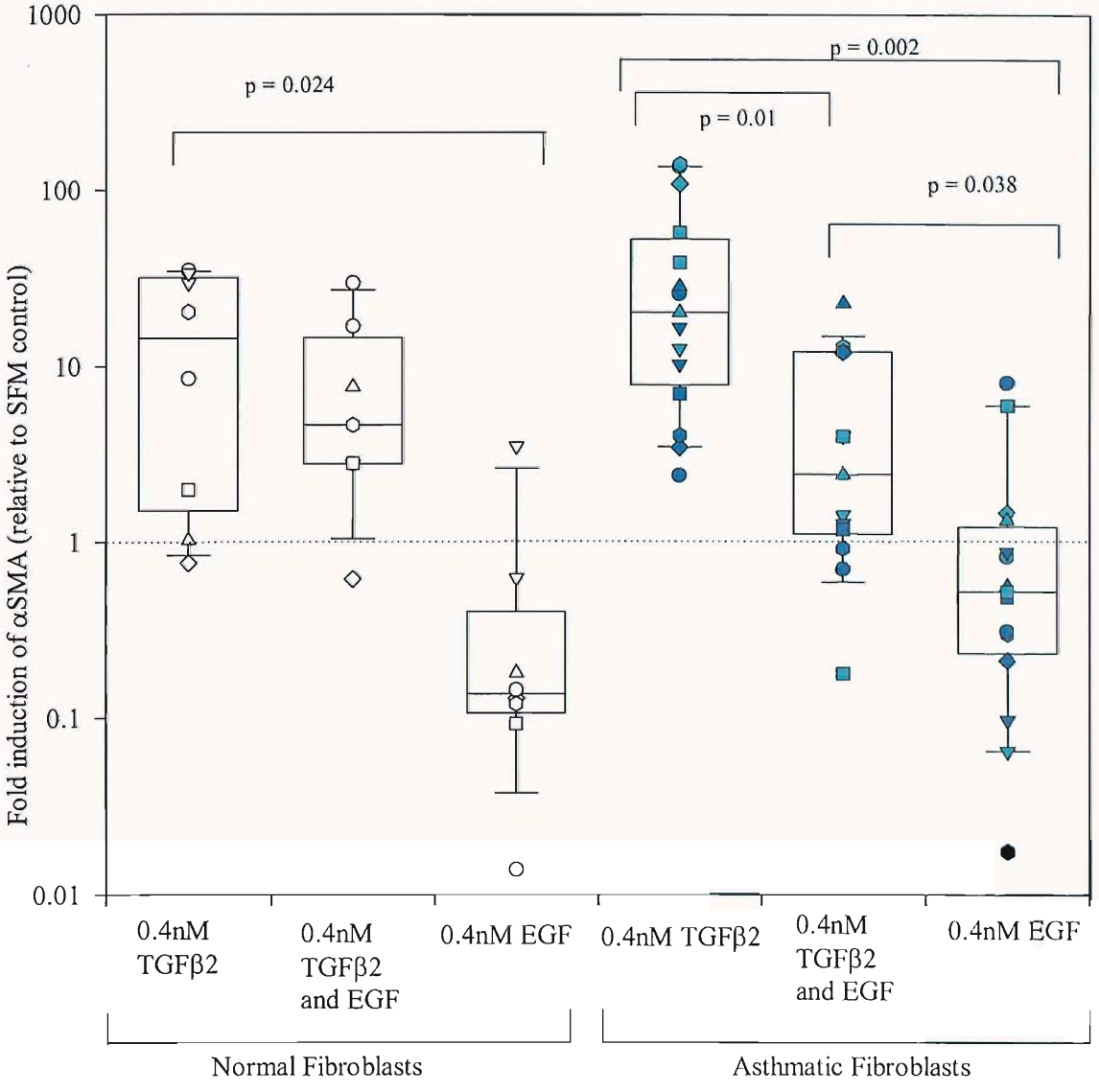


Figure 4.1. EGF suppresses TGFβ2 induced αSMA expression in fibroblasts

Normal (n=7), mild (n=7) and moderate severe asthmatic (n=8) fibroblasts were treated for 24 hours with TGFβ2 and EGF. αSMA mRNA expression was measured by RT-PCR with a Biorad Icycler and expression was normalised against 18S rRNA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile from normal and asthmatic fibroblasts. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols. A Mann Whitney U test was performed to determine inter group differences upon treatment with TGFβ2 and/or EGF. None of the treatment groups were significantly different.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.008$) and asthmatic ($p=0.000$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

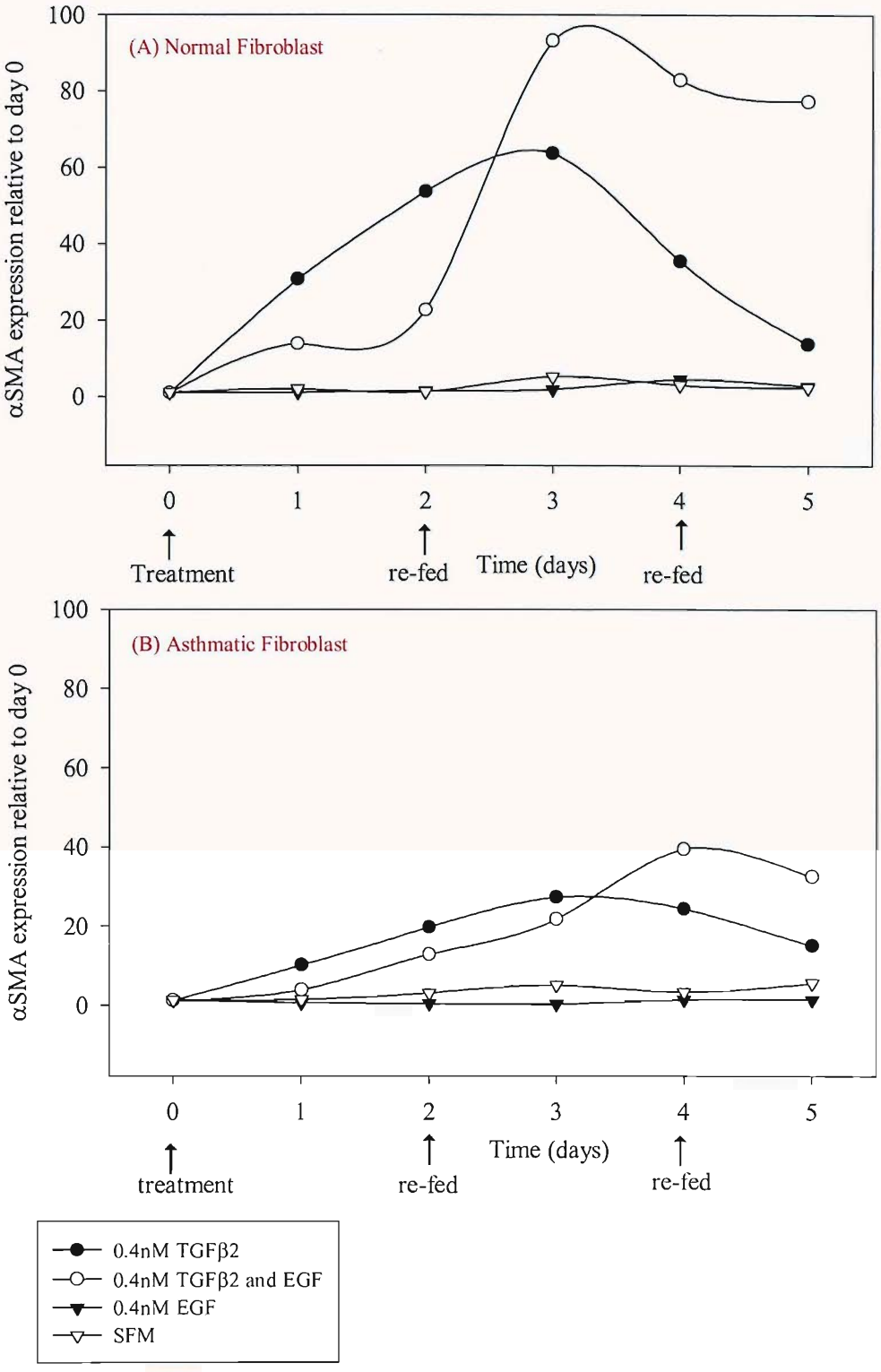


Figure 4.2: αSMA expression in response to prolonged stimulation with TGFβ2 and EGF

A representative normal (A) and asthmatic (B) fibroblast culture was treated for five days in total, re-feeding every two days. αSMA mRNA was measured by RT-PCR using a Biorad Icycler and gene expression normalised against 18SrRNA. The data plotted are the average of results repeated in duplicate. The data are internally consistent with data normalised to day 0.

higher than that experienced with TGFβ2 treatment alone. After 4 days the expression level decreased slightly and then began to plateau. Neither EGF alone nor SFM mediated any change in αSMA expression at any time point studied.

The expression pattern of αSMA induction in the asthmatic fibroblast culture (fig. 4.2B) by TGFβ2 and EGF was, again, similar to that described in the normal culture. The greatest induction with TGFβ2 and EGF occurred at day 4, with a 40-fold increase in αSMA. In both cases, the initial treatment of TGFβ2 and EGF appeared to prime the cells, allowing an exaggerated response to replenishing the media with growth factor two days later. This suggests that EGF delays rather than antagonises the TGFβ2 induced differentiation of fibroblasts. These results therefore do not support hypothesis that EGF antagonises all the actions of TGFβ2 in bronchial fibroblasts.

4.2. Effect of EGF on TGFβ2 induced αSMA Protein Expression

Results from chapter 3 (fig. 3.2), demonstrated that TGFβ2 induced filamentous αSMA protein expression by day 5. To determine the effect of EGF on TGFβ2 induced αSMA expression at the protein level, fibroblasts were treated with TGFβ2 and EGF, alone or in combination for up to 5 days. Immunofluorescent staining was performed on the fibroblasts using αSMA antibodies indirectly conjugated to FITC. It was postulated from the mRNA data that EGF would initially prevent the expression of αSMA but by day 5 the expression levels would be similar or even higher than that seen in fibroblasts treated with TGFβ2 and EGF.

Representative photomicrographs of the TGFβ2 treated normal fibroblasts revealed that there was a time dependent increase in the number of αSMA positive cells, with few αSMA cells at day 1 but after 5 days the majority of cells were αSMA positive (fig. 4.3). The induction of αSMA was delayed with the combination of TGFβ2 and EGF, with few αSMA positive cells present by day 3. By day 5, αSMA was strongly expressed, with levels comparable to treatment with TGFβ2

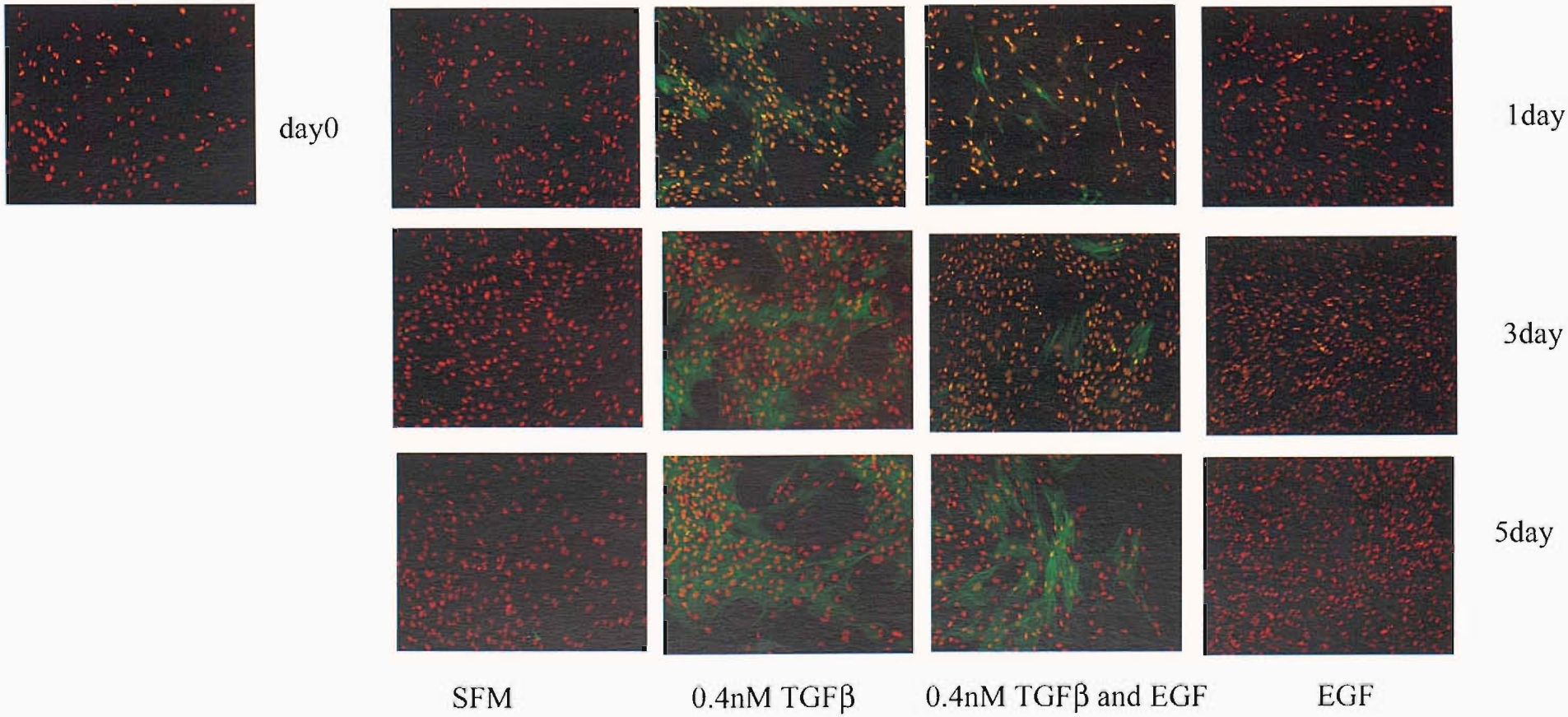


Figure 4.3: EGF initially suppresses TGFβ2 induced expression of αSMA in normal fibroblasts

A representative normal fibroblast culture was serum starved for 24 hours and then treated with TGFβ2, EGF or a combination of TGFβ2 and EGF for 1, 3 or 5 days, refeeding on days 2 and 4. αSMA protein was detected using an antibody raised against αSMA (Sigma A2547, diluted 1:500). A FITC conjugated secondary antibody was used to visulise the protein expression (green staining).Nuclei were counter-stained with 7-aminoactinomycin D (red staining). Digital images were taken with a Hamamatsu camera (x10 magnification) using a Leica DM-RBE microscope and analyzed using the Wasabi analysis programme (Hamamatsu, GMHB).

alone. In agreement with the mRNA time course data, there was no αSMA staining in either EGF treated fibroblasts or in the SFM control. In the representative asthmatic fibroblast culture, the expression of αSMA after treatment with TGFβ and EGF was very similar to that seen in the normal culture (see fig. 4.4). The main difference noted between the cultures was the presence of a small number of αSMA positive cells as a result of SFM treatment for 5 days. Subsequent staining experiments suggested that this was a subject dependent phenomena rather than a normal-asthma difference.

To summarise, EGF initially delayed the mRNA and protein expression of αSMA in bronchial fibroblasts. However, after prolonged exposure (5 days) this suppression was overcome and TGFβ2 induced αSMA expression despite the presence of EGF. The findings after 24 hours appear to support the hypothesis that EGF antagonises the effects of TGFβ2 in bronchial fibroblasts, however the day 5 results reveal that the suppression is time dependent.

4.3. Connective Tissue Growth Factor (CTGF) expression

Connective Tissue Growth Factor (CTGF) has been implicated in fibrosis and has been demonstrated to induce fibronectin and collagen in cultured fibroblasts (311). It has been shown previously that TGFβ can induce CTGF expression in fibroblasts (312), although the combined effects of TGFβ and EGF have not been studied in relation to CTGF expression. In light of the αSMA data it was postulated that over 5 days, EGF at an equimolar concentration would fail to suppress the TGFβ2 induced increase in CTGF.

A time course of CTGF mRNA expression (fig. 4.5) showed that TGFβ2 induced CTGF in normal fibroblasts, with an initial peak at 24 hours and then a further increase at day 3. The combination of TGFβ2 and EGF induced CTGF expression at 24 hours, at a level above that induced by TGFβ2 alone. A similar peak was seen at day 3, but unlike the effect induced by TGFβ2, the combination sustained the expression levels at both day 4 and 5. Neither EGF nor the SFM control altered the expression levels of CTGF during the 5 days in culture.

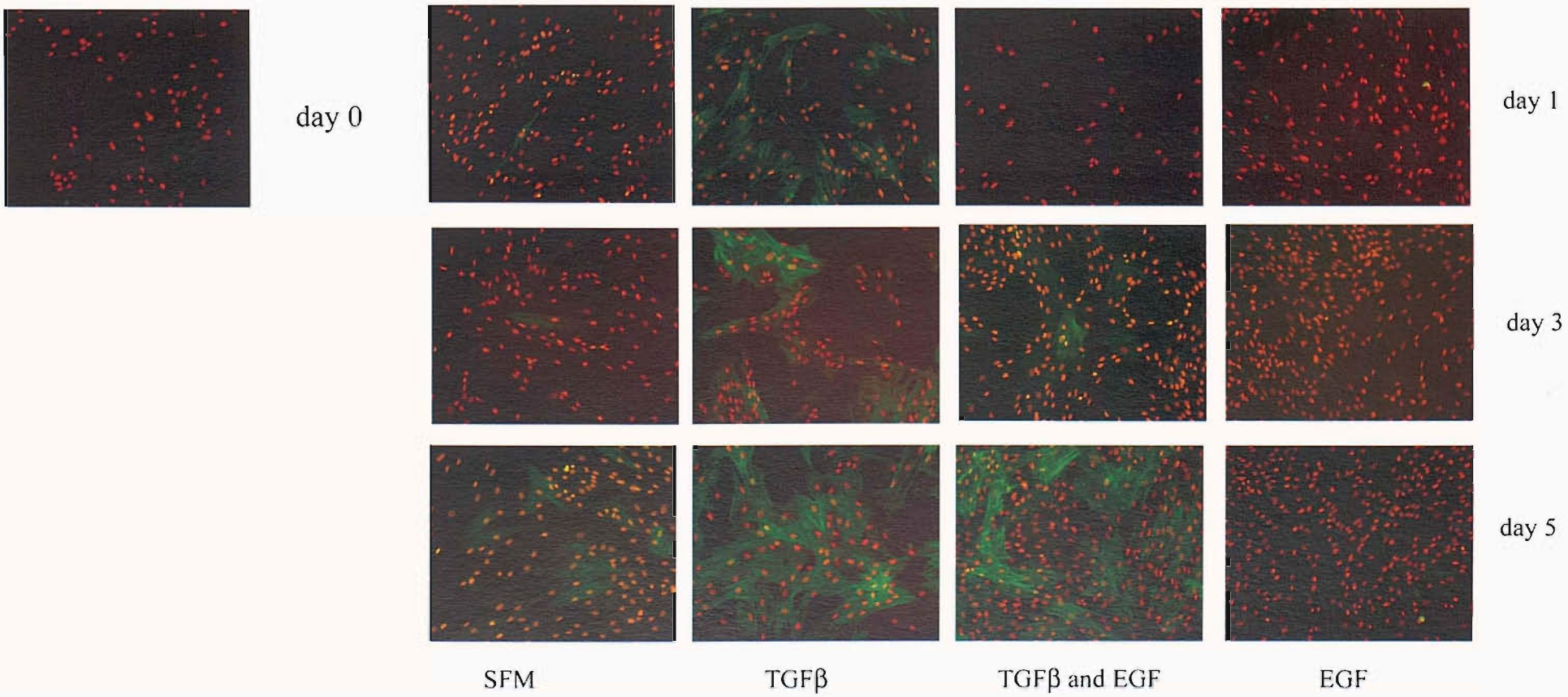


Figure 4.4: EGF initially suppresses TGFβ2 induced expression of αSMA in asthmatic fibroblasts

A representative asthmatic fibroblast culture was serum starved for 24 hours and then treated with TGFβ2, EGF or a combination of TGFβ2 and EGF for 1, 3 or 5 days, refeeding on days 2 and 4. αSMA protein was detected using an antibody raised against αSMA (Sigma A2547, diluted 1:500). A FITC conjugated secondary antibody was used to visulise the protein expression (green staining).Nuclei were counter-stained with 7-aminoactinomycin D (red staining). Digital images were taken with a Hamamatsu camera (x10 magnification) using a Leica DM-RBE microscope and analyzed using the Wasabi analysis programme (Hamamatsu, GMHB).

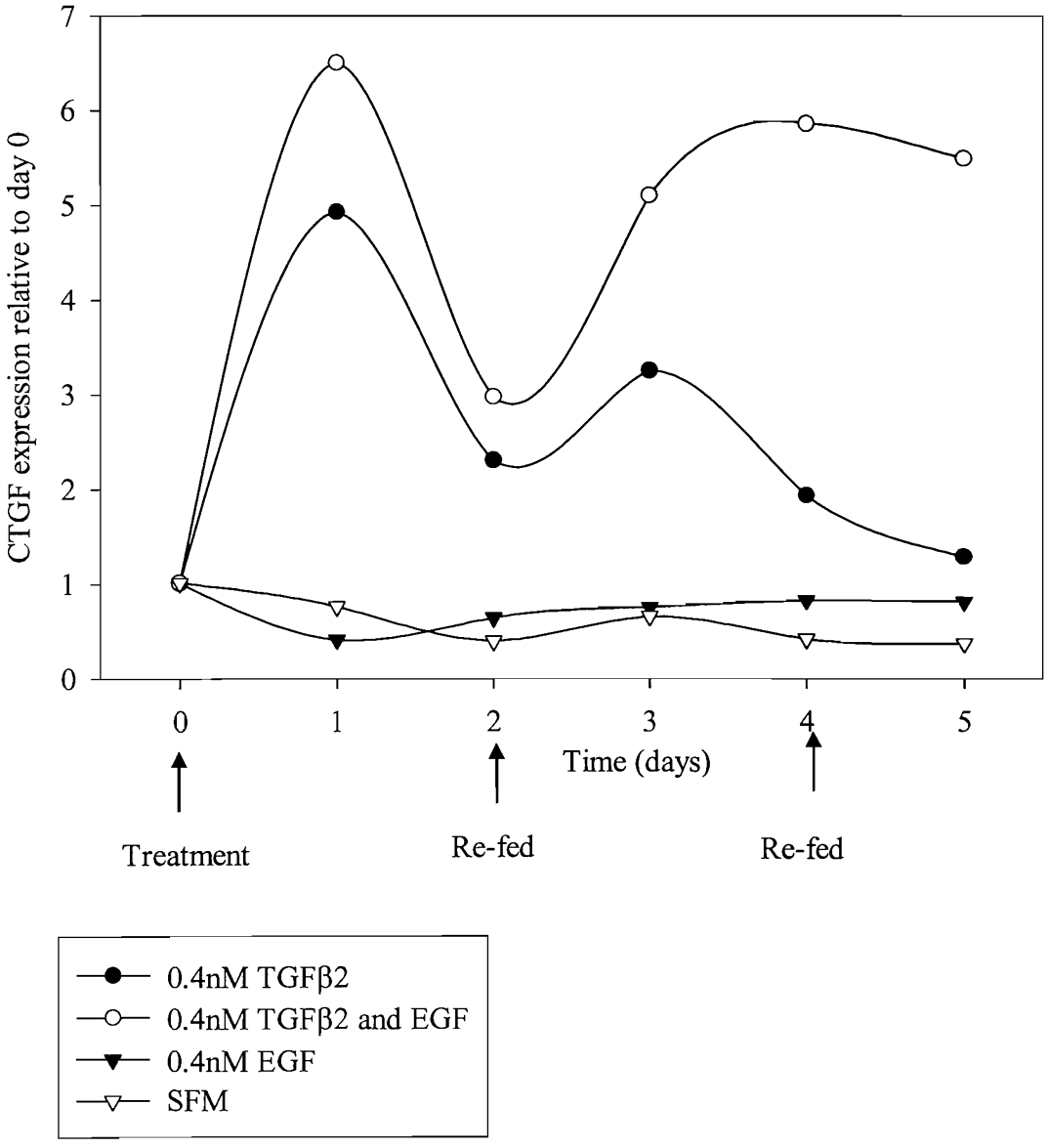


Figure 4.5: CTGF expression over time in representative normal fibroblast culture

A representative asthmatic fibroblast culture was treated for five days in total, refeeding every two days. CTGF mRNA was measured by RT-PCR using a Biorad Icycler and gene expression normalised against 18S rRNA. The data plotted are the average of results repeated in duplicate.

Induction of CTGF mRNA, in response to TGFβ2 and EGF for 24 hours, was further studied to elucidate disease related differences in response to the combination of growth factors (fig. 4.6). The results showed that TGFβ2 and EGF induced a significant increase in CTGF mRNA in both normal and asthmatic fibroblasts. Interestingly, the basal levels of CTGF were lower in the asthmatic fibroblasts compared to the normal fibroblasts ($p=0.029$), similar to the expression data for αSMA and TGFβ RI.

4.4. Effect of EGF on TGFβ Receptor expression

Data presented in chapter 3 revealed that normal and asthmatic fibroblasts express all three subtypes of TGFβ receptor and that TGFβ2 was demonstrated to regulate expression of the receptors at the mRNA level. There are no reports in the literature as to the effect of EGF on TGFβ receptor expression in fibroblasts. It was postulated that EGF could delay the early induction of αSMA by modulating TGFβ signalling at the receptor level.

Results from the previous chapter revealed that 0.4nM TGFβ2 caused a significant increase ($p=0.01$, fig. 3.6) in TGFβ RI in asthmatic fibroblasts ($n=15$). Neither treatment with EGF alone or in combination with TGFβ2, resulted in a significant change in the basal expression level of TGFβ RI mRNA in the normal or asthmatic fibroblasts (fig. 4.7). EGF appeared to suppress the TGFβ2 induced increase of TGFβ RI in the asthmatic fibroblasts, a finding that almost reached significance ($p=0.055$).

Previously (fig. 3.7), it was demonstrated that there was a trend for suppressed TGFβ RII expression in the normal and asthmatic fibroblasts in response to TGFβ2. The combined treatment of 0.4nM TGFβ2 and EGF (fig. 4.8) lifted the TGFβ2 induced suppression of TGFβ RII in the normal fibroblasts ($p=0.046$). Treatment with EGF did not alter the expression level of TGFβ RII above basal levels in either the normal or asthmatic fibroblasts.

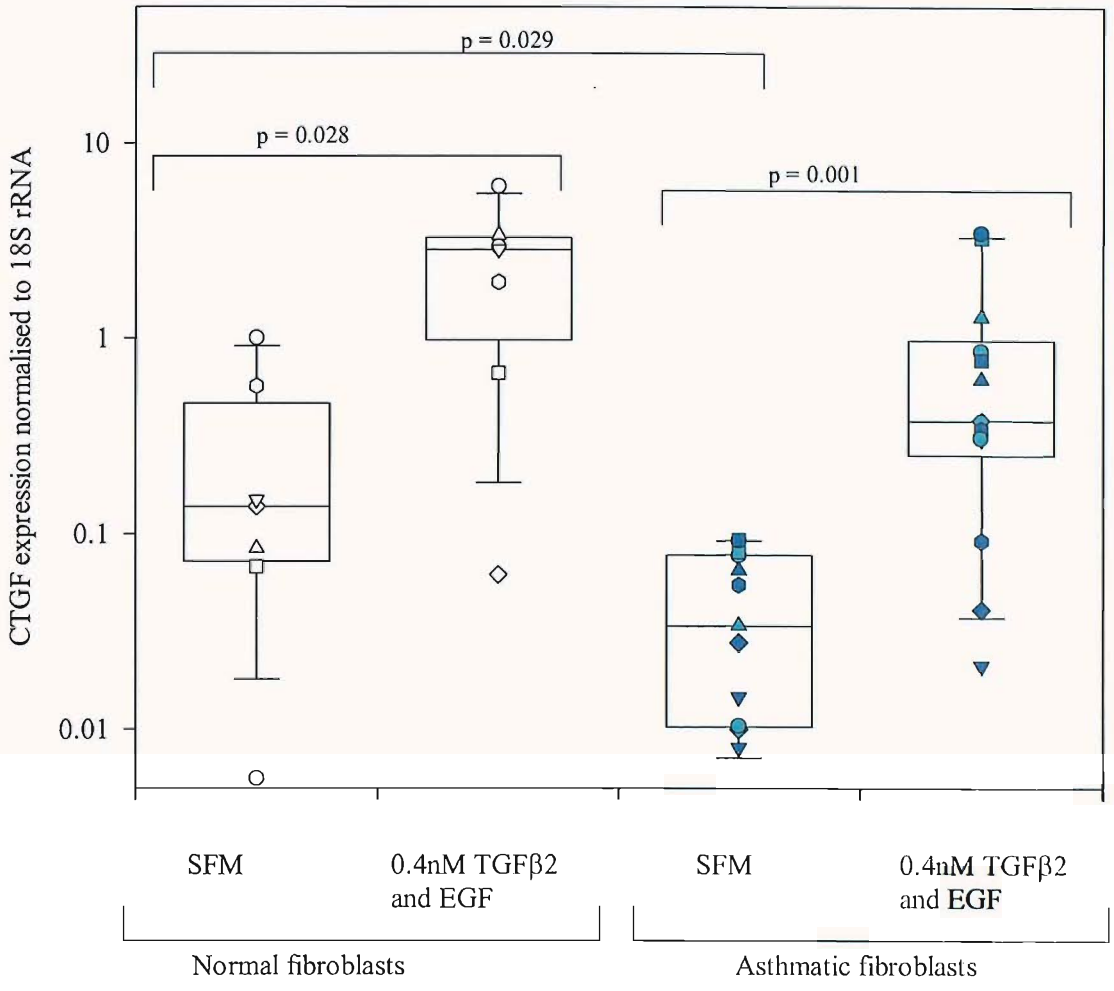


Figure 4.6: CTGF is induced by TGFβ2 and EGF in bronchial fibroblasts

Normal (n=7), mild (n=5) and moderate severe asthmatic (n=8) fibroblasts were treated for 24 hours with TGFβ2 and EGF. CTGF mRNA expression was measured by RT-PCR with a Biorad Icycler and expression was normalised against 18S rRNA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols. Wilcoxon Signed Ranks tests were utilised to determine intra-group statistical comparisons and Mann Whitney U tests were used to measure inter-group differences. Statistical comparisons where $p<0.05$ were noted on the graph.

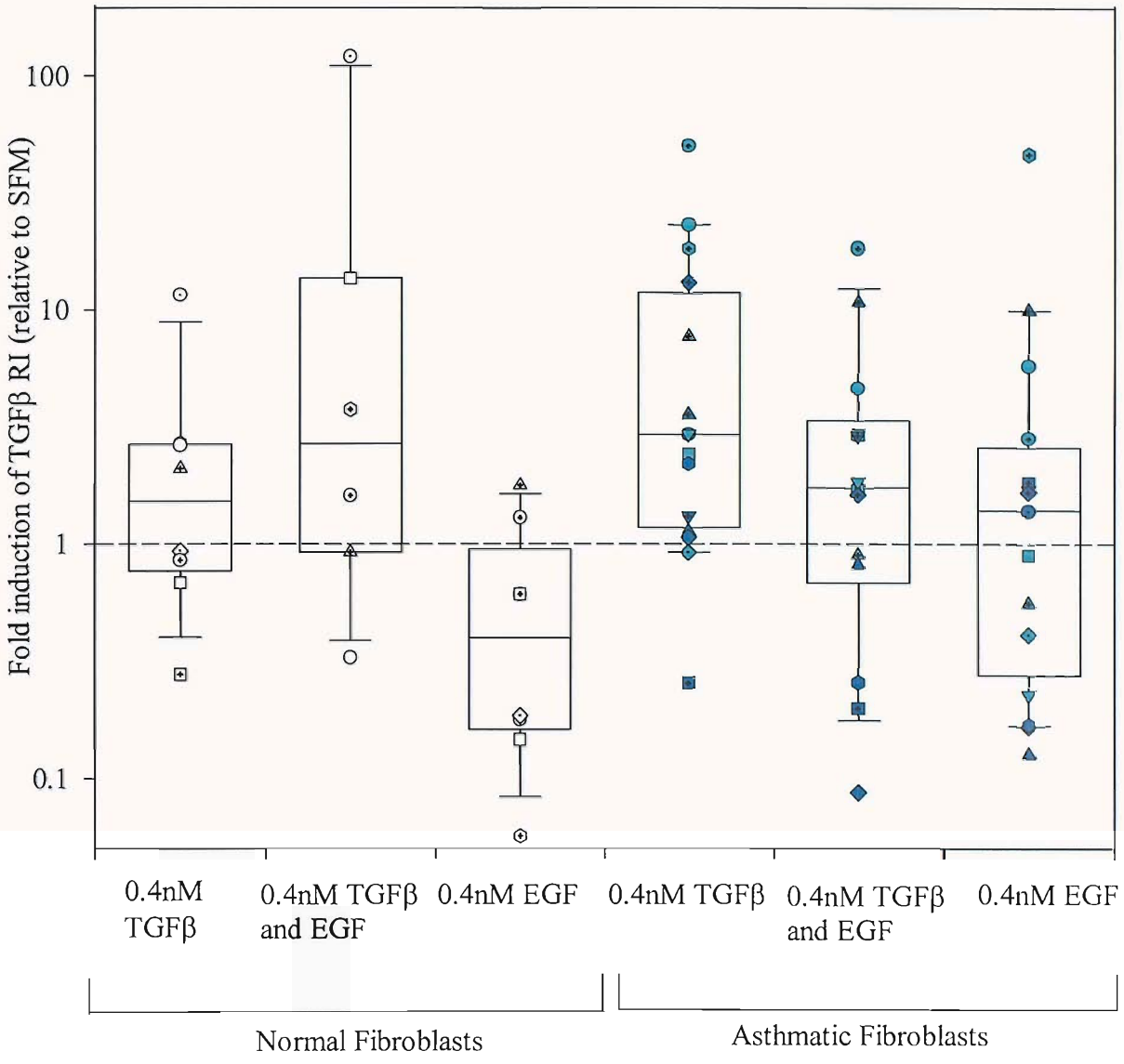


Figure 4.7: EGF does not significantly alter the expression levels of TGFβ RI

Normal and asthmatic fibroblasts were treated for 24 hours with TGFβ2 and/or EGF. TGFβ RI mRNA expression was measured by RT-PCR using a Biorad Icycler and normalised against 18S rRNA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile from normal (n=6-8), and mild asthmatic (n=7) and moderate severe (n=8) asthmatic fibroblasts. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols. A Mann Whitney U test was performed to determine inter group differences upon treatment with TGFβ2 and/or EGF. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there was no significant difference within either the normal (p=0.311) or the asthmatic group (p=0.166).

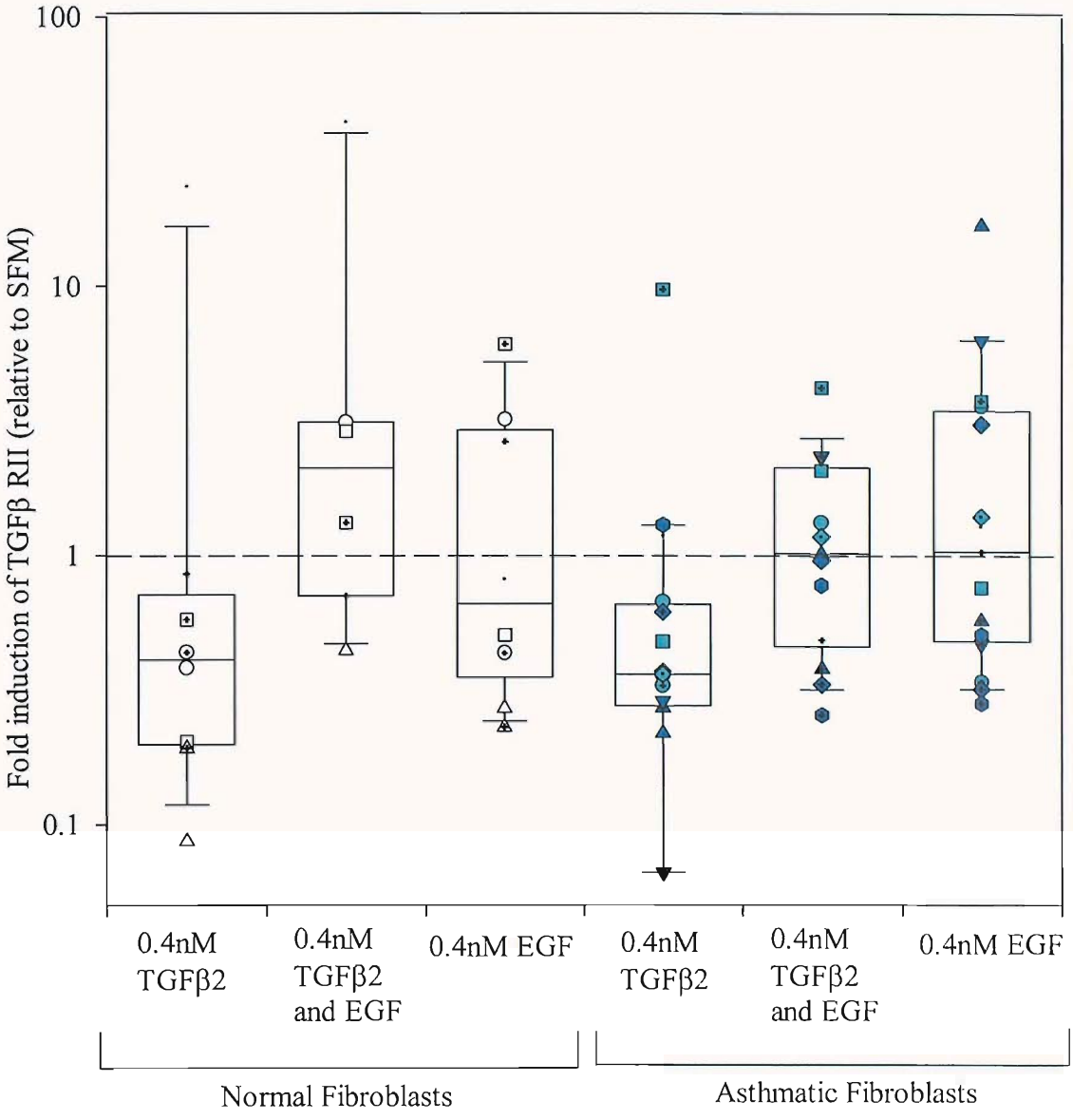


Figure 4.8: EGF does not significantly alter the expression of TGFβ RII

Normal and asthmatic fibroblasts were treated for 24 hours with TGFβ2 and/or EGF. TGFβ RII mRNA expression was measured by RT-PCR on a Biorad Icycler and normalised against 18SrRNA

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile from normal (n=6-8), mild asthmatic (n=5-7) and moderate severe (n=8) fibroblasts. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols. A Mann Whitney U test was performed to determine inter group differences upon treatment with TGFβ2 and/or EGF. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there was no significant difference within either the normal (p=0.135) or the asthmatic group (p=0.199).

Betaglycan (TGFβ RIII) is a binding protein for TGFβ. Previous results (fig. 3.8) showed that there was a non-significant trend whereby 0.4nM TGFβ2 caused a decrease in the level of betaglycan after 24 hours. The combined treatment of 0.4nM TGFβ2 and EGF appeared to further decrease in betaglycan expression (fig. 4.9) in normal fibroblasts when compared to the TGFβ2 treatment. EGF alone appeared to suppress the mRNA expression of betaglycan further still in the normal fibroblasts. These observations lacked statistical power due to the small number of samples analysed.

To summarise, the effect of TGFβ2 and EGF on receptor expression was dependent on receptor subtype. EGF alone did not alter the expression of TGFβ RI or RII. In combination with TGFβ2, EGF prevented the TGFβ2 dependent effects, revealing EGF as the dominant growth factor in this instance. These results show that EGF antagonised the effects of TGFβ RI and RII, supporting the hypothesis. Although EGF prevented the TGFβ2 induced suppression of betaglycan in asthmatic fibroblasts, EGF induced the greatest suppression of betaglycan in normal fibroblasts. These results show that treatment with both TGFβ2 and EGF independently can result in the same outcome, although in this example, EGF was more effective at equimolar concentrations of growth factor.

4.5. Effect of EGF on TGFβ ligand expression

Results from chapter 3 revealed that TGFβ1 is synthesised and released from bronchial fibroblasts although the intricacies surrounding the regulation of gene expression and release in response to EGF have not been elucidated. As the combined treatment of TGFβ2 and EGF promoted an enhanced induction of both αSMA and CTGF, it was postulated that this was in response to an exaggerated release of TGFβ1 in response to EGF and TGFβ2.

TGFβ1 mRNA and protein was induced upon treatment with TGFβ2 (fig. 3.12 and 3.15) in both normal and asthmatic fibroblasts. A kinetic experiment (day 0 - 5) was performed on a representative fibroblast culture from one normal and one

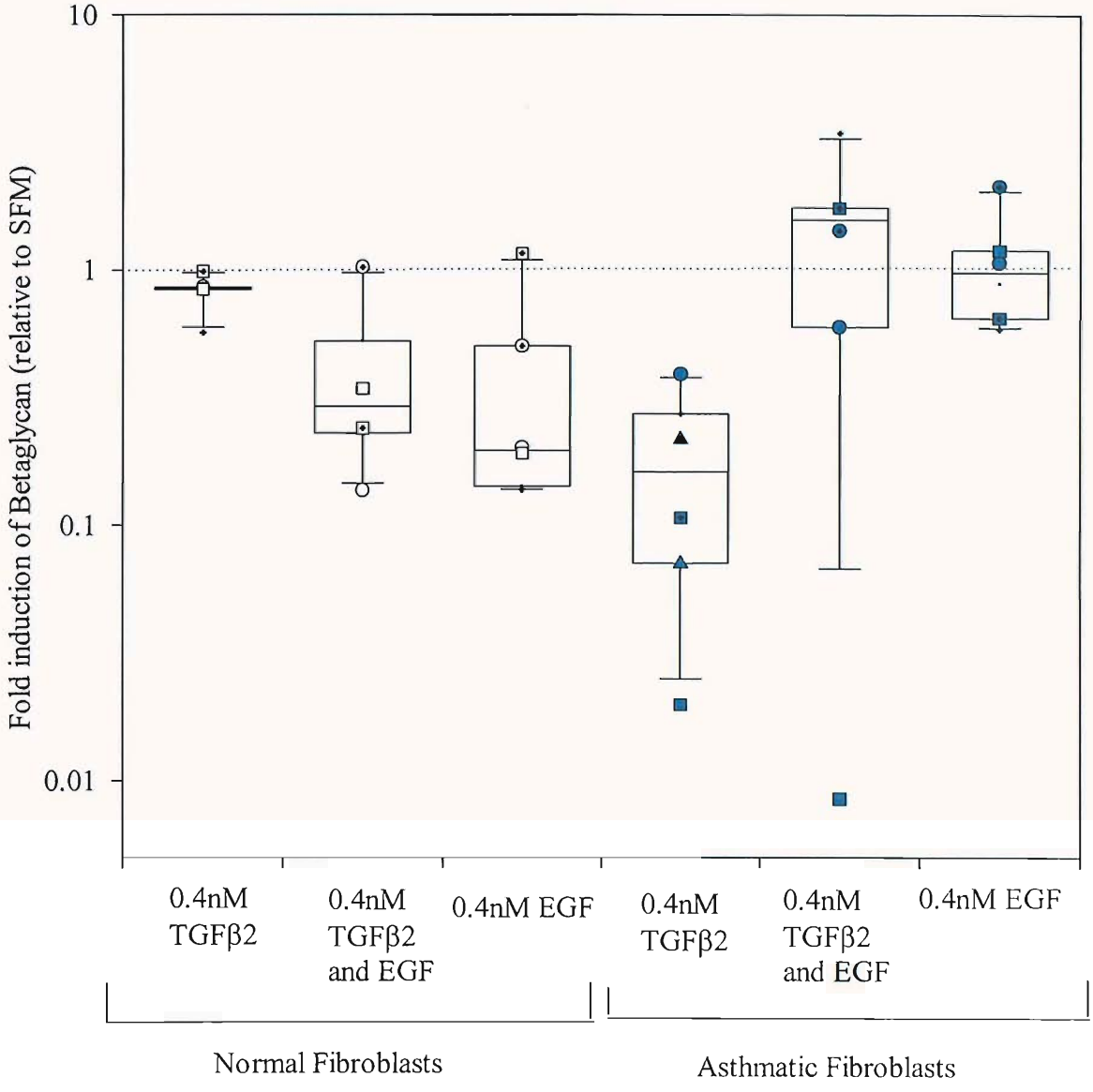


Figure 4.9: EGF does not significantly alter expression levels of betaglycan in fibroblasts

Normal (n=6) and asthmatic (n=6-7) fibroblasts were treated for 24 hours with TGFβ2. Betaglycan expression was measured using RT-PCR and normalised against 18S rRNA using the standard curve method (ABI Prism 7700 sequence detection Taqman machine).

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, data from asthmatic subjects as blue symbols. A Mann Whitney U test was performed to determine inter group differences upon treatment with TGFβ2 and/or EGF. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there was no significant difference within either the normal (p=0.115) or the asthmatic group (p=0.174).

asthmatic subject to determine the effect of EGF on TGFβ1 expression (fig. 4.10). The cultures were re-fed on day 2 and 4, consistent with the proliferation assays carried out in chapter 3. In accordance with previous results, TGFβ2 initially (24 hours) induced TGFβ1 expression by 2 or 3 fold. After replenishing the media on day 2, there was a slight increase in expression in the asthmatic fibroblasts, but not in the normal fibroblasts. As predicted, the strongest stimulus to induce TGFβ1 in both cultures was the combination of TGFβ2 and EGF. The combination resulted in an initial peak in expression at 24 hours and then again at 3 days in response to replenishing the media on day 2. The initial increase was approximately 2 fold in the normal fibroblasts and 4 fold in the asthmatic fibroblasts. The second peak was higher than the first peak, with a 3 fold induction in the normal fibroblasts and a 5 fold induction in the asthmatic fibroblasts. Neither EGF nor SFM induced TGFβ1 at any of the time points measured.

As the combination of TGFβ2 and EGF in the time course experiment induced the greatest increase in TGFβ1, the effect of TGFβ2 and EGF on TGFβ1 ligand expression was examined in more detail (fig. 4.11). In a group of 5 normals and 6 asthmatics studied, 0.4nM TGFβ and EGF induced a significant increase in TGFβ1 expression in normal ($p=0.043$) and asthmatic ($p=0.028$) fibroblasts after a 24-hour period.

4.6. EGF induced Proliferation

It was shown previously that EGF does not suppress the TGFβ-induced expression of αSMA after prolonged exposure. Indeed, I have provided evidence that EGF in combination with TGFβ2 can in fact exaggerate the induction of αSMA and CTGF, possibly due to an enhanced release of TGFβ1. EGF is a known mitogen for mesenchymal cells. It was postulated that EGF would induce bronchial fibroblasts to proliferate. As TGFβ2 was the dominant growth factor in respect to αSMA expression, it was predicted that TGFβ2 would suppress the EGF induced proliferation of bronchial fibroblasts.

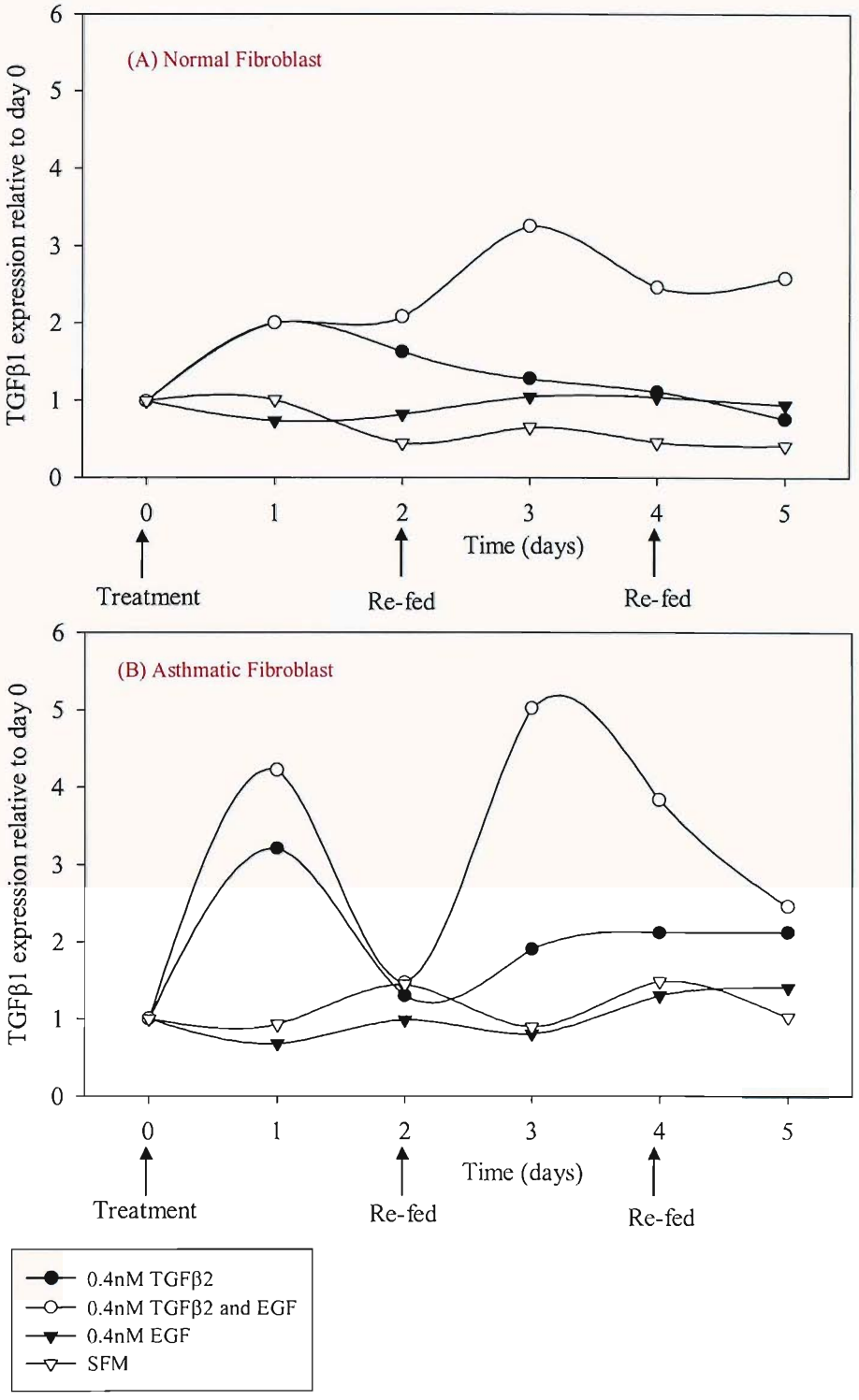


Figure 4.10: TGFβ1 expression in response to prolonged stimulation with TGFβ2 and EGF

A representative normal and asthmatic fibroblast culture were treated for five days in total, re-feeding every two days. TGFβ1 mRNA was measured by RT-PCR using a Biorad Icycler and gene expression normalised against 18SrRNA. The data plotted are the average of results repeated in duplicate. Each data set are internally consistent with data normalised to day 0.

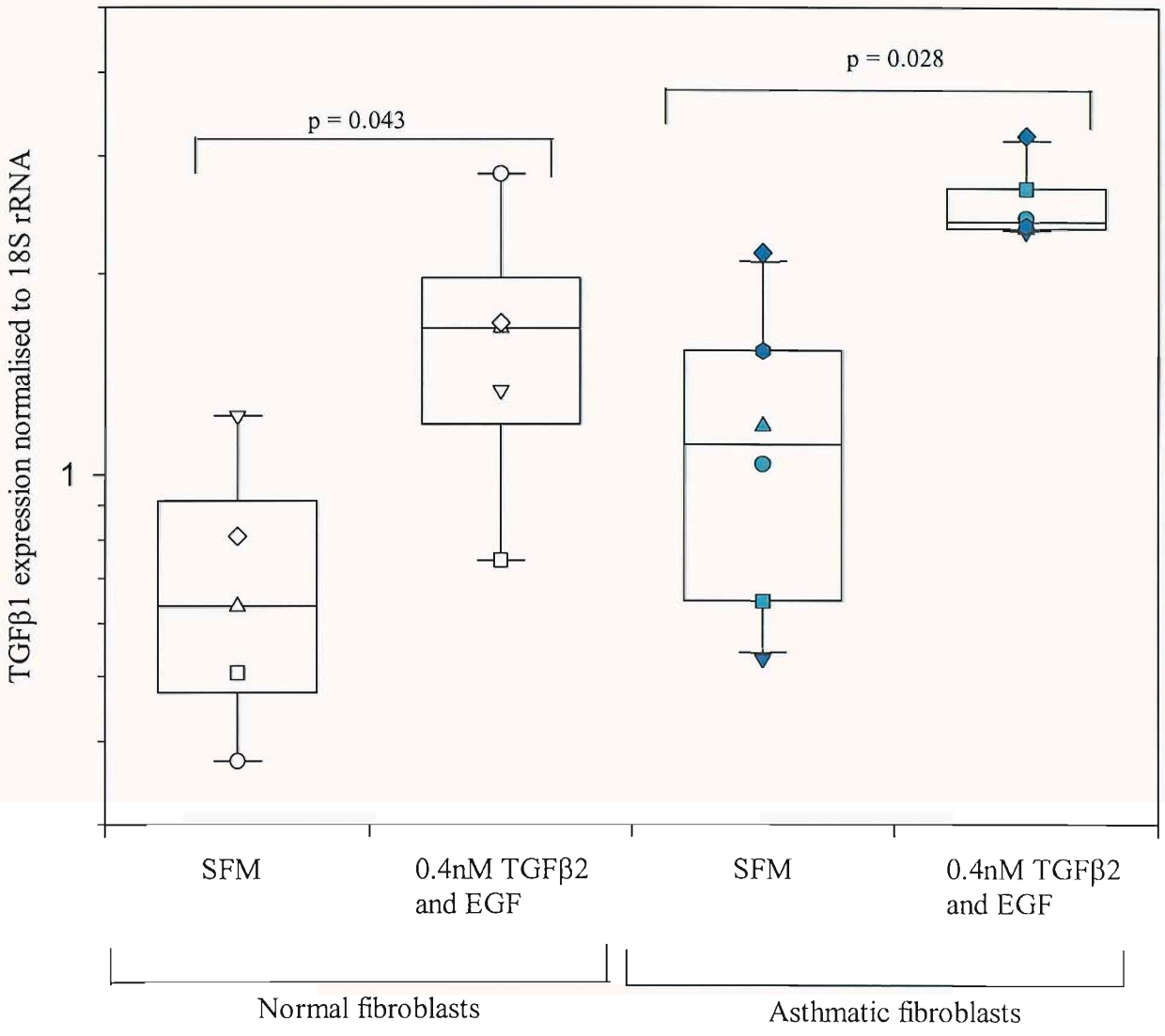


Figure 4.11: TGFβ1 is induced by the combination of TGFβ2 and EGF

Normal (n=5), mild asthmatic (n=3) and moderate severe asthmatic (n=3) fibroblasts were treated with TGFβ2 for 24 hours. TGFβ1 expression was measured using RT-PCR and normalised using 18S rRNA

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, mild asthmatics turquoise symbols and moderate asthmatics blue symbols.

Wilcoxon Signed Ranks tests were utilised to determine intra-group statistical comparisons, Mann Whitney U tests were employed to measure inter-group differences. Statistical comparisons where $p<0.05$ were noted on the graph.

4.6.1. Morphological effects of TGFβ2 and EGF on bronchial fibroblasts

Fibroblasts were fixed in formal saline after treatment and subsequently stained with methylene blue. Before the dye was eluted to determine a measurement of cell biomass, cultures were photo-documented to reveal changes in morphology.

There were no changes in morphology after growth factor treatment for 1 day. After 3 days, the fibroblasts were becoming morphologically distinct, although the greatest differences in morphology were apparent at day 5. After 5 days, TGFβ2 appeared to cause the cells to contract (fig. 4.12), suggestive of a transformation to a myofibroblast phenotype, as evidenced by the induction of αSMA. Contractile filaments were clearly present in the cells and were aligned in a parallel conformation along the length of the cell. EGF treated cells appeared elongated and spindle-shaped, and were randomly positioned. Fibroblasts treated with TGFβ2 and EGF in combination were myofibroblast-like in appearance with cultures from some subjects capable of contraction. The methylene blue stained cultures showed that the morphological effects promoted by both TGFβ2 and EGF were evident in all subjects, and were independent of disease status. The photomicrographs were also visible proof of the proliferative effects of TGFβ and EGF. In the example shown, there are clearly more fibroblasts after treatment with EGF and fewer cells upon treatment with TGFβ2.

4.6.2. Proliferative effects of EGF over time

Figure 4.13 shows changes in cell biomass of a representative asthmatic fibroblast culture, from day 0 to day 5. EGF acts as a potent mitogen of bronchial fibroblasts causing more than a 3-fold increase above the basal proliferation over 5 days. TGFβ2 had a weak inhibitory effect on basal proliferation. The combination of TGFβ2 and EGF resulted in an increased proliferative response, although to a lesser degree than EGF alone. As the greatest proliferative difference between the treatments was seen at 5 days, subsequent studies to compare growth related differences in fibroblast cultures isolated from normal or asthmatic subjects were performed at this later time point.

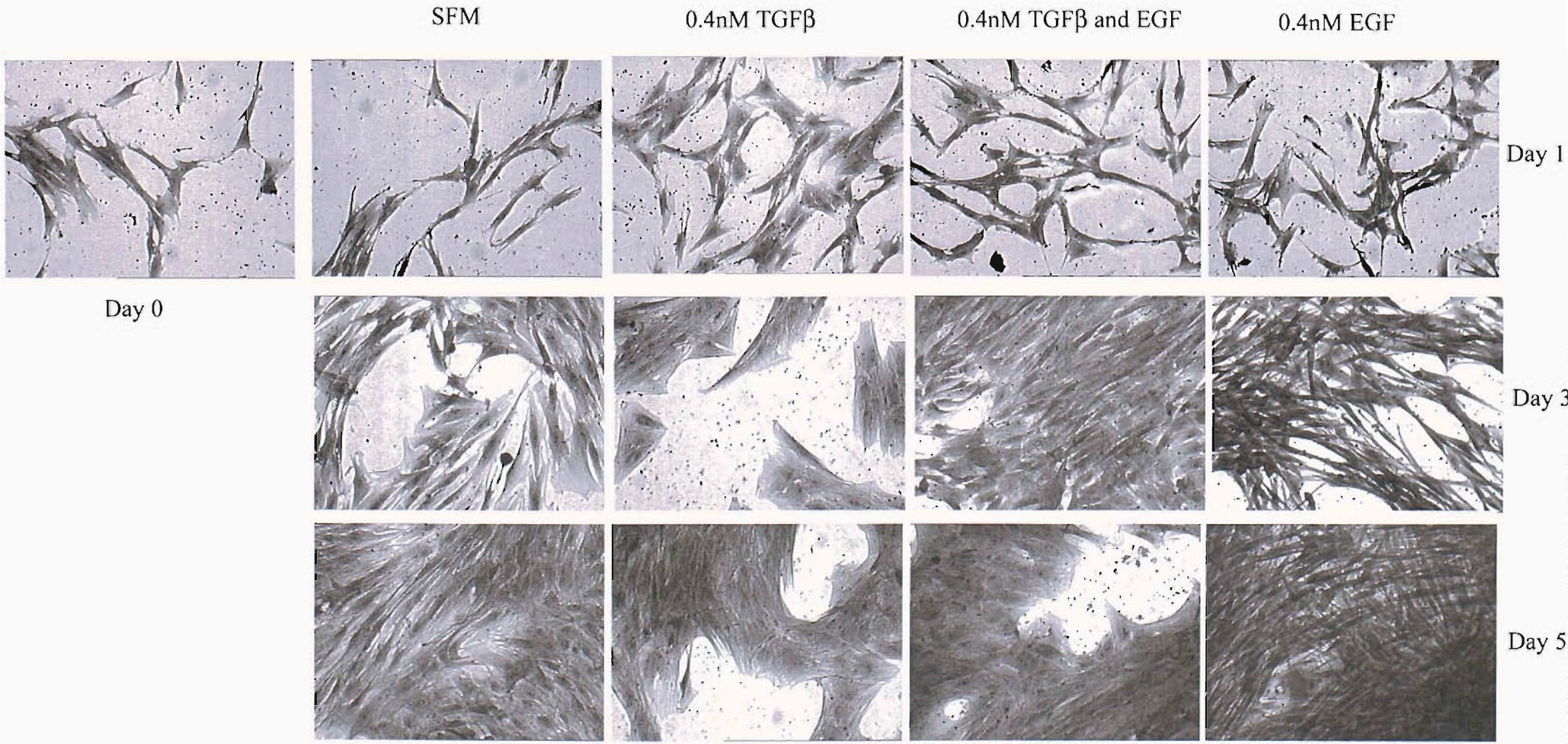


Figure 4.12: Growth factor induced morphological changes in bronchial fibroblasts

Fibroblasts were plated at 8,000 cells/well in a 24 well plate in DMEM (10% FBS). After 24 hours the fibroblasts were serum starved for 24 hours. The fibroblasts were then treated with TGFβ2, EGF or a combination of TGFβ2 and EGF for 1,3, or 5 days. The cells were refed on day 2 and day 4. The fibroblasts were then fixed in formal saline and then stained with methylene blue. Photographs were taken to record treatment induced morphological changes using a Nikon Coolpix digital camera.

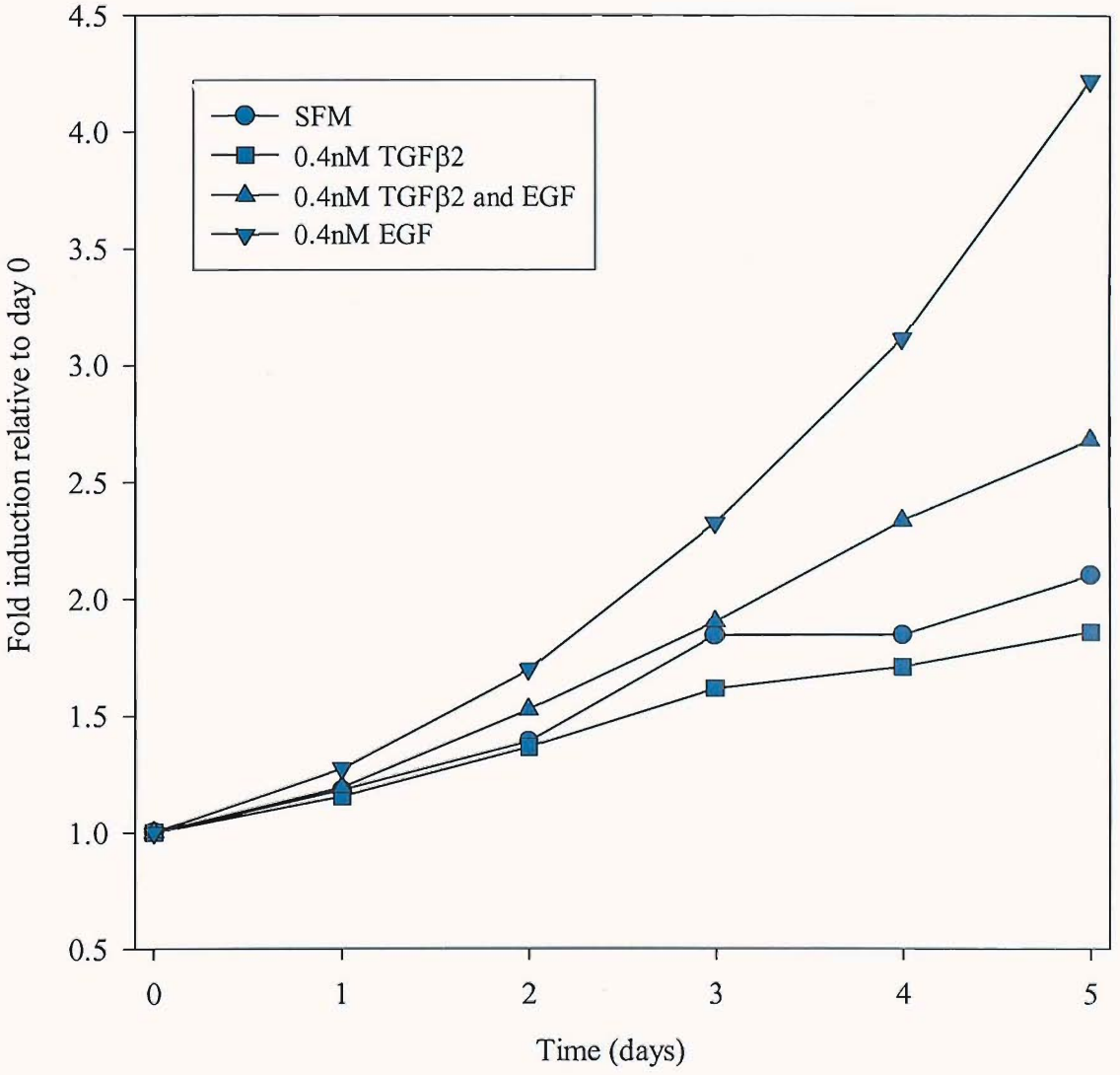


Figure 4.13: Co-ordinate effects of TGFβ2 on EGF induced proliferation

Graph shows the increase in cell biomass of one representative asthmatic fibroblast culture in response to TGFβ2 and EGF using methylene blue elution. Data points were performed in triplicate. Media were replenished on days 2 and 4 and data are plotted as fold induction relative to day 0.

4.6.3. Mitogenic effects of EGF on normal and asthmatic fibroblasts

The majority of studies carried out to determine cellular response to exogenous growth factors are often complicated by the contribution of autocrine/paracrine growth factors produced by the cells themselves. In order to determine the actual contribution of autocrine/paracrine growth factors on the proliferative response, the mitogenesis assay was performed in two ways. Initially the fibroblasts were treated on day 0, with growth factor enriched media replenished on day two and four. These results were compared to results obtained by only treating the cells once on day 0 with equivalent amounts of growth factor. The results show that the trends were similar regardless of the treatment regime (fig. 4.14 and 4.15).

As 0.4nM TGFβ2 was the only dose to cause growth suppression in primary fibroblasts (fig. 3.16), it was the molar concentration chosen to examine the equivalent growth modifying effects of EGF. As predicted, EGF significantly induced fibroblast proliferation (fig. 4.14 and 4.15) above basal levels in both normal and asthmatic fibroblasts. The combination of EGF and TGFβ2 together resulted in increased growth above control levels. Although the TGFβ2 and EGF induced proliferation was significantly less than EGF induced proliferation levels, EGF did overcome the inhibitory effect of TGFβ2. These results indicate that EGF is more dominant than TGFβ2, in that the outcome of the combined treatment was a significant proliferative response. Therefore, in a TGFβ2 and EGF rich environment fibroblasts can both differentiate and proliferate.

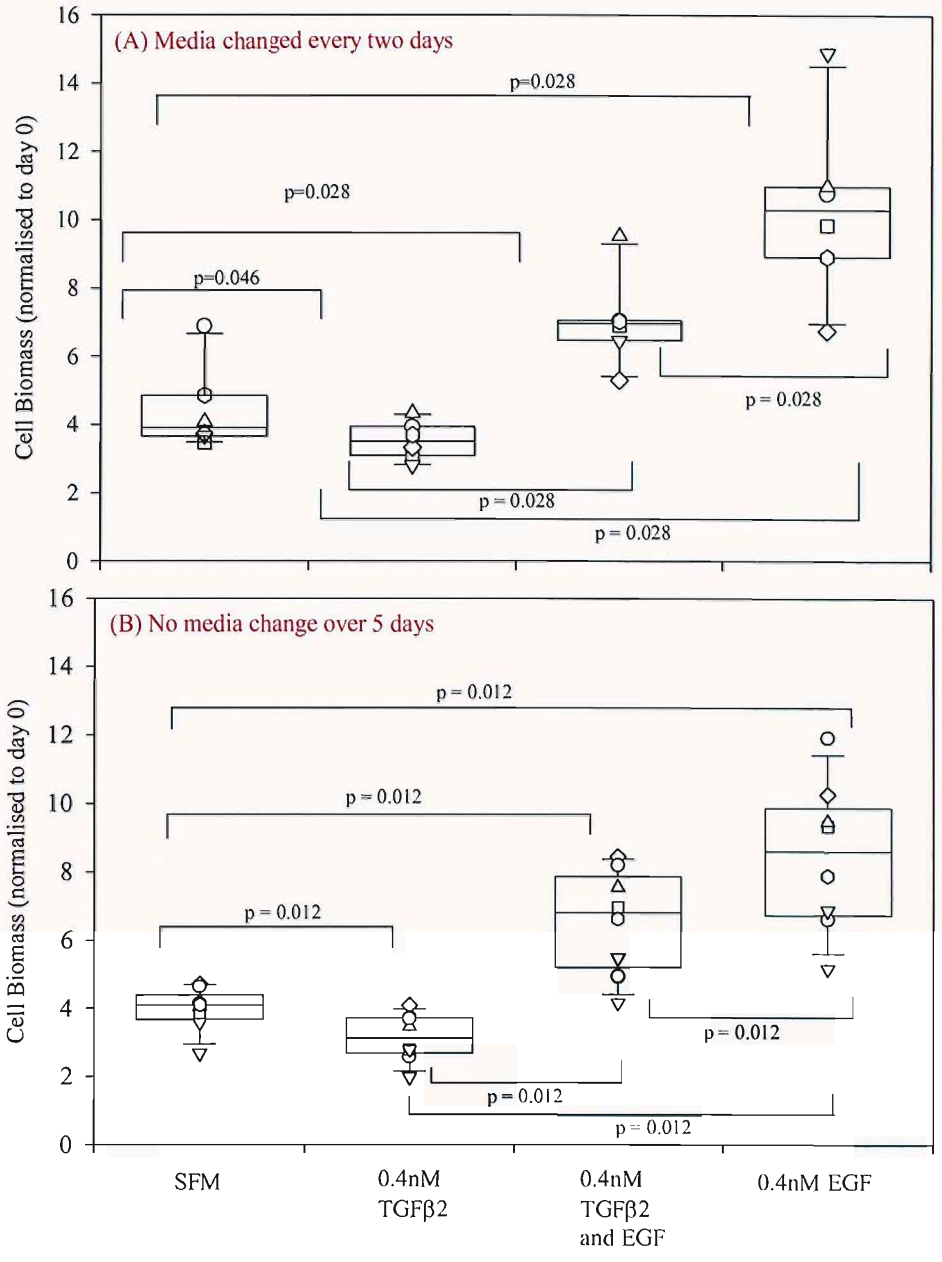


Figure 4.14: EGF overcomes the TGFβ2 induced suppression of proliferation in normal fibroblasts

Normal fibroblasts (n=6) were treated with TGFβ2 and EGF for 5 days. Media was replenished on days 2 and 4 (A). In comparison, normal fibroblasts (n=8) were treated with just one initial dose of growth factor (B) . The increase in cell biomass, measured by methylene blue elution assay, was comparible regardless of the treatment regime. Data are presented as fold induction relative to day 0 in box plots showing median, interquartile range, 10th and 90th percentile.

A Mann Whitney U test was performed to determine inter group differences upon treatment with TGFβ2 and/or EGF. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both feeding strategy (multiple feeds, p=0.001and one initial dose p=0.000) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

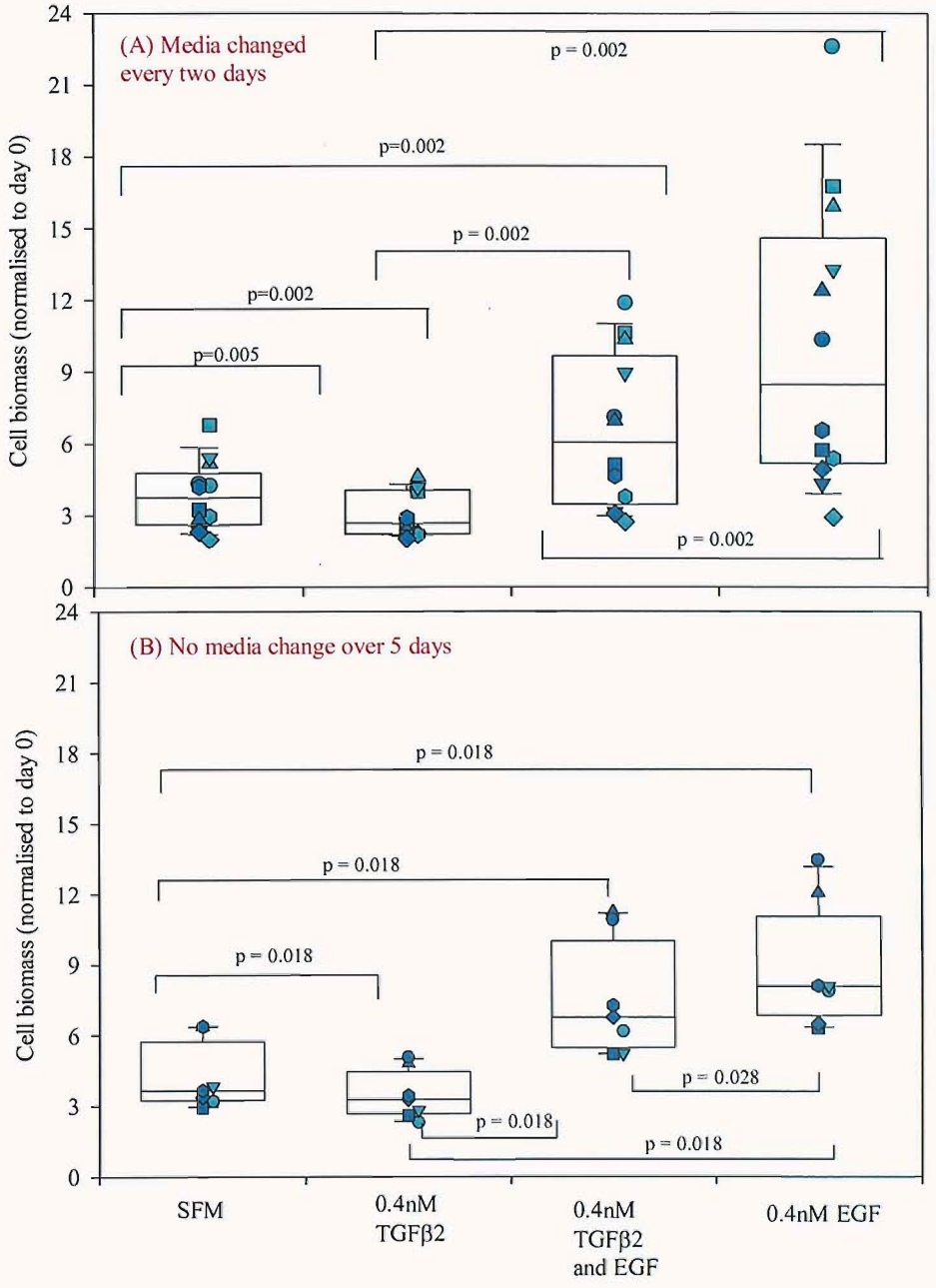


Figure 4.15: EGF overcomes the TGFβ2 induced suppression of proliferation in asthmatic fibroblasts

Mild asthmatic fibroblasts (n=6, turquoise symbols) and moderate severe asthmatic (n=6, blue symbols) fibroblasts were treated with TGFβ2 and EGF for 5 days. Media was replenished on days 2 and 4 (A). In comparison, mild asthmatic fibroblasts (n=2, turquoise symbols) and moderate severe asthmatic (n=5, blue symbols) were treated with just one initial dose of growth factor (B). The increase in cell biomass, measured by methylene blue elution assay, was comparible regardless of the treatment regime. Data are presented as fold induction relative to day 0 in box plots showing median, interquartile range, 10th and 90th percentile.

A Mann Whitney U test was performed to determine inter group differences upon treatment with TGFβ2 and/or EGF. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both feeding strategy (multiple feeds, p=0.000 and one initial dose p=0.000) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where p<0.05were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

4.7. Discussion

A number of studies have suggested that EGFR ligands are expressed at a higher level in asthmatic airways (167;275). This may be a consequence of the chronic inflammation concomitant with remodelled asthmatic airways. For example, EGF is bound to platelets and macrophages release TGFα. Potentially, this is further enhanced in asthma by the increase in angiogenesis in the submucosa which can facilitate the distribution of ligand. Alternatively, the structural cells of the airways such as the fibroblasts and epithelial cells may be responsible for additional expression of EGFR ligands (313;314).

There are many references in the literature describing the antagonistic effects of TGFβ2 and EGF. As a result it was postulated that EGF would antagonise the effects of TGFβ2 in bronchial fibroblasts. The results presented in this chapter suggest that this is not always the case. Indeed, the fibrotic effects of TGFβ2 appeared to be exaggerated by the presence of EGF, and the anti-proliferative effects reversed. It was hypothesised that EGF would prevent the TGFβ2 mediated transformation of fibroblasts into myofibroblasts. The results reveal that this is a very simplistic view. EGF in combination with TGFβ2 is capable of inhibiting αSMA induction at the gene level, after 24 hours. However over a period of 5 days, EGF and TGFβ2 caused the greatest induction of αSMA, above that seen with TGFβ2 alone. The delay in αSMA mRNA correlated with the induction of protein, although little difference could be seen between the number of αSMA positive cells treated with TGFβ2 +/- EGF after 5 days.

To further the study, quantitative data for αSMA expression in TGFβ2 and EGF treated fibroblasts are required. To achieve this aim, flow cytometry would be performed on normal and asthmatic fibroblasts treated initially for 24 hours and then for a longer time point of five days. It is predicted from the immunofluorescent staining data that αSMA protein would be suppressed at the early time point and then expressed at similar levels to TGFβ2 alone at the later time point. Although no differences in expression between the normals and

asthmatics were seen with the immunofluorescent staining, analysis of a greater number of subjects may reveal subtle differences.

Methylene blue proliferation assays were performed throughout the thesis to measure changes in proliferation over time. Methylene blue elution is an excellent way to measure changes in cell biomass although a certain degree of care should be used in interpreting the results. Certain treatments, such as TGFβ2, can induce a morphological change in the appearance of the fibroblast. As myofibroblasts tend to be larger cells than fibroblasts, more methylene blue can be taken up by the cell. As a result, the TGFβ2 mediated inhibition of proliferation is probably greater than that suggested by elution of the dye.

This study revealed that TGFβ2 is growth inhibitory for primary lung fibroblasts, irrespective of disease status. EGF is a known mitogen for fibroblasts and, unsurprisingly induced fibroblasts to proliferate in the present study. Although 0.4nM TGFβ2 attenuated the mitogenic effects of EGF, there were still significantly more cells present with the combined treatment of TGFβ2 and EGF compared to control levels. This suggests that in an environment where EGF predominates, the fibroblasts will proliferate. In a TGFβ rich environment, the fibroblasts will tend to differentiate. The results from this chapter have shown that TGFβ2 treated cells are still capable of proliferation over 5 days. The αSMA immunofluorescent staining showed that only approximately 50% of the TGFβ2 fibroblasts differentiated into myofibroblasts over this time period. These findings suggest that a proportion of TGβ2 treated cells retain their capacity to proliferate in order to replenish the population of myofibroblasts which are postulated to be a terminal phenotype (315).

The results also reveal conflicts of dominance between TGFβ2 and EGF, in that either TGFβ2 or EGF can act as the dominant growth factor, a phenomenon unaffected by disease status. For example, EGF is the dominant growth factor in regulation of TGFβ receptor expression whereas TGFβ2 is dominant in the regulation of αSMA expression. The study also showed that EGF fails to suppress the TGFβ2 induction of TGFβ1 and CTGF expression. The kinetic data for TGFβ1

suggested that the delayed increase in αSMA protein expression after 5 days with TGFβ2 and EGF might be an indirect response to the induction of TGFβ1.

Results from this chapter show that in the presence of TGFβ2 and EGF, fibroblasts can still differentiate into myofibroblasts. *In vivo*, the relative balance of TGFβ2 and EGF will ultimately determine the functional outcome. It is postulated that in asthma there is an enhanced expression of both TGFβ and EGF. This is the worse possible case scenario as TGFβ2 and EGF together can promote a fibrotic environment, with increased numbers of myofibroblasts and fibroblasts, which can potentially contribute to the fibrosis associated with airway remodelling.

4.7.1. Novel Findings

- EGF suppresses TGFβ2 dependent αSMA mRNA and protein at early time points
- At later time points (5days), EGF in combination with TGFβ2 potentiates aSMA mRNA and protein
- EGF and TGFβ2 potentiates CTGF mRNA, no difference between normal and asthmatic fibroblasts
- EGF has no effect on TGFβ receptor mRNA, no difference between normals and asthmatics
- EGF and TGFβ2 potentiates TGFβ2 mRNA, no difference between normals and asthmatics
- 0.4nM TGFβ2 suppresses EGF stimulated proliferation in normal and asthmatic fibroblasts, although not to basal levels

CHAPTER 5

Amphiregulin Processing in Bronchial Fibroblasts

CHAPTER 5

AMPHIREGULIN PROCESSING IN BRONCHIAL FIBROBLASTS

It has been proposed that the Epithelial-Mesenchymal Trophic Unit (EMTU) is set up during branching morphogenesis, enabling bidirectional communication between the epithelium and underlying mesenchyme (316). During wound healing the embryonic signalling pathways are recapitulated. In emulating the developmental pathways, TGFβ2 is released from the damaged epithelial cells, promoting the migration of fibroblasts into the area and inducing their differentiation into myofibroblasts. The myofibroblasts are more biosynthetic in nature than the migratory fibroblast and can release a plethora of soluble mediators which can in turn modulate epithelial activity.

The previous two chapters have shown how bronchial fibroblasts can respond to TGFβ2 and EGF in terms of proliferation and differentiation. The aim of this chapter was to study how fibroblasts can communicate with the epithelium by secreting a factor known to specifically cause epithelial mitogenesis. Amphiregulin is an EGFR ligand that exhibits lower potency than other EGFR ligands due to its C-terminal truncation (317). As a result, amphiregulin requires the presence of heparan sulphate proteoglycans, such as syndecans and glypicans, which aid its presentation to the receptor (318;319). The presence of a heparin binding domain (HBD) promotes the specificity of the ligand as it can only mediate its effects on those cells types expressing the correct heparan sulphates. Polosa *et al* (273), demonstrated the presence of amphiregulin in the submucosal glands and bronchial epithelium of the lung, where it co-localised with EGFR.

It is postulated that in response to wounding, AR is released from fibroblasts to selectively induce epithelial repair with minimal fibrosis. This is achieved by binding selectively to the exposed EGFR expressed basolaterally on damaged epithelial cells. In asthma, epithelial damage is observed without immediate repair suggesting that communication between the epithelium and the mesenchyme is compromised. Although there are no published studies identifying differences in

regulation of EGFR ligand release between normal and asthmatic fibroblasts, it is hypothesised that there is an altered regulation of AR production in asthma.

Hypothesis: Asthmatic fibroblasts release less amphiregulin

5.1. EGF induces amphiregulin

The autoinduction of EGFR ligands in fibroblasts has not been studied previously, although there are a number of references in the literature showing EGFR family members to be capable of auto- and cross-induction in other cell types. A study by Barnard *et al* in 1994 revealed that TGFα, AR, HB-EGF and EGF could each induce the expression of TGFα, AR, HB-EGF and EGF mRNA within 30 minutes in an intestinal epithelial cell line, keratinocytes and colon adenocarcinoma cells. Another example of cross induction is provided by a study by Coffey *et al* (320) which showed that EGF can induce TGFα expression in human keratinocytes. The current study was designed to measure the expression of amphiregulin, in response to EGF, in bronchial fibroblasts.

Using RT-PCR, it was demonstrated that amphiregulin mRNA was expressed by both normal (n=5) and asthmatic (n=7) fibroblasts (fig. 5.1). 0.4nM EGF caused a significant induction in amphiregulin mRNA after a 24 hour incubation in fibroblasts from both normals ($p=0.043$) and asthmatics ($p=0.028$), providing evidence of cross-induction. Contrary to expectations, asthmatic fibroblasts produced significantly more amphiregulin ($p=0.019$) in response to EGF stimulation than the normal fibroblasts at the mRNA level.

To determine whether the protein data followed the same trends as mRNA data, release of amphiregulin was measured in fibroblast conditioned medium by ELISA (fig. 5.2). Media collected from both normal and asthmatic fibroblasts, after stimulation with EGF for 24 hours, contained low levels of amphiregulin. In the small set of samples measured, no increase in amphiregulin was seen in response to EGF from either the normal or the asthmatic fibroblasts.

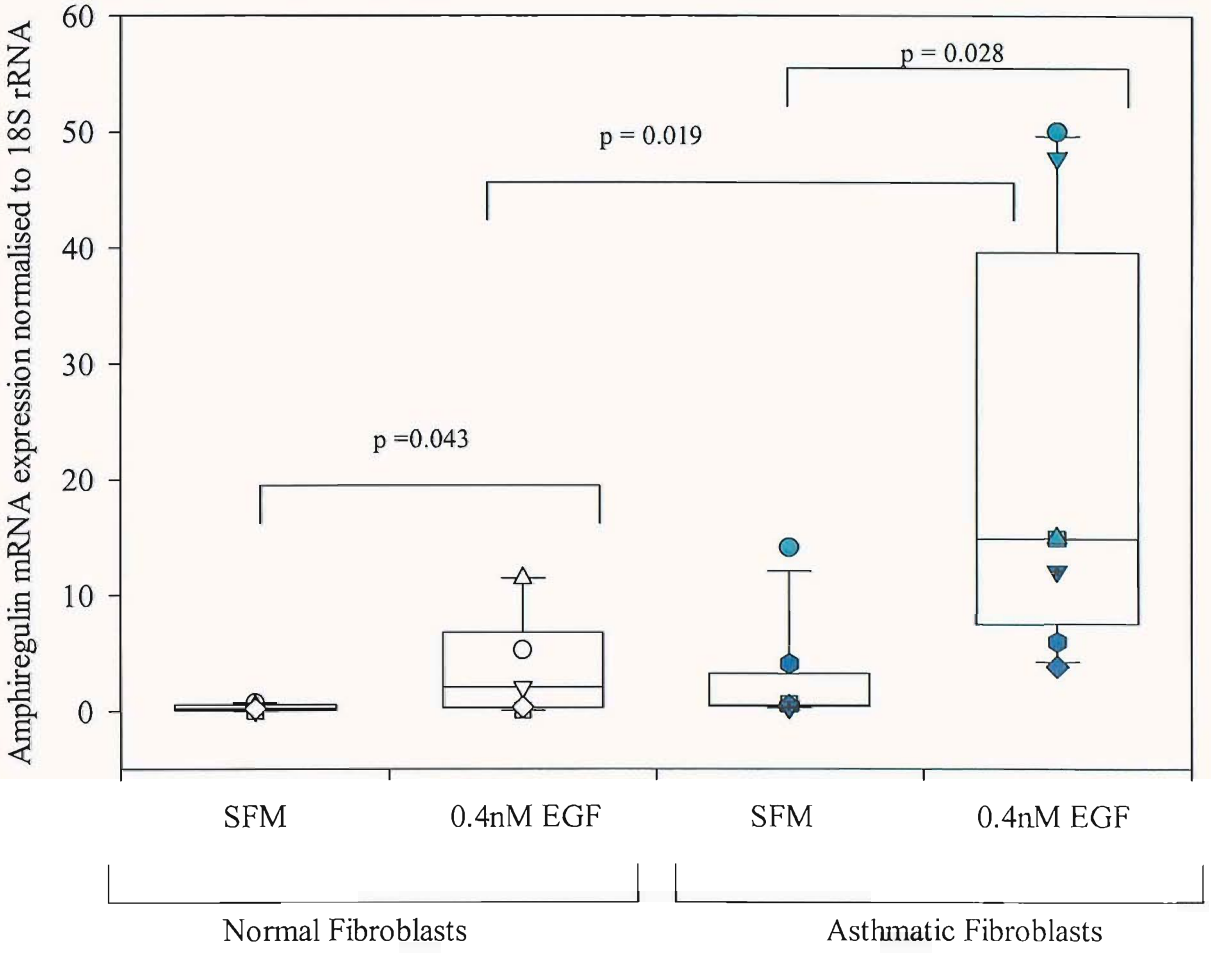


Figure 5.1: EGF induces amphiregulin mRNA in bronchial fibroblasts

Normal (n=5) and mild asthmatic (n=4) and moderate severe asthmatic (n=3) fibroblasts were treated with EGF for 24 hours. Amphiregulin expression was measured by RT-PCR and normalised using 18S rRNA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, data from mild asthmatics by turquoise symbols and moderate severe asthmatic subjects as dark blue symbols. Wilcoxon Signed Ranks tests were used to assign significance to intra-group comparisons, Mann Whitney U tests were used to measure inter-group differences.

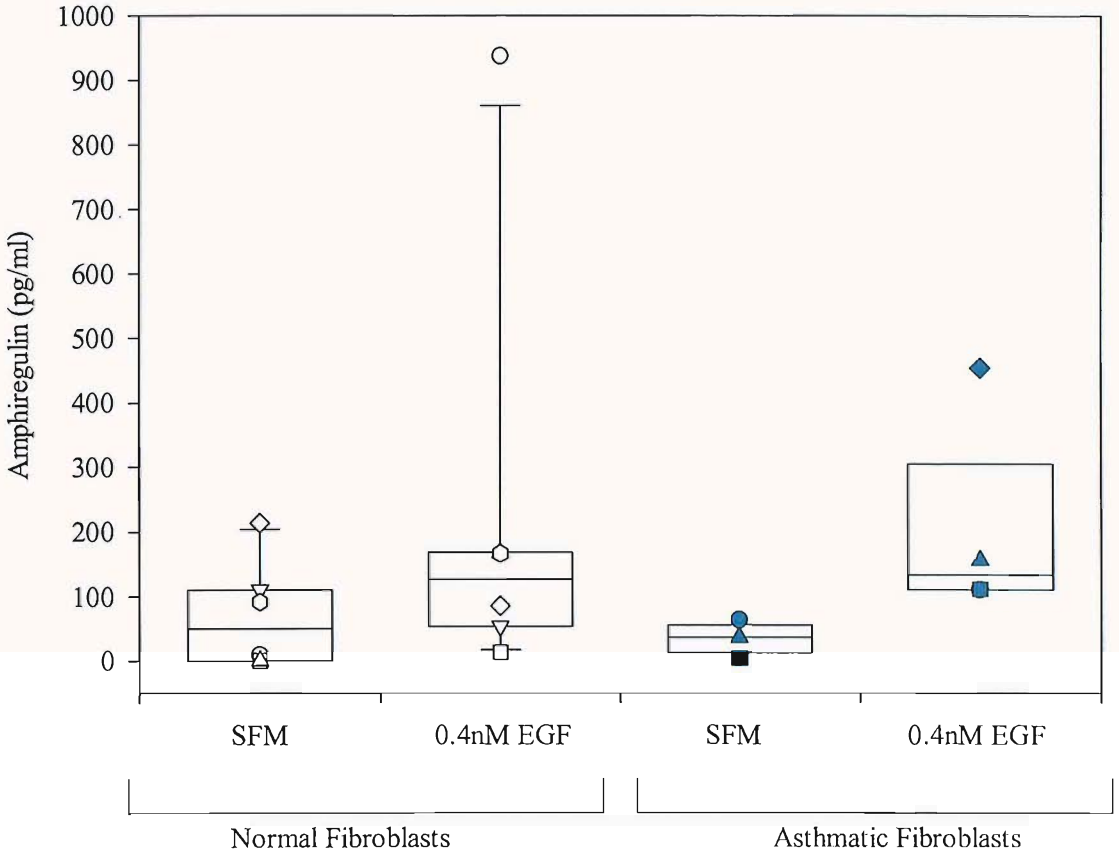


Figure 5.2: Bronchial fibroblasts release amphiregulin

Confluent normal (n=6) and asthmatic (n=3-4) fibroblasts were treated with 0.4nM EGF for 24 hours. Release of mature amphiregulin into the medium was measured using an ELISA purchased from R&D.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data from normal subjects are indicated by unfilled symbols, data from asthmatic subjects as blue symbols. There was no significant increase in release of amphiregulin after treatment with EGF, as determined by a Wilcoxon Signed Ranks Test.

5.2 TGFβ2 prevents EGF induced amphiregulin expression

TGFβ1 has been shown to reduce the expression and release of amphiregulin in the A549 cell line (286). In light of this observation, it was predicted that TGFβ2 would suppress the basal expression of amphiregulin in bronchial fibroblasts. In the current study it was shown that TGFβ2 did not alter the basal expression of amphiregulin at the mRNA level after 24 hours, in either the normal or asthmatic bronchial fibroblasts (fig. 5.3). Furthermore, the fibrogenic growth factor prevented the EGF induced increase in amphiregulin mRNA.

Amphiregulin release was measured in TGFβ treated samples (0.4nM) and in the corresponding SFM control over a time course between 4 and 66 hours in fibroblasts from one normal subject (data not shown). No induction was noted upon treatment with TGFβ2 at any of the time points tested.

5.3. Phorbol Ester (PMA) induces cleavage of amphiregulin

ErbB receptor family ligands, such as amphiregulin, are expressed as membrane bound precursors and undergo cleavage before they are released in their fully active soluble form. Amphiregulin was originally isolated and characterised from phorbol ester (PMA) treated MCF-7 cells (321). PMA has been suggested to induce the translocation of PKCδ from the cytoplasm to the cell membrane where it has a role in activating sheddases such as ADAM proteins (322), although the precise sequence of events leading to the shedding of the growth factor is unknown.

EGF alone was not capable of inducing the cleavage of pro-amphiregulin. It was hypothesised that EGF together with PMA would induce the release of amphiregulin, EGF to stimulate the transcription of amphiregulin mRNA, PMA to stimulate cleavage. Initially, a concentration response was a carried out in the bronchial fibroblasts to determine the concentration of PMA that would cause the greatest release of AR without inducing cell death. A concentration of 10nM was shown to induce optimal release of amphiregulin from both the normal and asthmatic cells (fig. 5.4). At this concentration, minimal change in morphology

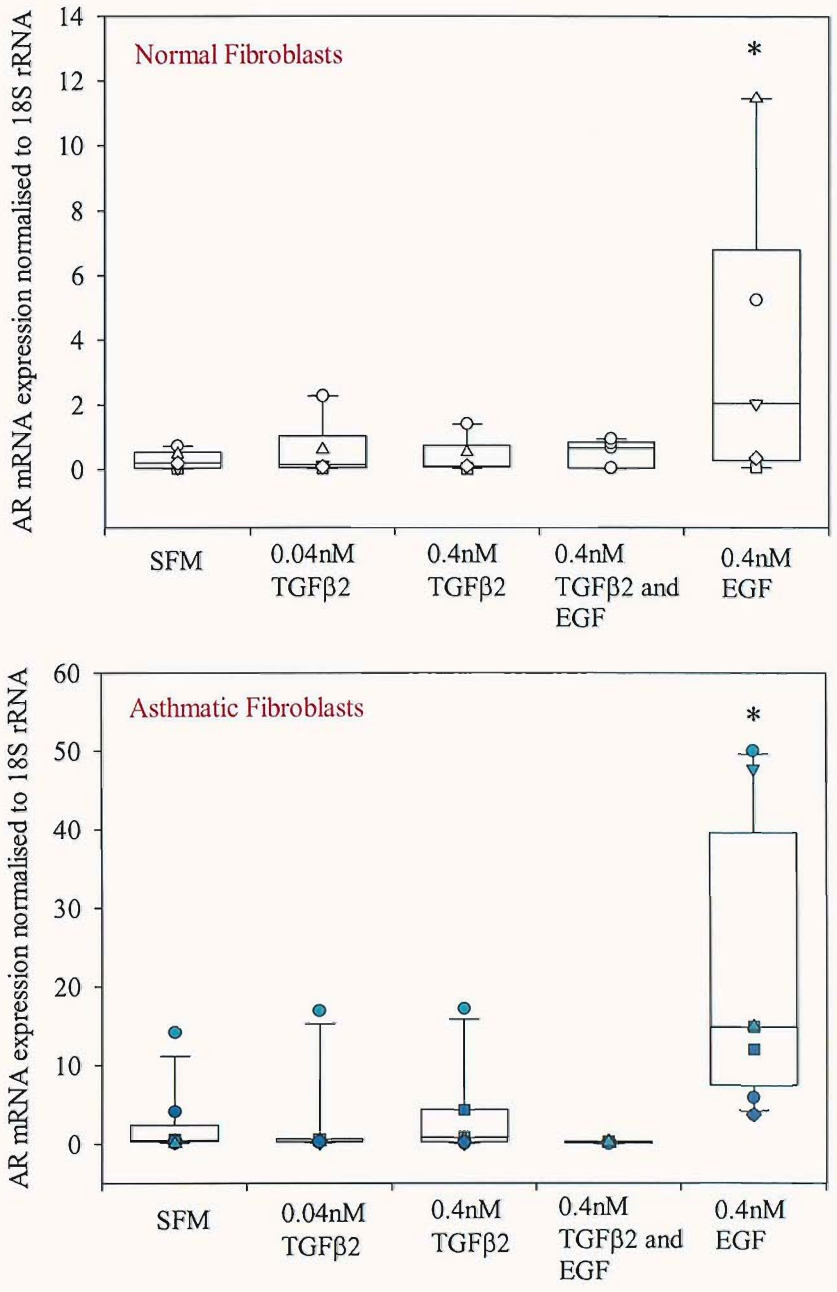


Figure 5.3: EGF induced amphiregulin mRNA

Normal (n=5, unfilled symbols), mild asthmatic (n=3-5, turquoise symbols) and moderate severe asthmatic (n=2-3, blue symbols) fibroblasts were subjected to TGFβ2 and EGF, alone or in combination for 24 hours. Amphiregulin mRNA was measured by Taqman PCR and normalised to 18S rRNA using the standard curve method. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile.
* represents significance ($p<0.05$) according to Wilcoxon Signed Ranks Test comparing treatment to SFM control.

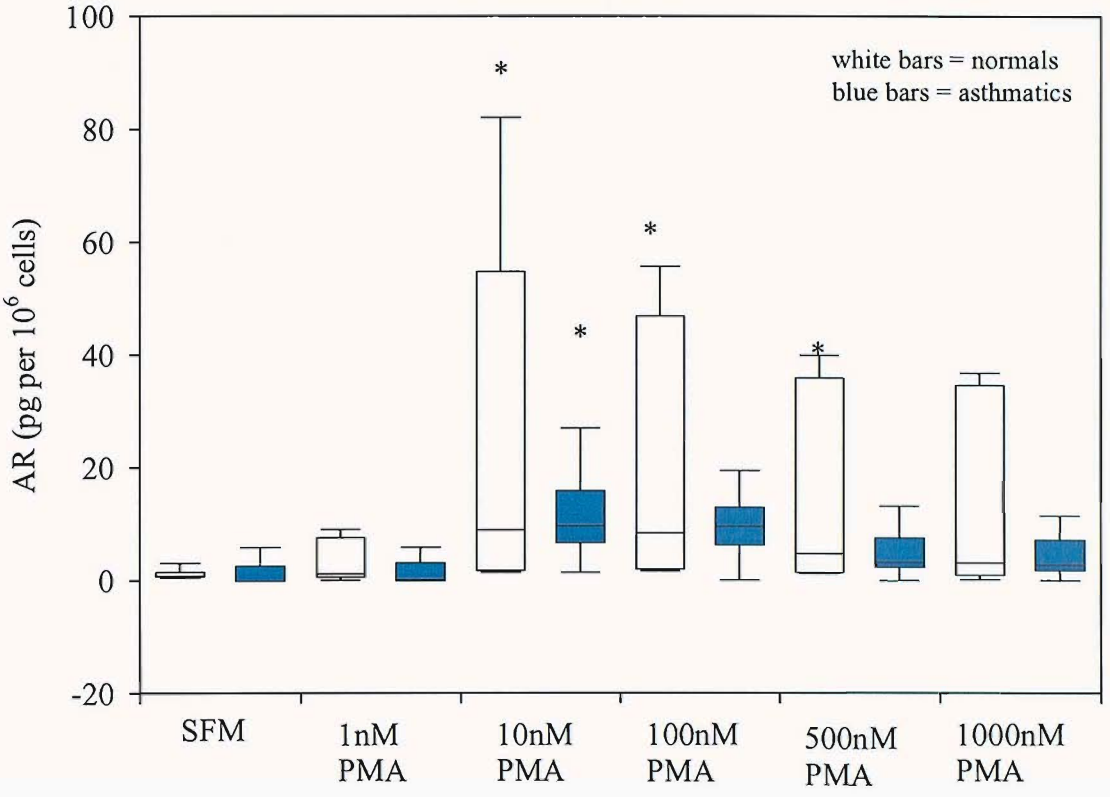


Figure 5.4: PMA induces release of amphiregulin

Normal (unfilled bars, n=6) and asthmatic (blue bars, n=5) fibroblasts were treated with increasing doses of PMA for 24 hours. Amphiregulin release was measured by ELISA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data are normalised to cell number by methylene blue elution assay.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with PMA. None of the treatment groups were significantly different.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.000$) and asthmatic ($p=0.001$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant compared to the SFM control. Statistical comparisons where $p<0.05$were noted on the graph (* represents significance).

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was noted. An initial experiment was performed on a representative fibroblast culture to access the effects of PMA and growth factor on amphiregulin release after 24 hours (fig. 5.5). EGF in combination with PMA induced amphiregulin release. TGFβ2 did not increase cleavage of amphiregulin above SFM control levels. Therefore only the effects of EGF on ligand release were considered henceforth.

5.4. PMA induced amphiregulin release is lower from asthmatic fibroblasts

After optimising the conditions to measure amphiregulin release in conditioned medium, normal (n=10), mild asthmatic (n=4) and moderate severe asthmatic (n=8) fibroblasts were serum starved for 24 hours and then treated with EGF (0.4nM) in combination with PMA (10nM) for 24 hours. The amphiregulin release was normalised to cell number by methylene blue elution assay. EGF, in the presence of PMA, was shown to significantly induce the release of amphiregulin in both normal ($p=0.009$) and asthmatic ($p=0.002$) fibroblasts (fig. 5.6). Surprisingly, the release was significantly lower both with PMA alone ($p=0.002$) and after EGF and PMA stimulation ($p=0.018$) in the asthmatic fibroblasts compared to the normal fibroblasts. These results were the converse of that expected in view of the mRNA data which showed that EGF induced a higher level of amphiregulin expression in the asthmatic fibroblasts compared to the normal fibroblasts (fig 5.3).

5.5. Cleavage of amphiregulin by PMA is a MP mediated response

GM6001 (Galardin) was originally synthesized as an inhibitor of human skin collagenase (323) but is now recognised to be a broad spectrum metalloproteinase inhibitor that inhibits both MMPs and ADAMs. To determine whether the PMA induced release of amphiregulin was mediated by a metalloprotease (MP), GM6001 (Calbiochem, San Diego, U.S.A.) was added to the medium to prevent the cleavage of EGFR ligands. The fibroblasts were pre-treated with GM6001 for 30 minutes prior to the addition of PMA and GM6001 (5μM) together. The metalloproteinase inhibitor significantly reduced the EGF/PMA mediated release of amphiregulin in the normal ($p=0.009$) and asthmatic ($p=0.028$) fibroblasts (fig. 5.7).

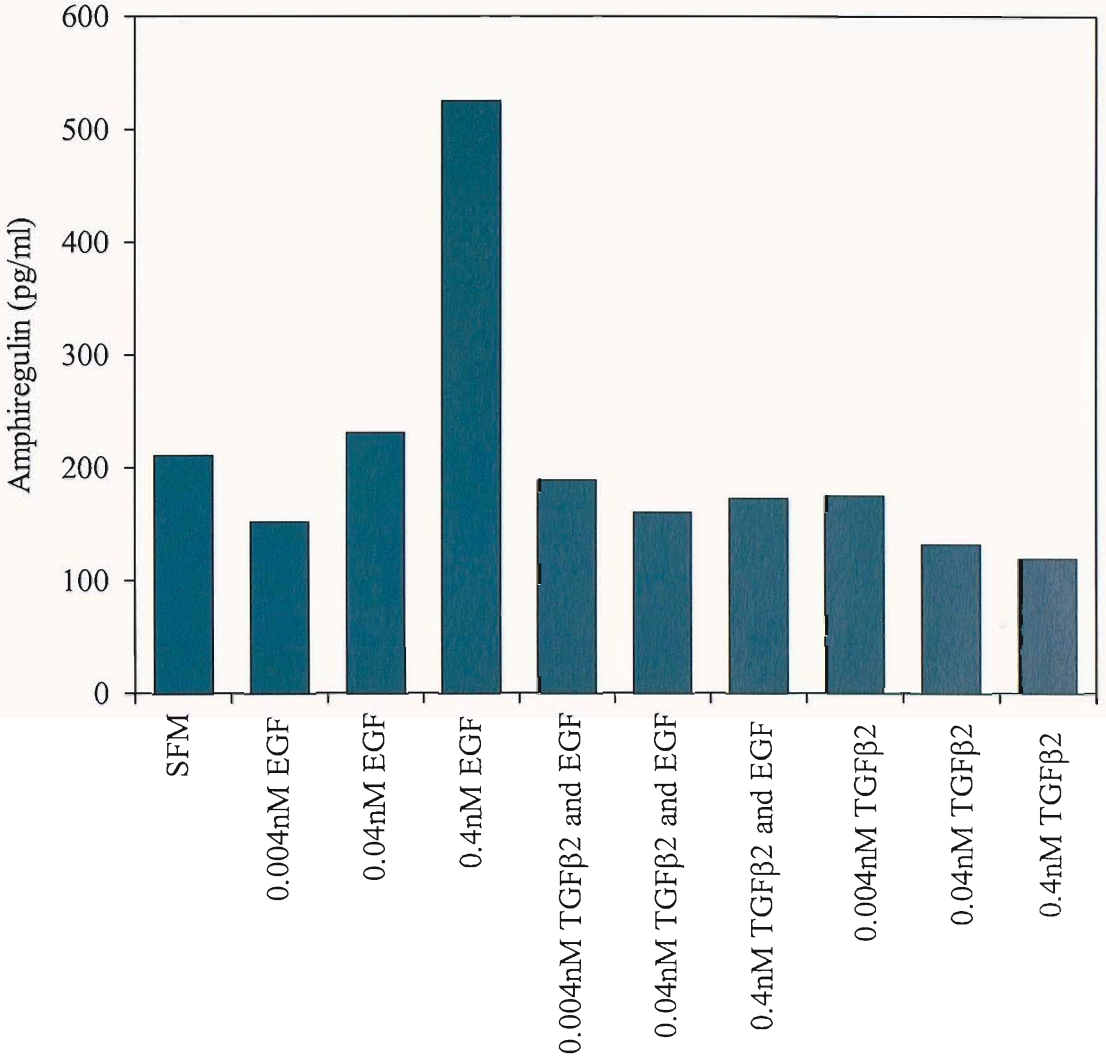

Figure 5.5: Growth factor stimulated amphiregulin release is enhanced by co-stimulation with PMA

A representative mild asthmatic fibroblast culture was treated with EGF and TGFβ2 for 24hrs in the presence of 10nM PMA. Amphiregulin release into the medium was measured in duplicate by ELISA.

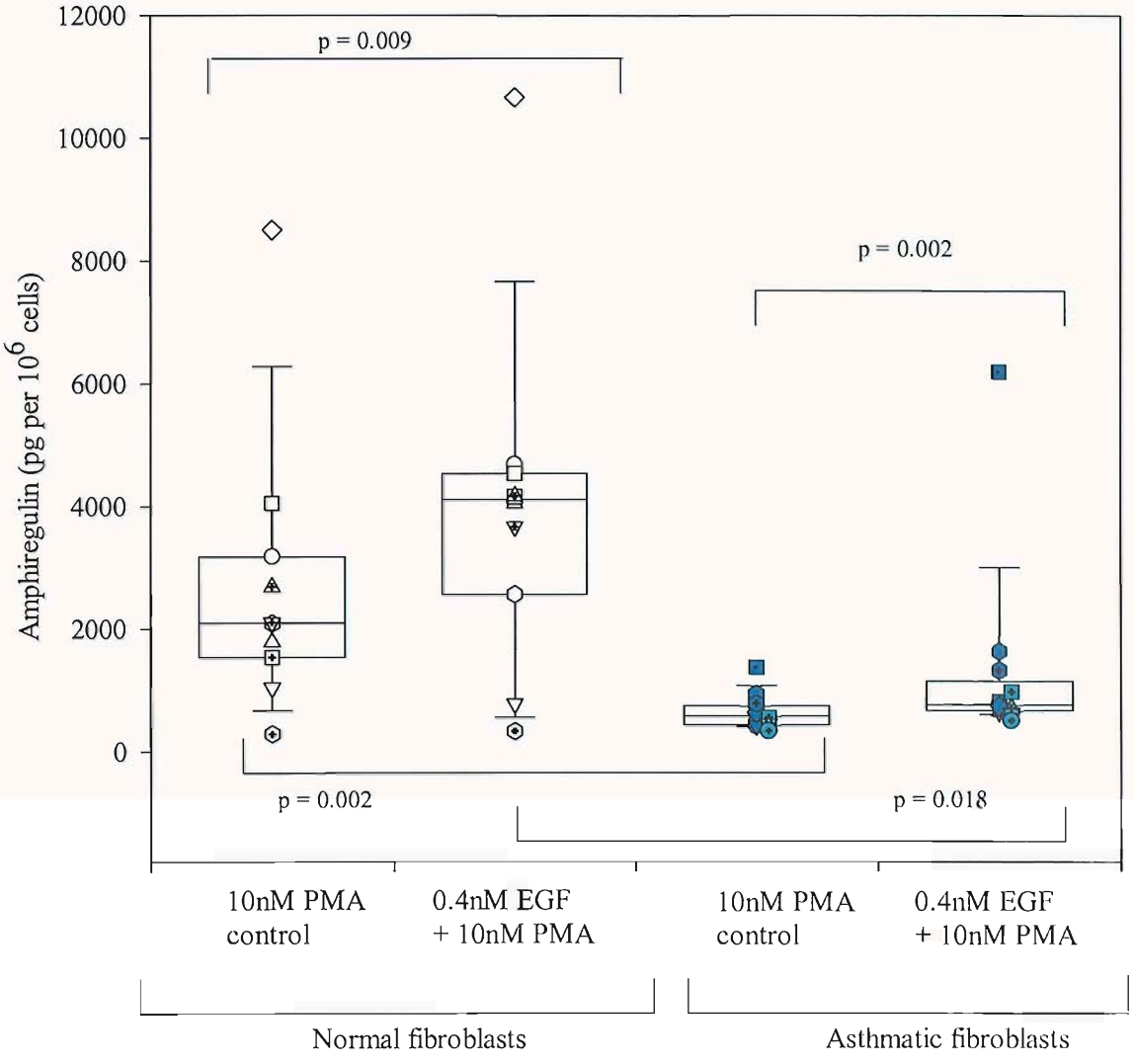

Figure 5.6: EGF, in the presence of PMA, induces amphiregulin release

Normal (unfilled symbols, n=10), mild asthmatic (turquoise symbols, n=4) and moderate severe asthmatic (blue symbols, n=8) fibroblasts were treated with 0.4nM EGF in combination with 10nM PMA for 24 hours. Amphiregulin release was measured by ELISA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data are normalised to cell number by methylene blue elution assay.

Significance ($p<0.05$) was determined according to a Wilcoxon Signed Ranks Test comparing treatment to SFM control, a Mann Whitney U test determined inter-group variation.

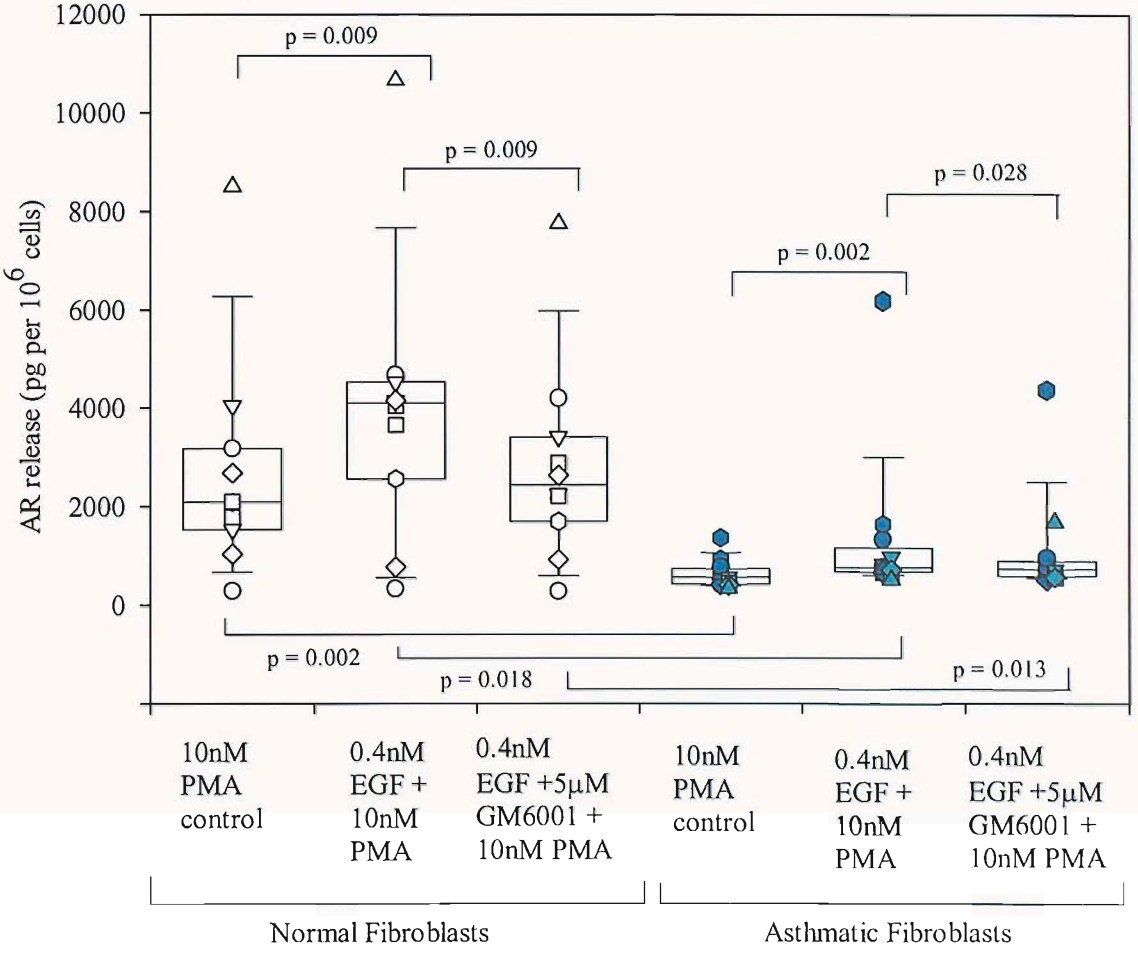

Figure 5.7: GM6001 suppresses EGF induced cleavage of amphiregulin (in the presence of PMA)

Normal (unfilled symbols, n=10), mild asthmatic (turquoise symbols, n=4) and moderate severe asthmatic (blue symbols, n=8) fibroblasts were treated with EGF +/- GM6001 in the presence of PMA for 24 hours. Amphiregulin release was measured by ELISA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Data are normalised to cell number by methylene blue elution assay.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment (statistical comparisons where $p<0.05$were noted on the graph). To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.007$) and asthmatic ($p=0.000$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

These data suggest that PMA in combination with EGF induces the release of amphiregulin by a MP mediated mechanism.

As EGF is a mitogen for fibroblasts, the amphiregulin release was normalised to cell number. By examining the proliferation data alone, it was shown that EGF, after only 24 hours, induced both normal and asthmatic fibroblasts to proliferate (fig. 5.8).

5.6. ADAM 17 expression levels are similar in normal and asthmatic fibroblasts

In order to determine a reason for the disparity between the mRNA and protein data for amphiregulin, the expression level of ADAM 17 was measured. ADAM 17 has been implicated in the cleavage of amphiregulin and other EGFR ligands by Gschwind *et al* (231). In the current study, ADAM 17 mRNA expression, normalised to 18S rRNA, was measured after treatment with 0.4nM EGF in both normal and asthmatic fibroblasts (fig. 5.9). After 24 hours, EGF did not alter the expression level of ADAM 17 mRNA expression and there was no difference between normal and asthmatic fibroblasts.

5.7. TIMP-3 expression levels are similar in normal and asthmatic fibroblasts

Proteolytic ADAM activity can be inhibited *in vivo* by Tissue Inhibitors of MetalloProteases (TIMPs). TIMP-3 has been reported to inhibit ADAM 17 (220) activity in cell based assays. TIMP-3 expression was therefore measured in fibroblasts treated with 0.4nM EGF (fig. 5.10). As with the data for ADAM 17, TIMP-3 expression did not change upon stimulation with EGF or between the normal and asthmatic cultures. Neither result gave any indication of altered processing of EGFR ligands between normal and asthmatics.

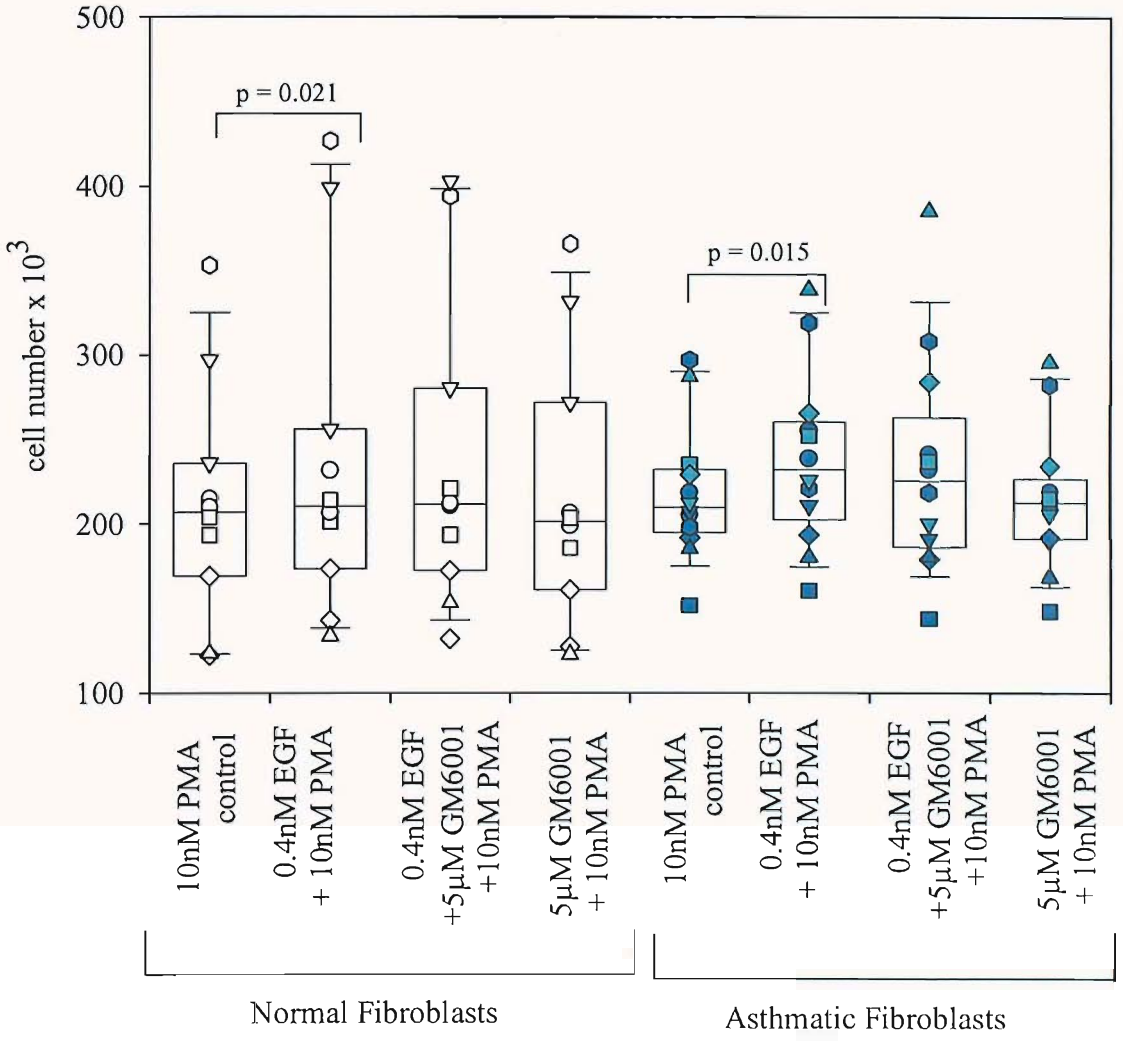

Figure 5.8: EGF (in the presence of PMA) induced the proliferation of fibroblasts over 24hrs

Normal (unfilled symbols, n=10), mild asthmatic (turquoise symbols, n=4) and moderate severe (blue symbols, n=8) fibroblasts were treated with EGF +/- GM6001 in the presence of PMA for 24 hours. Cell number was calculated by methylene blue elution assay. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.001$) and asthmatic ($p=0.001$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$ were noted on the graph, Bonferoni corrections were applied to correct for multiple testing.

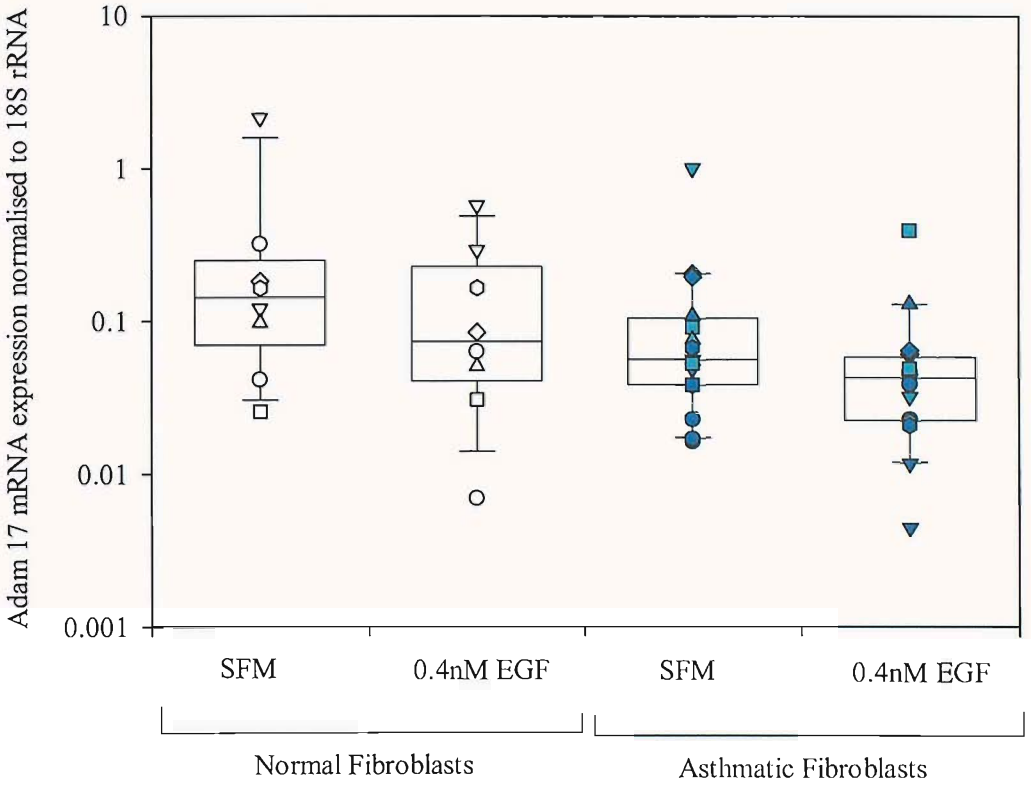

Figure 5.9: EGF did not alter the expression of ADAM 17 in bronchial fibroblasts

Normal (n=8, unfilled symbols), mild asthmatic (n=7, turquoise symbols) and moderate severe asthmatic (n=8, blue symbols) fibroblasts were treated with EGF for 24 hours.

ADAM 17 expression was measured using RT-PCR and normalised using 18S rRNA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Wilcoxon Signed Ranks tests were used to determine intra-group statistical comparisons, Mann Whitney U tests were used to measure inter-group differences.

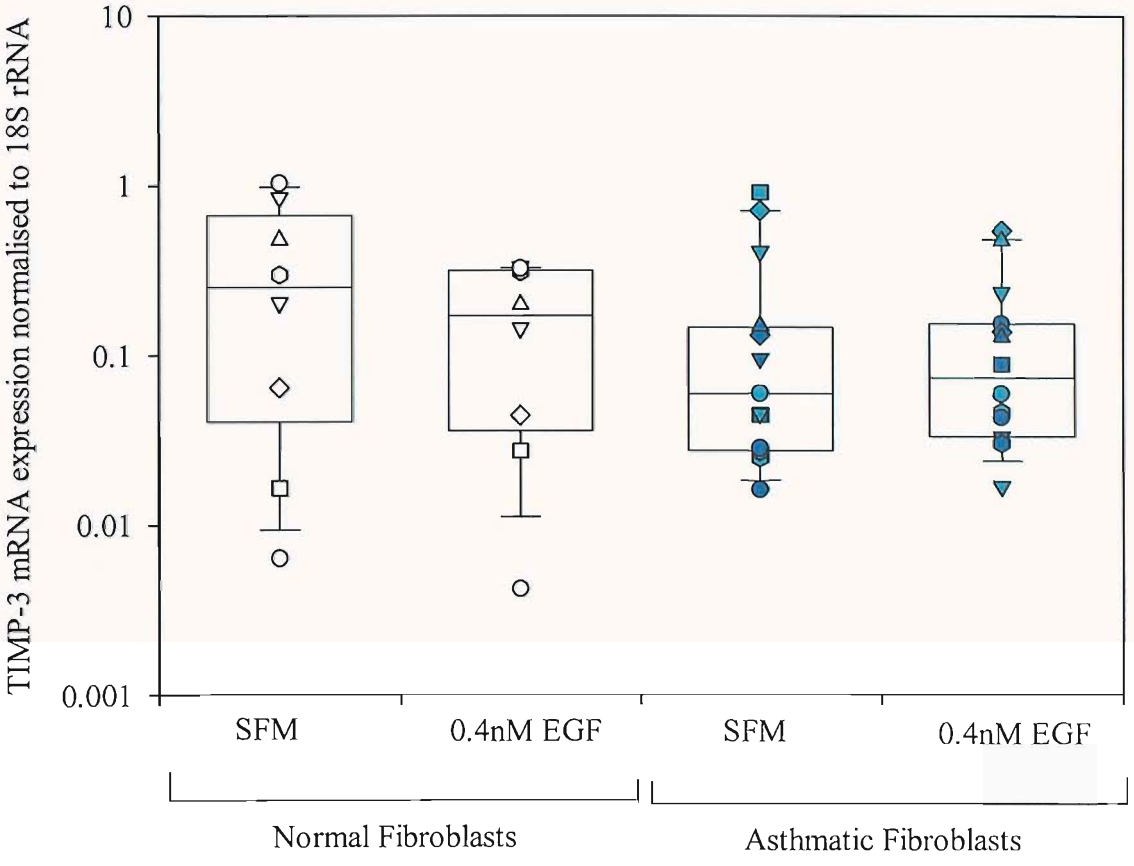

Figure 5.10: TIMP-3 expression is not altered by EGF in bronchial fibroblasts

Normal (n=8, unfilled symbols), mild asthmatic (n=7, turquoise symbols) and moderate severe asthmatic (n=8, blue symbols) fibroblasts were treated with EGF for 24 hours. TIMP-3 expression was measured using RT-PCR and normalised using 18S rRNA

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. Wilcoxon Signed Ranks tests were used to determine intra-group statistical comparisons, Mann Whitney U tests were used to measure inter-group differences.

5.8. EGFR mRNA levels are increased in asthmatic fibroblasts

As analysis of the enzymes involved in processing amphiregulin had not yielded any differences between the normal and asthmatic fibroblasts, the study continued by examining how the cells themselves can respond to amphiregulin. The expression level of EGFR, the receptor for amphiregulin, was measured. EGF did not alter the expression of EGFR in the normal fibroblasts (fig. 5.11). Surprisingly, EGF caused an increase in the mRNA levels of EGFR in the asthmatic fibroblasts ($p=0.009$). The EGF stimulated ($p=0.002$) levels of EGFR were higher in the asthmatic fibroblasts compared to the normal fibroblasts.

5.9. Amphiregulin is a weak mitogen for asthmatic fibroblasts

It was predicted from the above observation that asthmatic fibroblasts may have a greater ability to respond to EGFR ligands compared to normal fibroblasts. As the most cited response of amphiregulin is in regard to proliferation, a mitogenesis assay was performed on bronchial fibroblasts treated with amphiregulin.

The fibroblasts were seeded at $8x10^3$ cells/well in a 24 well plate, with each treatment repeated in triplicate. The fibroblasts were serum starved for 24 hours prior to treatment and then treated with 0.4nM amphiregulin for a total of 5 days. This was a concentration of amphiregulin that was observed to be released by some of the normal cultures after 24 hours with EGF and PMA. EGF at an equimolar concentration was used as a positive control in the assay. As with previous proliferation assays, cell biomass was determined by methylene blue elution assay with results normalised to day 0.

As amphiregulin was considered to be a selective mitogen for epithelial cells, it was predicted that amphiregulin would have no effect on the proliferation of fibroblasts. The results showed that amphiregulin did not induce the normal fibroblasts to proliferate (fig. 5.12). Surprisingly, amphiregulin was shown to induce a small but significant increase ($p=0.028$) in the cell biomass of the asthmatic fibroblasts over 5 days. This data suggests that the reduced release of amphiregulin into the conditioned medium may be due to an increased rate of utilisation in the fibroblasts

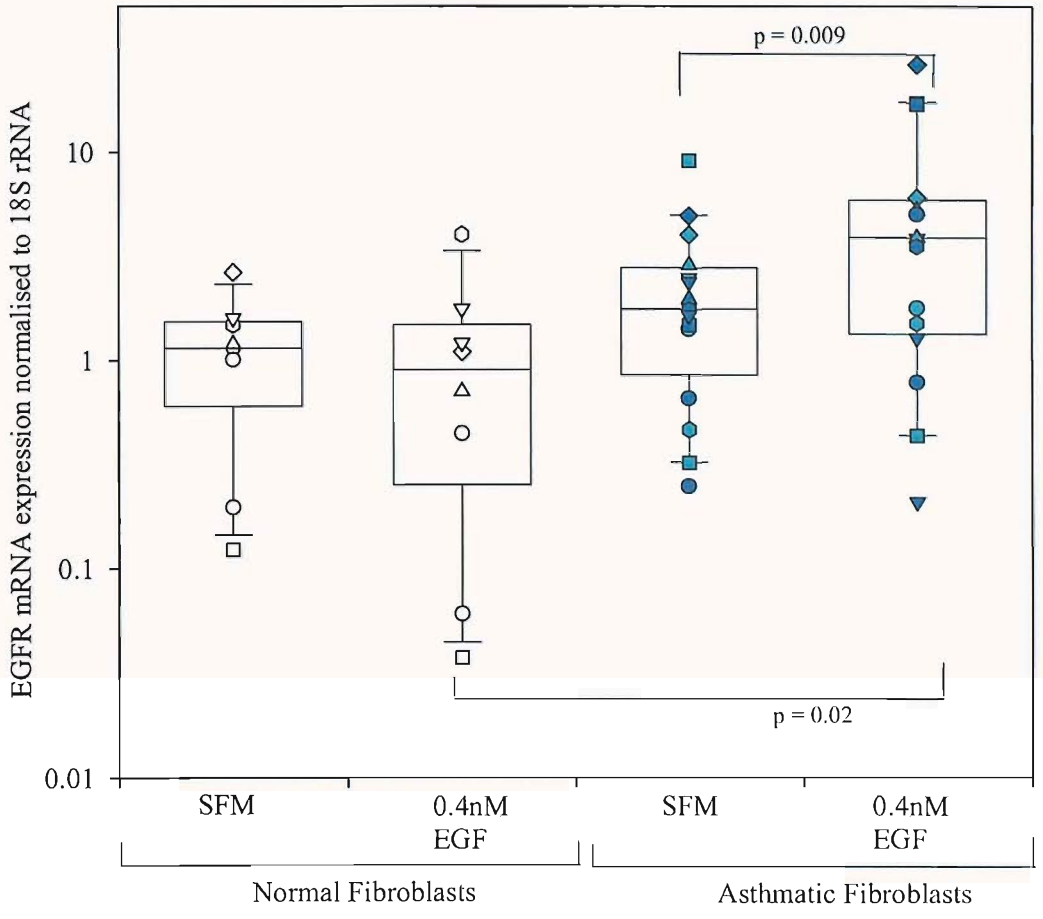

Figure 5.11: EGF increased EGFR expression only in the asthmatic fibroblasts

Normal (n=8, unfilled symbols), mild asthmatic (n=7, turquoise symbols) and moderate severe asthmatic (n=8, blue symbols) fibroblasts were treated with EGF for 24 hours. EGFR expression was measured using RT-PCR and normalised using 18S rRNA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile.

Wilcoxon Signed Ranks tests were used to determine intra-group statistical comparisons, Mann Whitney U tests were used to measure inter-group differences.

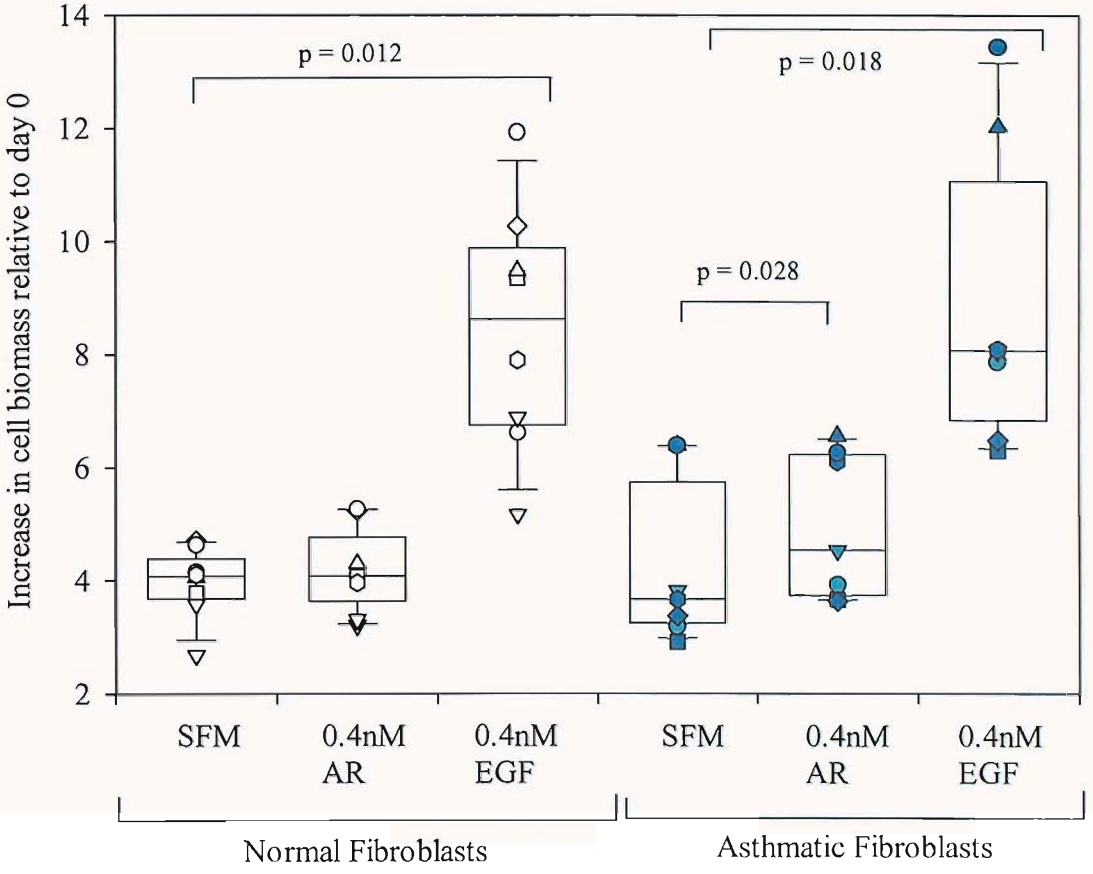

Figure 5.12: Amphiregulin is a mitogen for asthmatic fibroblasts

Normal (n=8, unfilled symbols), mild asthmatic (n=2, turquoise symbols) and moderate severe asthmatic (n=5, blue symbols) fibroblasts were treated with 0.4nM EGF or amphiregulin for 5 days. Increase in cell biomass was measured by methylene blue elution assay, with data normalised to day 0. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment. None of the treatment groups were significantly different. To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.002$) and asthmatic ($p=0.002$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph.

isolated from the biopsies of asthmatic subjects. The increased expression of EGFR, if translated to protein in the asthmatic fibroblasts, may be in part responsible for the increased mitogenic activity in response to amphiregulin. As amphiregulin requires the presence of heparan sulphate in order to bind to EGFR and elicit a response, this data also is suggestive of a different proteoglycan expression profile in asthmatic compared to normal fibroblasts.

5.10. Discussion

To conclude, this study has shown that the expression, release and utilisation of amphiregulin are altered in asthmatic fibroblasts. Asthmatic fibroblasts express higher levels of EGFR mRNA than normal fibroblasts. This may be responsible for the increased expression of amphiregulin mRNA in response to EGF. The increased receptor expression may also explain the ability of asthmatic fibroblasts to utilise amphiregulin, resulting in an increase in proliferation in the asthmatic fibroblasts stimulated with 0.4nM amphiregulin. The only concentration used to study proliferation in this study was 0.4nM amphiregulin, as it was the equimolar concentration of TGFβ2 and EGF used throughout the study. To fully appreciate the potency of the growth factor in normal compared to asthmatic fibroblasts, a dose response could have been performed.

Although RT-PCR data suggest that upon EGF stimulation, EGFR mRNA levels are increased in asthmatic fibroblasts compared to normals, quantitative protein data were not performed. Immunofluorescent staining for the receptor revealed widespread membrane staining but there was insufficient time to carry out flow cytometric analysis for EGFR. It was intriguing that EGF stimulated asthmatic fibroblasts did not proliferate quicker than normals. This would suggest that EGFR protein expression may not vary between normal and asthmatic fibroblasts.

This study highlights not only the importance of protein data but also the importance of selecting the correct readout for the protein data. This study has measured amphiregulin mRNA and release. There are however a number of steps that are involved between the translation of the mRNA, the expression of the pro-amphiregulin at the cell membrane and the binding of mature amphiregulin to the heparin sulphate side chains of proteoglycans. The current study reported an increase in mRNA for amphiregulin but a reduced release in the conditioned medium by asthmatic fibroblasts. This, although initially may be considered to be a conflicting observation, in reality reflects the complexity of the growth factor production and utilization.

The proliferation data suggested that only the asthmatic fibroblasts can utilise amphiregulin, a phenomenon which can only take place in the presence of an accessory molecule, heparin or heparan sulphate. PMA was added to the cells to promote the cleavage of pro-amphiregulin. The ELISA was designed however on the assumption that amphiregulin was not used by the fibroblasts. Addition of a neutralising antibody to EGFR would have prevented uptake by the cell, increasing accumulation of EGFR ligands in the medium.

The difference in heparan sulphate proteoglycan expression between the cell types can determine whether a growth factor acts in an autocrine or paracrine fashion. Lories *et al* (324) reported that mammary epithelial cells and lung fibroblasts both express glypigan, but that fibroglycan was expressed only by the fibroblasts. It was also demonstrated that proteoglycan expression can vary according to confluence, with higher levels of syndecan mRNA expressed in confluent fibroblasts than exponentially growing fibroblasts. The proteoglycan profile has been reported to differ in fibroblasts isolated from asthmatic subjects to those in healthy controls. Westergren-Thorsson *et al* (45) reported that subjects with the most hyperresponsive airways produce up to four times more total proteoglycan than subjects with less responsive airways. Venkatesan *et al*, (325) demonstrated that bleomycin-exposed lung fibroblasts expressed higher levels of versican, heparan sulphate proteoglycan and biglycan compared with normal lung fibroblasts. The increased expression of proteoglycans by asthmatic fibroblasts may go some way to explain the altered function of amphiregulin in disease. An increased expression of proteoglycan could theoretically increase the bioavailability and half life of growth factors such as amphiregulin, HB-EGF and FGF-2, all of which have heparin-binding domains.

In vitro observations give invaluable information on the activity of a growth factor in the absence of other cell types and other growth factors. In trying to interpret the data, it important to place it in context. Firstly, bronchial fibroblasts *in vivo* would not be exposed to PMA, this was used as a pharmacological tool to bypass the G-protein coupled receptor activation. It has been reported that *in vivo*, ligands for G-protein coupled receptors such as thrombin, endothelin, LPA, angiotensin and bombesin can induce the release of EGFR ligands (326-329). It has been

reported that there is an enhanced expression of G-protein coupled ligands in asthmatic airways (330-332), potentially leading to an enhanced cleavage of EGFR ligands. Additionally, Amishima *et al*, (275) reported that EGF was expressed at higher levels in the bronchial epithelium and glands of asthmatic subjects compared to normals. These observations in light of the current study suggest that there may be an increased expression of amphiregulin in the mesenchyme of asthmatic airways.

As the majority of studies have focused on the mitogenic activity of amphiregulin in adult cells, very little is known about other roles of the growth factor. Even less is known about the functional activity of pro-amphiregulin which may have the ability to signal in its own right. However there are a few recent studies suggesting that amphiregulin may play a role in inflammation. It was reported by Blanchet *et al* (333) that amphiregulin can stimulate the release of GM-CSF, a pro-inflammatory cytokine involved in the recruitment of dendritic cells. Furthermore, overexpression of amphiregulin in basal keratinocytes (272) has been associated with a psoriasis-like phenotype. Psoriasis is an inflammatory disorder resulting in epidermal hyperplasia. This suggests an increased expression of amphiregulin might play a part in propagating the chronic inflammation already present in asthmatic airways.

In relation to asthma, it has been shown that respiratory irritants such as diesel exhaust particles (333) and cigarette smoke extract (334)can induce the expression and release of amphiregulin respectively. A recent study by Kumar *et al* (335) has further implicated amphiregulin in asthma by demonstrating a small induction in amphiregulin release shortly after allergen challenge in BALB/c mice sensitized to ovalbumin.

The increased concentration of TGFβ in the airways of asthmatics also has to be taken into consideration. The suppressive effect of TGFβ2 on the expression and release of amphiregulin is not surprising in view of previous findings in chapters 3 and 4, suggesting that the TGFβ and EGF signalling pathways interlink and regulate each other. As TGFβ2 prevented the EGF induced release of

amphiregulin, the increased levels of TGFβ may suppress amphiregulin expression in the asthmatic lung. The lack of *in vivo* studies undertaken on the mesenchymal expression of amphiregulin in asthma has prevented a fuller understanding of the interaction of the two growth factors in the lung.

In summary, the results from this study are very exciting as the data suggests that amphiregulin may act as a paracrine factor in healthy control fibroblasts, but as an autocrine growth factor for asthmatic fibroblasts. The data reported here shows that only the asthmatic fibroblasts can respond to amphiregulin, inducing a small but significant mitogenic effect. In utilising the growth factor, the asthmatic fibroblasts are essentially preventing the epithelial cells from using it, as shown by the reduced levels seen in the conditioned medium from asthmatic fibroblasts. In view of this data, amphiregulin may be considered to play a role in remodelling of the airways. Not only is there suppressed paracrine activity in the asthmatic fibroblasts which may prevent the repair of the damaged epithelium but amphiregulin may also act as a weak mitogen for asthmatic fibroblasts, potentially contributing to the fibrosis associated with remodelled airways.

5.10.1. Novel Findings

- EGF induces amphiregulin mRNA but was unable to stimulate release of mature protein into the conditioned media alone.

- Asthmatic fibroblasts express more amphiregulin mRNA than normal fibroblasts

- TGFβ2 suppressed EGF dependent amphiregulin mRNA, no difference was observed between the normals and asthmatics

- EGF in combination with PMA increased release of amphiregulin into the media after 24 hour stimulation

- EGF and PMA induced a significantly greater release of amphiregulin in normal fibroblasts compared to asthmatics.

- Expression levels of ADAM 17 and TIMP-3 mRNA were unaltered by EGF

- EGF induces EGFR in asthmatic fibroblasts

- 0.4nM amphiregulin induced proliferation of asthmatic fibroblasts but not normal fibroblasts.

CHAPTER 6

Processing of Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) in Bronchial Fibroblasts

CHAPTER 6

PROCESSING OF HEPARIN-BINDING EPIDERMAL GROWTH FACTOR-LIKE GROWTH FACTOR (HB-EGF) IN BRONCHIAL FIBROBLASTS

HB-EGF was originally identified in the conditioned medium of Macrophage-like U-937 cells (336) but has now been found in smooth muscle (337) (338;339) (340), epithelial cells (340;341), CD4+ T cells (342), fibroblasts (314) and eosinophils (343). HB-EGF, like amphiregulin, has heparin binding domains (HBDs) allowing local accumulation at the cell surface (344). It is a mitogen and chemoattractant for a number of cell types including fibroblasts and smooth muscle cells (338;345-347). It has been suggested that HB-EGF can induce FGF-2 in fibroblasts and smooth muscle cells, which in part accounts for its mitogenic activity (314;348;349).

The previous chapter examined the processing of amphiregulin by bronchial fibroblasts. To complement the amphiregulin data, the processing of HB-EGF, the other heparin binding EGFR ligand, was studied. Asthmatic fibroblasts were shown to express a higher level of amphiregulin than normal fibroblasts at the mRNA level. Although normal fibroblasts were shown to release more amphiregulin, the asthmatic fibroblasts were shown to utilise the growth factor, potentially accounting for the difference in release between the control and asthmatic fibroblasts.

The potent mitogenic activity of HB-EGF for fibroblasts and smooth muscle cells makes it a prime candidate for a role in remodelling of the asthmatic lung. It was postulated therefore that asthmatic fibroblasts express more HB-EGF than normals.

Hypothesis: Asthmatic fibroblasts express more HB-EGF than normal fibroblasts.

6.1. HB-EGF is a potent fibroblast mitogen

There are a number of references in the literature suggesting that HB-EGF can induce fibroblasts to proliferate. Dluz *et al* (350) reported that HB -EGF can mediate proliferation in an autocrine and paracrine manner. Furthermore, Higashiyama *et al* (351) demonstrated that soluble HB-EGF, and the promembrane form in association with CD9 (352), have mitogenic activity.

In order to determine whether soluble HB-EGF is a potent mitogen for bronchial fibroblasts, the mitogenic activity of HB-EGF was compared to that of other EGFR ligands. Normal (n=8) and asthmatic (n=7) bronchial fibroblasts (fig. 6.1) were subjected to an equimolar concentration (0.4nM) of EGF, TGFα, HB-EGF or amphiregulin on day 0. Increases in cell biomass were compared after 5 days in culture, with all measurements internally normalised to day 0.

As reported in the previous chapter, amphiregulin at 0.4nM only induced the asthmatic fibroblasts to proliferate. The EGFR ligands appeared to follow the same order of potency in both the normal and asthmatic fibroblasts, with EGF being the most potent, followed by TGFα, HB-EGF and then amphiregulin. The HB-EGF induced proliferation reached significance in both the normal ($p=0.012$) and asthmatic ($p=0.018$) fibroblasts, causing a 6-7 fold increase in cell biomass compared to day 0. There were no significant differences reported between the fibroblasts isolated from normal or disease tissue.

6.2. TGFβ2 induces HB-EGF mRNA

TGFβ has been reported to induce HB-EGF mRNA in the intestinal epithelial cell line RIE-1 (353). It was postulated that HB-EGF expression is inducible upon stimulation with TGFβ2 in bronchial fibroblasts as well.

Normal (n=8) and asthmatic (n=15) bronchial fibroblasts (fig. 6.2) were treated with 0.04nM or 0.4nM TGFβ2 for 24 hours. RT-PCR analysis of the fibroblast cDNA revealed that TGFβ2 can increase the mRNA for HB-EGF in a

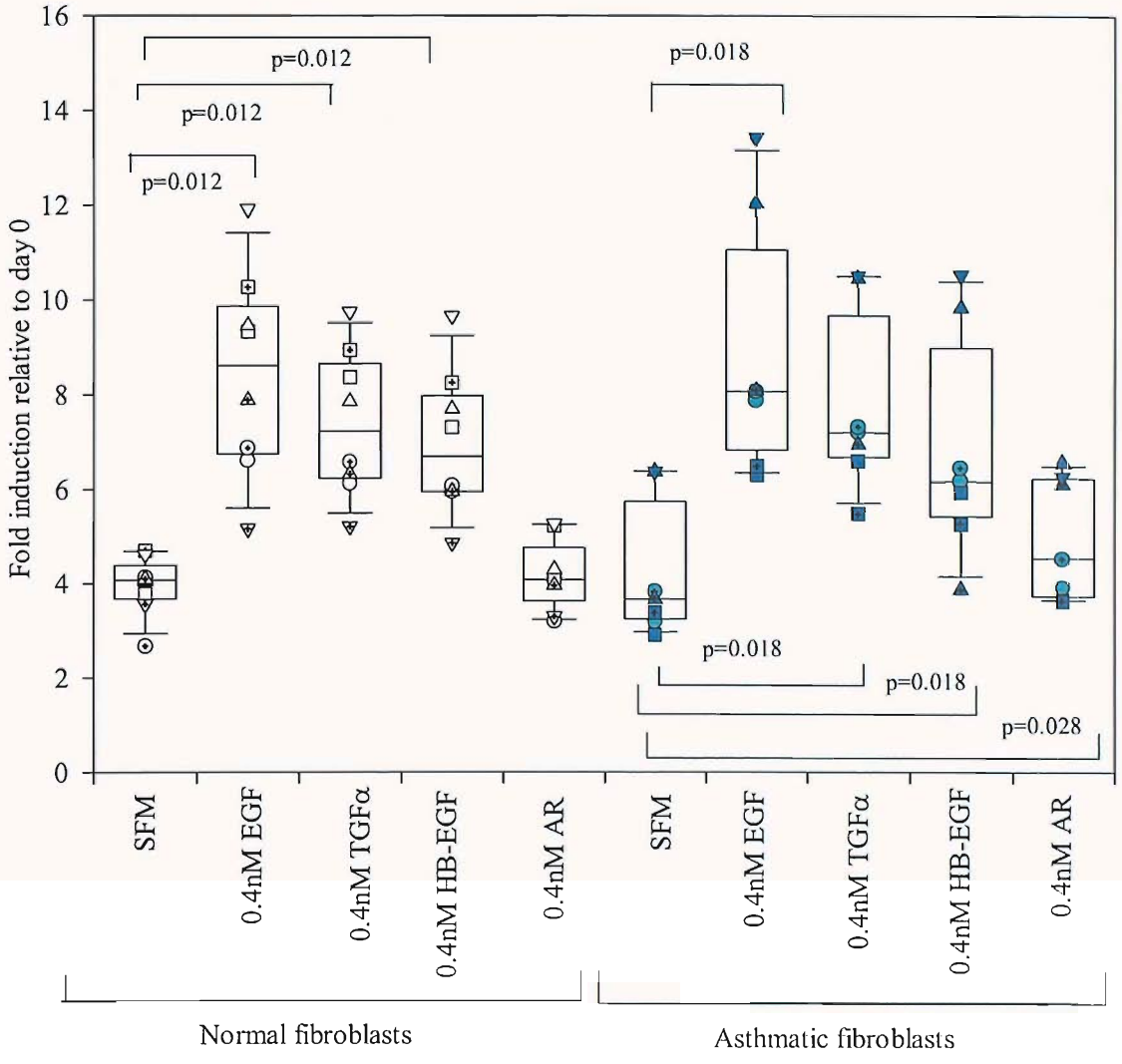

Figure 6.1: EGFR ligand induced proliferation of bronchial fibroblasts

Normal (n=8, unfilled symbols), mild asthmatic (n=2, turquoise symbols) and moderate severe asthmatic (n=5, blue symbols) fibroblasts were treated with 0.4nM EGF, TGFα, HB-EGF or amphiregulin for 5 days. Changes in cell biomass were measured by methylene blue elution assay. Each subject was normalised to day 0. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with EGFR ligands. None of the treatment groups were significantly different.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal (p=0.000) and asthmatic (p=0.000) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph.

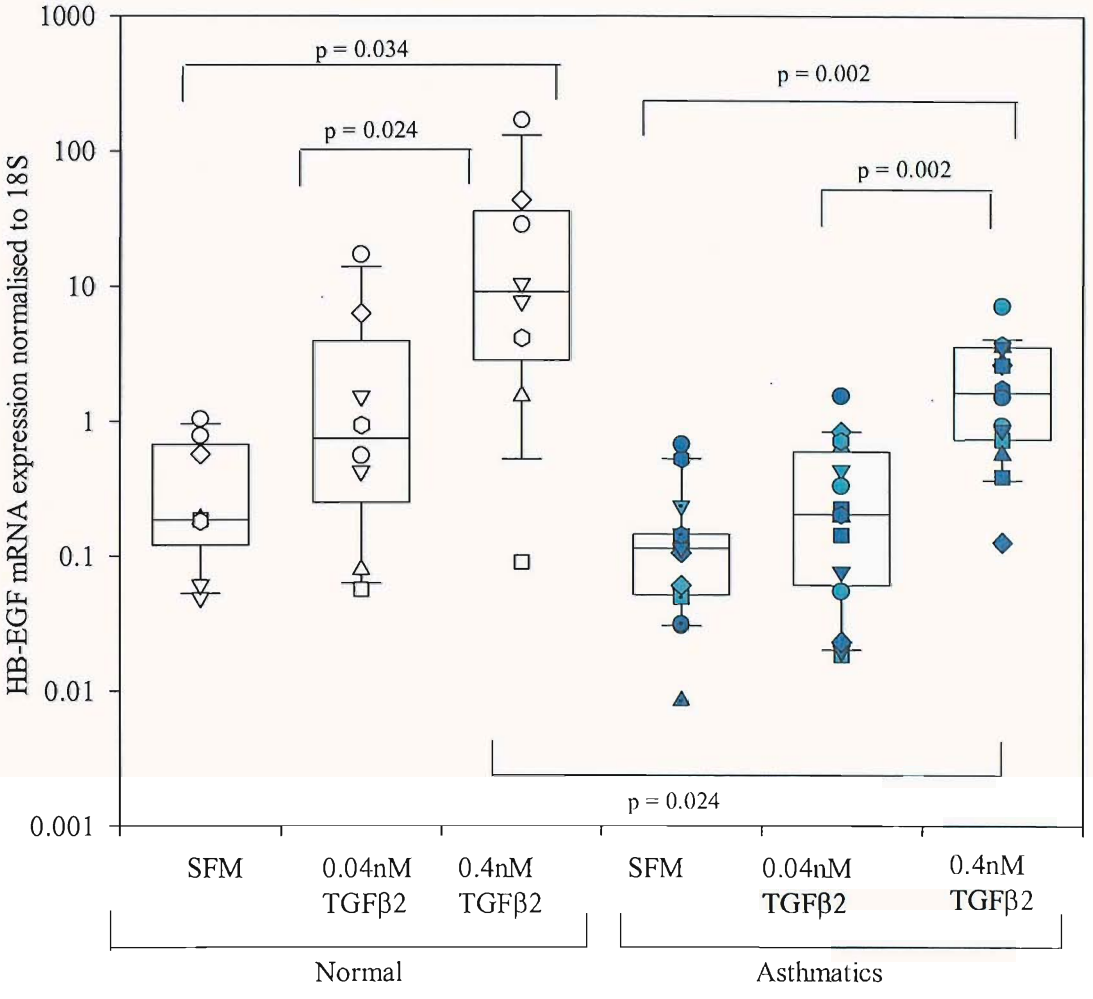

Figure 6.2: TGFβ2 induces HB-EGF mRNA expression in a dose dependent manner

Normal (n=8, unfilled symbols), mild asthmatic (n=7, turquoise symbols) and moderate severe asthmatic (n=8, blue symbols) fibroblasts were subjected to TGFβ2 and EGF, alone or in combination for 24 hours. HB-EGF mRNA was measured by Taqman PCR and normalised to 18S rRNA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile.

A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with EGFR ligands. Statistical comparisons where $p<0.05$were noted on the graph.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.01$) and asthmatic ($p=0.000$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph.

concentration dependent manner. Although similar in the unstimulated cultures, 0.4nM TGFβ2 induced a significantly higher expression of HB-EGF mRNA in the normal fibroblasts compared to the asthmatic fibroblasts. This observation does not support the hypothesis that asthmatic fibroblasts express more HB-EGF than normal fibroblasts.

6.3. TGFβ2 induces HB-EGF mRNA to a greater extent than EGF

Previously (Chapter 5) it was shown that EGF could induce amphiregulin mRNA, demonstrating that EGFR family ligands can alter the expression levels of one another. It was predicted that EGF could also increase the expression of HB-EGF. Using RT-PCR it was shown that EGF could induce HB-EGF in normal and asthmatic fibroblasts to a similar degree (fig. 6.3). However, the increase was much less than that induced by TGFβ2. Indeed, the TGFβ2 induction was markedly reduced in the presence of an equimolar concentration of EGF. In view of the fact that TGFβ2, a profibrogenic growth factor, was a much better stimulus for HB-EGF expression, further work centred on TGFβ2.

6.4. TGFβ2 induces ADAM 12 expression

HB-EGF, like other EGFR family ligands, is initially expressed as a membrane bound pro-form (354). The exact mechanism whereby HB-EGF is shed from the membrane is unknown although a number of proteases, such as ADAM 12 have been suggested to play a role in its cleavage. Asakura *et al* (237) reported that KB-R7785, a specific HB-EGF inhibitor, suppressed G-protein coupled receptor induced HB-EGF processing by ADAM 12, preventing cardiac hypertrophy in cultured rat neonatal cardiomyocytes.

In the current study, ADAM 12 was expressed in quiescent bronchial fibroblasts (fig. 6.4). Upon TGFβ2 stimulation, the expression of ADAM 12 increased in both normal and asthmatic bronchial fibroblasts in a dose dependent manner. TGFβ2 can therefore induce the mRNA of both HB-EGF and ADAM 12, the protease proposed to cleave pro-HB-EGF.

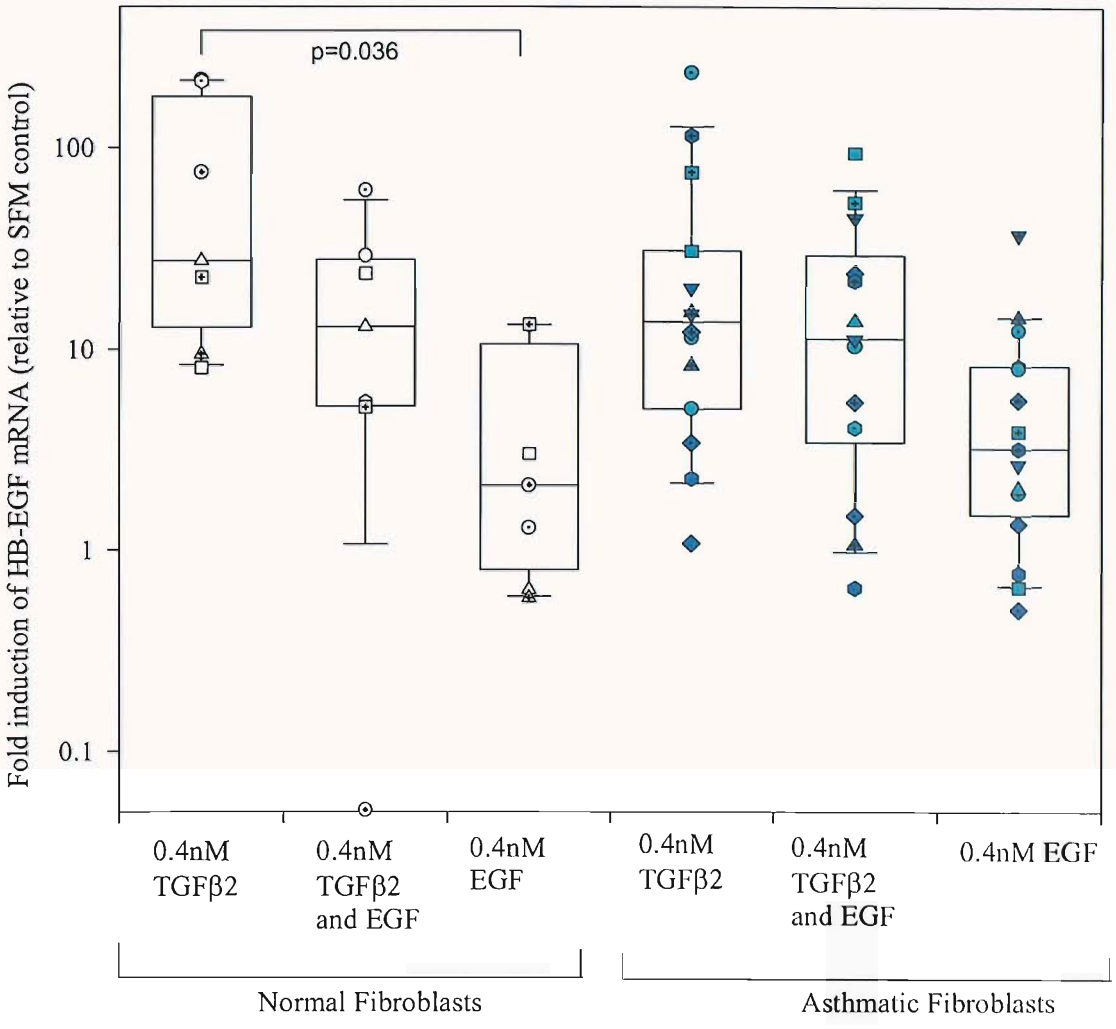

Figure 6.3: TGFβ2 induces a greater increase in HB-EGF expression than EGF

Normal (n=8, unfilled symbols), mild asthmatic (n=7, turquoise symbols) and moderate severe asthmatic (n=8, blue symbols) fibroblasts were treated with EGF for 24 hours. HB-EGF expression was measured by RT-PCR and normalised using 18S rRNA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within the normal ($p=0.042$) group but not the asthmatic ($p=0.174$) group. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$were noted on the graph, Bonferoni corrections were applied to account for multiple testing.

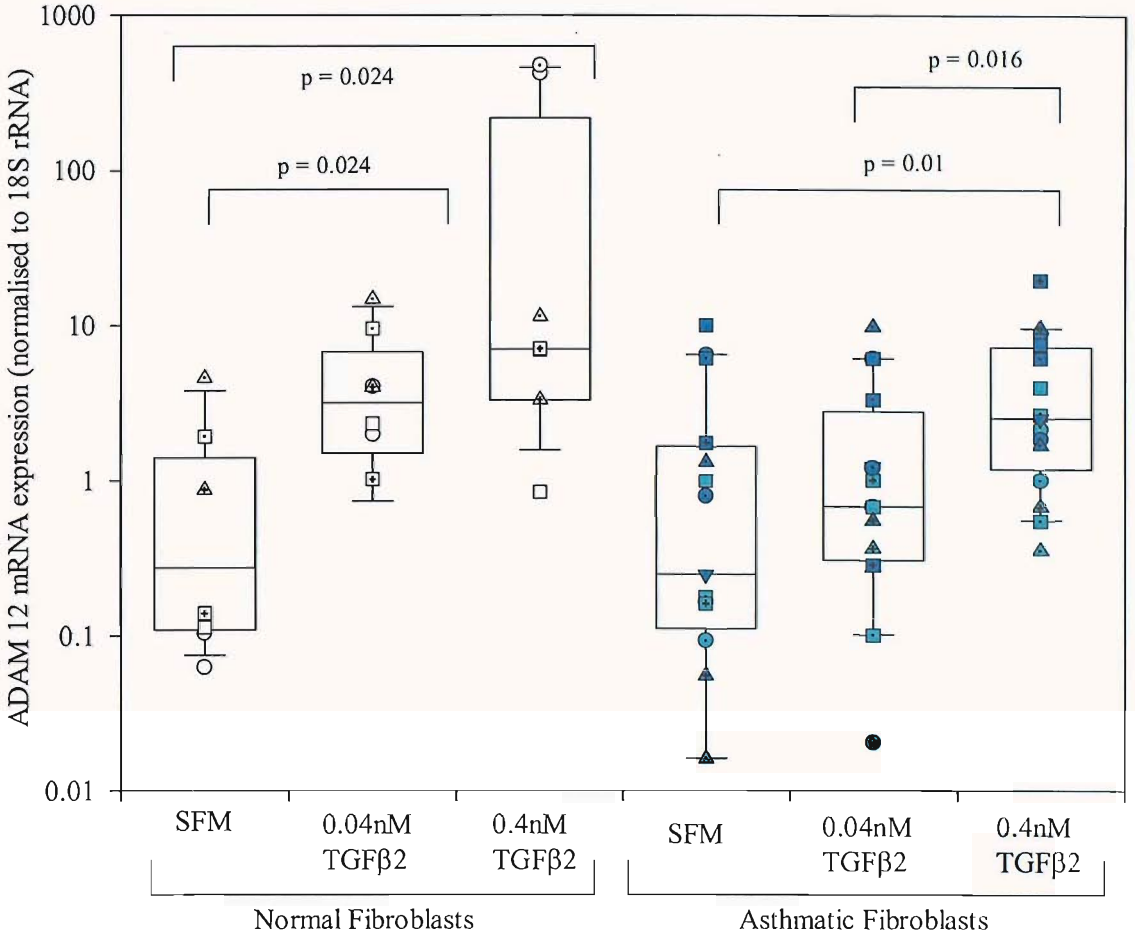

Figure 6.4: TGFβ2 induces ADAM12 expression in bronchial fibroblasts

Normal (n=8, unfilled symbols), mild asthmatic (n=7, turquoise symbols) and moderate severe asthmatic (n=8, blue symbols) fibroblasts were treated with TGFβ2 for 24 hours. ADAM 12 expression was measured using RT-PCR and normalised using 18S rRNA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. A Mann Whitney U test was performed to determine inter group differences basally and upon treatment with TGFβ2. None of the treatment groups were significantly different.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences within both the normal ($p=0.002$) and asthmatic ($p=0.000$) groups of data. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$ were noted on the graph, Bonferoni corrections were applied to account for multiple testing.

6.5. TGFβ2 can induce TIMP-3 expression

It has been shown that EGF does not appear to alter the expression level of TIMP-3 (Chapter 5). As TIMP-3 has been implicated in inhibiting the activity of both ADAM 12 and ADAM 17 (220), the effect of TGFβ2 on TIMP-3 expression was measured.

There was a trend whereby the basal expression level of TIMP-3 was higher in the non-stimulated normal fibroblasts compared to the asthmatic fibroblasts (fig. 6.5). The subsequent increase in expression in response to TGFβ2 did not reach significance in the normal fibroblasts, possibly due to the higher baseline. TGFβ2 induced a concentration dependent increase in the expression of TIMP-3 in the asthmatic fibroblasts after 24 hours. The expression level of TIMP-3 after stimulation with TGFβ2 (0.4nM) however was very similar, regardless of disease.

6.6. Setting up an HB-EGF ELISA

The mRNA data suggests that TGFβ2 induces both HB-EGF and the protease involved in its cleavage from the cell surface. The increase in TIMP-3 levels possibly represents a negative feedback mechanism to regulate the amount of HB-EGF that is shed from the surface. To determine whether TGFβ2 can induce the shedding of HB-EGF *in vitro*, an ELISA was developed (section 2.5.3) to measure the amount of mature cleaved HB-EGF in the conditioned medium of TGFβ2 treated fibroblasts.

6.7. The hunt for HB-EGF

Initially, conditioned medium from TGFβ2 treated fibroblasts was assayed for release of soluble HB-EGF. Disappointingly, no HB-EGF was detected in any sample, regardless of treatment or disease. To promote the cleavage of HB-EGF into the medium, PMA was added at a concentration of 10nM, the optimal concentration for amphiregulin release. As HB-EGF was shown to be utilised by the fibroblasts (fig. 6.1), a neutralising EGFR antibody was added at 10μg/ml; a

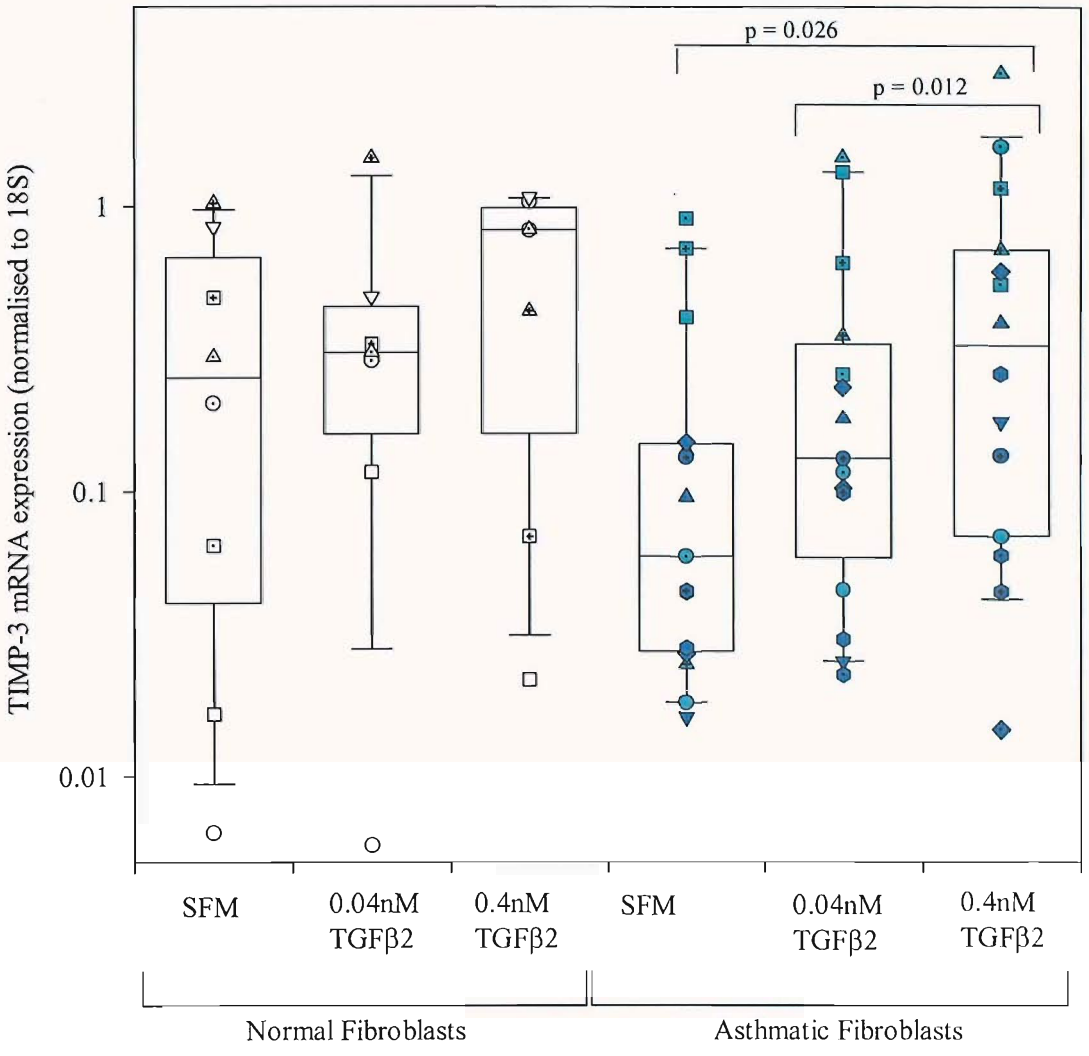

Figure 6.5: TGFβ2 induces TIMP 3 expression in asthmatic fibroblasts

Normal (n=8, unfilled symbols), mild asthmatic (n=7, turquoise symbols) and moderate severe asthmatic (n=8, blue symbols) fibroblasts were treated with TGFβ2 for 24 hours. TIMP-3 expression was measured using RT-PCR and normalised using 18S rRNA. The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences between the asthmatic ($p=0.004$) groups but not in the normal ($p=0.565$) data sets. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within each group. Statistical comparisons where $p<0.05$ were noted on the graph, Bonferoni corrections were applied to account for multiple testing.

concentration shown to prevent the EGF mediated proliferation of bronchial fibroblasts (data not shown). As no HB-EGF was detected under these conditions, hexadimethrine bromide was added to the cultures in addition to the PMA and neutralising EGFR antibody. Hexadimethrine bromide is a heparin antagonist and acts to displace growth factors from heparin and heparan sulphates on the cell surface. With 100μg/ml hexadimethrine bromide, HB-EGF was finally detected in the medium.

To determine the optimal time point to measure the release of HB-EGF, a kinetic study was performed on the bronchial fibroblasts. Two representative cultures were chosen to measure the release of HB-EGF. The fibroblasts were subjected to PMA and hexadimethrine in addition to TGFβ2 +/- neutralising EGFR antibody. The greatest release of HB-EGF occurred after 6 hours in the presence of TGFβ2 and neutralising EGFR antibody (fig. 6.6). HB-EGF levels appeared to decrease after 24hrs, suggestive of either receptor internalisation after antibody binding followed *by de novo* synthesis of EGFR, or degradation of HB-EGF. In further experiments, the release of HB-EGF was shown to be slightly improved by pre-stimulating the fibroblasts with TGFβ2 for 24 hours prior to exposure of PMA, hexadimethrine and EGFR neutralising antibody.

Normal (n=5) and asthmatic (n=8) fibroblasts were stimulated for 24 hours with +/- TGFβ2 and then treated with hexadimethrine and EGFR neutralising antibody +/- PMA for 6 hours (fig. 6.7). As predicted, there was a trend for PMA and TGFβ2 increased release of HB-EGF into the medium. The PMA induced release of HB-EGF reached significance in the TGFβ2 treated normal fibroblasts ($p=0.028$). There was a trend for a higher release of HB-EGF from the healthy control fibroblasts compared to the asthmatics.

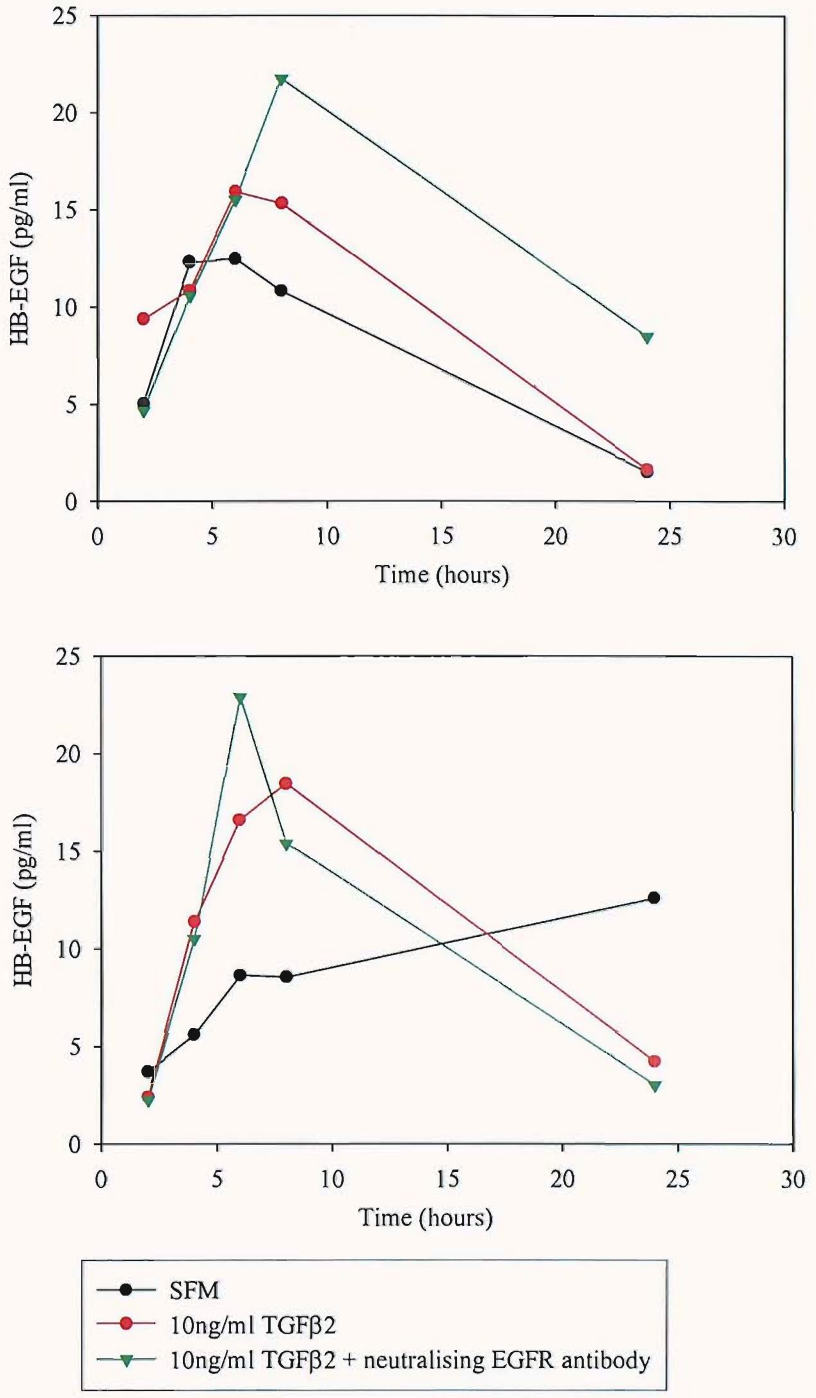

Figure 6.6: TGFβ2 induces HB-EGF release after 6 hours

Two representative fibroblast cultures were treated with TGFβ2+/- EGFR neutralising antibody, in the presence of 10nM PMA and 10mg/ml Hexadimethrine, for 2-24 hours. Media was collected at each time point and HB-EGF expression was measured by HB-EGF ELISA.

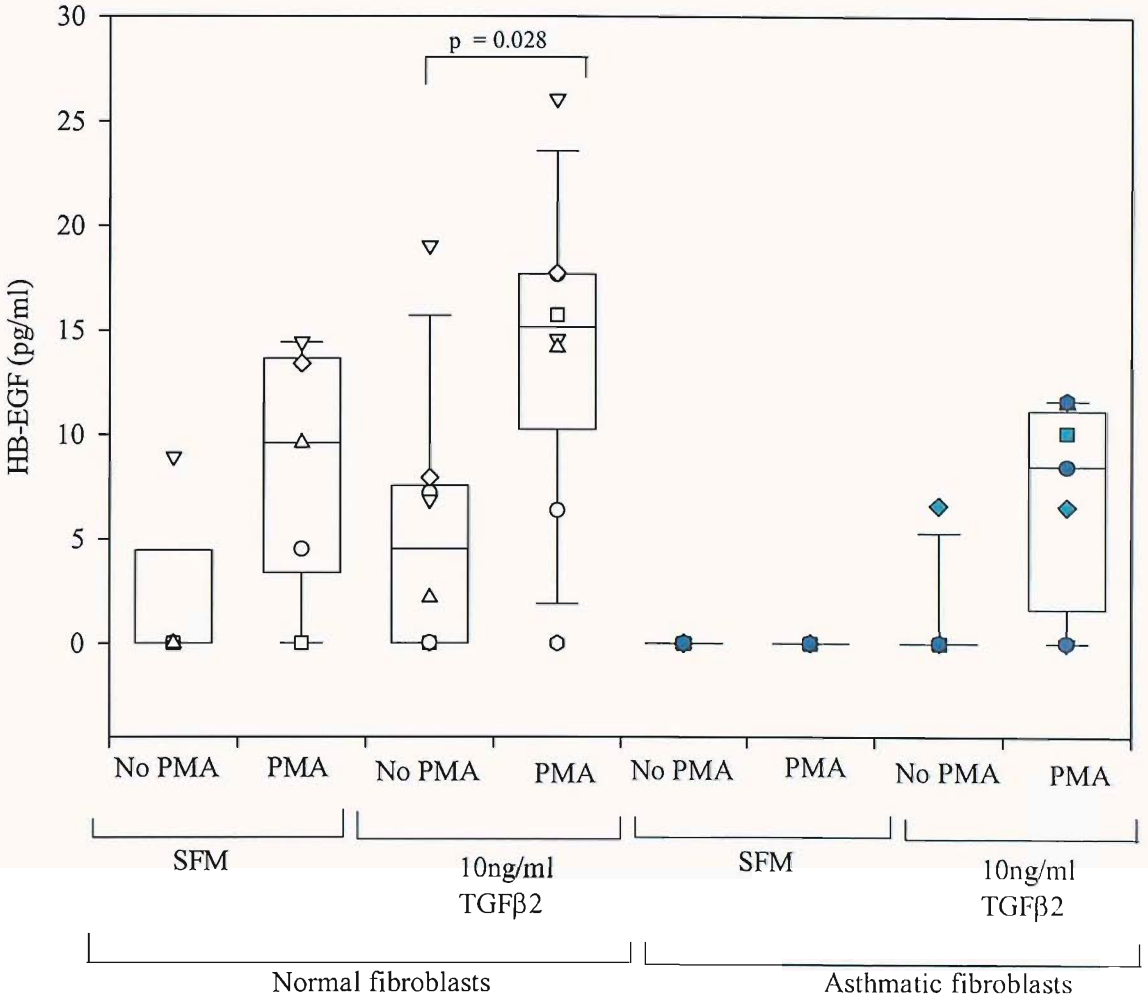

Figure 6.7: HB-EGF release from bronchial fibroblasts

Normal (n=5, unfilled symbols), mild asthmatic (n=3, turquoise symbols) and moderate severe asthmatic (n=4, blue symbols) fibroblasts were stimulated for 24 hours with or without TGFβ2 and then treated with hexadimethrine and EGFR neutralising antibody with or without PMA for 6 hours. HB-EGF was detected in the conditioned media using an in-house ELISA.

The results are displayed as a box plot showing median, interquartile range, 10th and 90th percentile. The sensitivity of the ELISA is 4pg/ml, therefore conditioned media that contained less than 4pg/ml HB-EGF have been represented graphically as a symbol at baseline.

To test for multiple intra-group differences, a Friedman test was performed. The results revealed that there were significant differences in the normal ($p=0.034$) data set. A Wilcoxon Signed Ranks test was used to determine which conditions were significant within this group. Statistical comparisons where $p<0.05$ were noted on the graph.

6.8. Discussion

HB-EGF is a potent smooth muscle chemoattractant and mitogen. It has been implicated in atherosclerotic plaque formation (339), myocardial infarction (355;356), neointimal accumulation induced by arterial injury (357), pancreatic fibrosis (358) and bleomycin induced lung fibrosis (359). Although high expression levels have been associated with the above remodelling processes, there has not been a reported link with asthma.

The hypothesis that HB-EGF is up-regulated in asthma is based on a number of observations. The primary rationale behind linking HB-EGF to the pathogenesis of asthma is the observation that HB-EGF is a mitogen and chemoattractant for fibroblasts and smooth muscle cells. An increase in the expression of HB-EGF in asthma may play a role in the fibroblast and smooth muscle hyperplasia associated with airway remodelling. It has been suggested the epithelium is more susceptible to damage in asthmatic airways, resulting in denuded areas of the epithelium and the detection of creola bodies in the BAL fluid. HB-EGF has been detected in wound fluid in a porcine model (360) and after scrape-wounding epithelial cell monolayers (Ellis *et al*, 2001), suggestive of a role in epithelial repair. In parallel, there are a couple of references suggesting that HB-EGF is anti apoptotic towards epithelial cells, possibly acting to suppress NF-κβ activity (361-363).

I have shown that HB-EGF is a mitogenic factor for bronchial fibroblasts, regardless of their origin. I have also shown that TGFβ2 induces both HB-EGF mRNA and protein. The data in chapter 1 suggest that TGFβ2 can act to suppress the basal proliferation of bronchial fibroblasts. This conflicting data represents something of a conundrum. This study has concentrated on measuring the release of soluble HB-EGF and it should be noted that TGFβ2 alone can not induce the cleavage of soluble HB-EGF. Indeed, even after optimising the *in vitro* conditions to induce the greatest release of HB-EGF, the highest concentration of growth factor measured was 26pg/ml. It is unlikely that such a low concentration of growth factor could induce a significant proliferative response.

Contrary to expectations, HB-EGF expression and release was highest in the normal fibroblasts, observations which do not support the hypothesis. As with the amphiregulin data, in attempting to interpret the significance of the data, it is important to comprehend how the results were obtained. The data presented here reveal how TGFβ2 is a potent inducer of HB-EGF mRNA expression. A number of studies have shown that TGFβ levels are higher in asthmatic airways compared to normals. In view of this, although HB-EGF expression is lower in the asthmatic fibroblasts, only the asthmatic fibroblasts are growing in a microenvironment rich in TGFβ2.

This study suggests that HB-EGF is not constituently released from bronchial fibroblasts. HB-EGF could only be detected in the medium after the cells were treated with hexadimethrine bromide, suggesting the proHB-EGF is present on the cell surface. The release of HB-EGF from the cell surface requires the presence of PMA to induce sheddase activity, possibly ADAM 12. In asthmatic airways, where higher levels of TGFβ and GPCR ligands have been reported, fibroblasts may be induced to release higher levels of HB-EGF, thus contributing to remodelling (326-329).

The HB-EGF ELISA only detected small quantities of soluble HB-EGF in fibroblast conditioned media although it could detect the recombinant HB-EGF used for the standard curve. Conditioned media from both primary fibroblasts and epithelial cells was used to determine whether the ELISA could detect native HB-EGF, although only very low levels were detected. Post translational modifications such as glycosylation may have hidden the epitope recognised by the antibodies used in the ELISA. It should be appreciated therefore that although small levels of soluble HB-EGF were detected, the cells may have produced alternatively modified HB-EGF which was not detected. Other reports in the literature surrounding HB-EGF detection have suggested similar problems in detection. For example, Wu *et al* (364) collected culture medium and then precipitated it using heparin-sepharose beads overnight at 4°C. Future work would include methods to concentrate the HB-EGF in the conditioned media to enhance the detection in the ELISA.

In conclusion, both TGFβ2 and EGF can induce bronchial fibroblasts to express HB-EGF mRNA, although TGFβ2 was more potent at eliciting a response at equimolar concentrations. The normal fibroblasts expressed and released more HB-EGF than asthmatic fibroblasts. This was a surprising observation, although in the microenvironment of the asthmatic lung, *in vivo* observations may not correlate to the *in vitro* disease-related trends. The results presented in this chapter further highlight the intricacies and overlapping roles of the TGFβ and EGF signalling pathways.

6.8.1. Novel Findings

- HB-EGF induces the proliferation of fibroblasts. No difference was observed between the asthmatic and normal fibroblasts in terms of proliferation

- TGFβ2 induces HB-EGF mRNA to a greater extent in normal fibroblasts than asthmatics

- EGF induces HB-EGF although to a lesser extent than TGFβ2. No difference was observed between the normal and the asthmatics

- TGFβ2 induces ADAM 12 mRNA

- TGFβ2 induces TIMP-3 expression in asthmatic fibroblasts

- HB-EGF protein release is increased by TGFβ2 and PMA in normal fibroblasts

CHAPTER 7

Final Discussion

CHAPTER 7

FINAL DISCUSSION

7.1. Major findings

- TGFβ2 induces differentiation of fibroblasts but suppresses proliferation.

- EGF lifts the TGFβ2 mediated suppression of proliferation but fails to prevent the TGFβ2 induced differentiation of fibroblasts.

- EGF induces amphiregulin mRNA expression, and in combination with PMA, promotes release of mature protein.

- TGFβ2 induces HB-EGF mRNA. Although culture conditions were optimised to induce HB-EGF release, mature protein was only just detectable (<20pg/ml).

- Although asthmatic fibroblasts expressed lower basal levels of αSMA, CTGF and TGFβRI mRNA, no differences in the number of cells containing filamentous αSMA or in the ability of the cells to bind TGFβ1 were noted.

- Although EGF induced EGFR mRNA expression in the asthmatic fibroblasts and not the normals, no differences in EGF induced proliferation were noted between the two groups.

- Normal fibroblasts release more amphiregulin than asthmatics

7.2 TGFβ2 and EGF

Proliferation and differentiation are considered to be mutually exclusive events. In order for a cell to differentiate, the cell must first stop dividing. The equimolar concentration of TGFβ2 and EGF gave a mixed population of cells, with some cells differentiating whilst retaining a proliferative compartment. Both normal and asthmatic fibroblasts responded similarly to TGFβ2 and EGF in terms of proliferation and differentiation. The response initiated by the combination of TGFβ2 and EGF appeared to prime the normal and asthmatic fibroblasts for both proliferation and differentiation. Initially, after 24 hours, EGF appeared to antagonise the fibrotic effects of TGFβ2 by suppressing αSMA expression at the mRNA and protein level. After prolonged stimulation, EGF potentiated the TGFβ2 response to promote an exaggerated induction of TGFβ1, TGFβ2, αSMA and CTGF, indicative of a fibrotic response.

The complexities of growth factor interactions were further studied in chapters 5 and 6. For example, TGFβ2 antagonised the EGF induced expression of amphiregulin whereas both TGFβ2 and EGF could induce HB-EGF mRNA expression. To summarise, these data show that EGF can modify TGFβ2 responses in fibroblasts. Not only can EGF can antagonise TGFβ2 responses, but EGF can have the same effect as TGFβ2 or indeed potentiate the TGFβ2 response.

7.3. Effect of disease severity?

There are a number of references to suggest that fibroblast and myofibroblast number increases with disease severity. There were, however, no significant differences in the current study between mild asthmatic and moderate/severe asthmatic fibroblasts in terms of receptor and ligand expression or functional readout. In view of this, mild asthmatic and moderate/severe asthmatic data were plotted together for the purposes of graphical representation throughout the thesis, although individual data points were colour coded to allow the reader to quickly differentiate between the mild and moderate/severe data.

These results are surprising in view of the fact that all the moderate/severe asthmatic subjects were taking corticosteroids for a significant period of time prior to bronchoscopy. These results suggest that it is the local tissue environment that affects myofibroblast number, rather than an intrinsic defect.

7.4. Do fibroblasts from asthmatic airways respond differently to TGFβ2?

One aim of this thesis was to determine whether normal and asthmatic fibroblasts, cultured under identical conditions *in vitro*, would behave in a similar manner when exposed to TGFβ2. Results from chapters 3 and 4 highlighted a number of disease related differences in baseline mRNA expression. For example, TGFβ RI, CTGF and αSMA expression were all expressed at a significantly lower level in quiescent asthmatic fibroblasts compared to normal fibroblasts.

In response to growth factor stimulation, the interpretation of the results obtained at the mRNA level became extremely complex. TGFβ2 induced αSMA and CTGF expression, as well as supporting a trend for further TGFβ1 and TGFβ2 expression in normal and asthmatic fibroblasts. The lower baseline appeared to make the asthmatic fibroblasts more sensitive to the fibrotic activity of TGFβ2, as illustrated by the expression of TGFβ RI and TIMP-3, where levels were increased only in disease after stimulation with TGFβ2. To summarise, a lower mRNA expression level of TGFβ RI in asthmatic fibroblasts may account for an enhanced ability to respond to TGFβ2 in some cases where the normal fibroblasts are already maximally induced (e.g. TGFβ RI and TIMP-3).

Although the differences in mRNA expression are very interesting, few differences were noted between the normal and asthmatic fibroblasts at the protein level. Normal and asthmatic fibroblasts responded similarly to TGFβ2 stimulation, in terms of the number of αSMA positive cells after treatment. The increase in the amount of αSMA per cell only significantly increased in the asthmatic fibroblasts, although the trend was similar in both cases. Neither the proliferation data nor the TGFβ1 release data suggested any significant differences between the two subject

groups. Overall the data from this study suggest that asthmatic fibroblasts do not respond differently to TGFβ2 compared to the healthy control fibroblasts.

7.5. Do fibroblasts from asthmatic airways respond differently to EGF?

EGF did not alter the expression levels of the TGFβ receptors, ADAM 17 or TIMP3. Interestingly, after 24 hours, EGF only induced EGFR mRNA expression in the asthmatic fibroblasts. This short term effect of EGF on EGFR mRNA expression was not obvious over 5 days. If the increase in mRNA was translated to protein, the asthmatic fibroblasts might have demonstrated a higher proliferative response. This was not evident. To summarise, the data from this study suggest that asthmatic fibroblasts do not respond differently to EGF compared to the healthy control fibroblasts. Future work on mRNA expression would further identify whether EGFR mRNA could be induced at a different time point, if at all in the normal fibroblasts upon EGF treatment.

7.6. Do asthmatic fibroblasts release more EGFR ligands?

One major difference between the normal and asthmatic fibroblasts was observed in relation to amphiregulin expression and release. mRNA expression data suggested that asthmatic fibroblasts express more amphiregulin transcripts after stimulation with EGF compared to normal fibroblasts. There was no difference in the release of amphiregulin between the two subject groups after stimulation with EGF alone. EGF in combination with PMA however induced a greater release of amphiregulin from the normal fibroblasts compared to the asthmatics suggesting differences in the ADAM or TIMP profiles. At the mRNA level no differences in ADAM 12, ADAM 17 or TIMP-3 expression were observed.

TGFβ2, and EGF although to a lesser extent, induce HB-EGF mRNA expression. Very small quantities of HB-EGF were detected in the conditioned media from fibroblasts. This suggests that either the cells really only release small amounts or that they secrete HB-EGF with post translational modifications which were undetectable by the ELISA. It was interesting that HB-EGF was detected in more

of the normal conditioned media samples than the asthmatic conditioned media. To summarise, the data from this study suggest that asthmatic fibroblasts release less EGFR ligands compared to the healthy control fibroblasts.

7.7. Do asthmatic fibroblasts respond differently to amphiregulin?

The study revealed that only asthmatic fibroblasts were able to utilise amphiregulin, inducing a small but significant mitogenic response. HB-EGF induced both the asthmatic and the normal fibroblasts to proliferate. To further the study it would have been interesting to study the dose effect of amphiregulin on normal fibroblasts with regard to proliferation. Potentially the normal fibroblasts may be able to proliferate with higher doses of amphiregulin.

7.8. Critical analysis of data and assay design

7.8.1. Matrix effects

In attempting to reproduce the *in vivo* conditions *in vitro*, the contribution of the matrix was considered. Not only does the matrix act as a biological sink for growth factors but it provides structural support for cells, permitting them to anchor themselves in a particular location. Fibroblasts *in vivo* grow within a complex mesh of ECM proteins, the bulk of which is laid down by the fibroblasts and myofibroblasts themselves. The fibroblasts used in this thesis were not grown in plastic ware coated with collagen, although the FBS supplemented growth medium contained fibronectin to aid their initial attachment onto the plastic. As a result, the cells were able to grow in an environment of their own making. Johnson *et al* (36) have revealed that asthmatic fibroblasts synthesise different ECM proteins to normal fibroblasts, therefore if work was to proceed using a synthetic matrix, which one should be used? Should asthmatic fibroblasts be grown on a matrix which is abundant in asthmatic airways, or should normal and asthmatic fibroblasts be grown on the same, but non-physiologically relevant matrix? Although not ideal, I believe that in allowing fibroblasts to synthesise their own matrix, I have prevented bias which may have significantly influenced my results.

7.8.2. The interplay of fibroblasts with other cell types

It is impossible to precisely simulate the airway environment *in vitro*, with the complex interplay between the different cell types present. In trying to determine the effect of a growth factor on a single cell type it is unfortunately necessary to study that event in isolation. We have, however, been fortunate to be able to work with a supply of primary cells allowing us to make comparisons between normal and asthmatic derived fibroblasts, without having to rely on cell line models. To further interpret the observations made in the last two chapters concerning the proposed paracrine activity of amphiregulin and HB-EGF, it would have been interesting to continue the work using co-cultures.

Primary bronchial epithelial cells grown on collagen coated transwells can be differentiated at an air liquid interface in the presence of retinoic acid over 21 days (365). These transwells inserts can be then be placed on the top of wells containing a monolayer of primary fibroblasts. This model is one of the best alternatives available to study signalling between the epithelium and the underlying fibroblasts. Similar work has been performed by Chakir *et al* (366), where human bronchial epithelial cells were seeded over a collagen gel containing bronchial fibroblasts. Results from this study showed that collagen IV and laminin are laid down in a layer between the epithelial cells and fibroblasts, suggestive of basement membrane formation. Morphologically, epithelial cells took on more disorganised structure when grown on asthmatic fibroblasts. Recently, Paquette *et al* (367) showed that asthmatic bronchial epithelial cells detach more readily from the matrix over time compared to normals, when grown in the presence of fibroblasts. These results are very exciting as it appears that features of airway remodelling can be observed *in vitro* as a result of signalling abnormities between the epithelial cells and fibroblasts.

7.8.3. mRNA and protein data

A number of observations presented in this thesis are at the mRNA level and not the protein level. Although changes in transcript levels are very enlightening, it is the changes at the protein level that ultimately affect the cellular response to a growth factor. A number of attempts were made throughout the thesis to obtain more protein data, although this work was largely hindered by the lack of specific TGFβ antibodies on the market. One major disadvantage with relying on Taqman PCR data is that it is impossible to determine whether a small population of cells within the culture are responsible for a change in mRNA levels, or whether all the cells are responding in the same way.

Additionally, a number of the growth factor induced changes at the mRNA level did appear relatively small. These small changes in transcript levels may not significantly alter the functional outcome after 5 days *in vitro*. However, over a number of years, *in vivo*, these small changes maybe very relevant. *In vitro* it is

only possible to study events in isolation without appreciating the complex interplay of the other cell types present. In the asthmatic lung, the cumulative changes may be more effective in inducing a fundamental change in fibroblast behaviour which may account for the fibrosis associated with remodelling.

7.8.4. Only two growth factors

A common limiting factor in the design of *in vitro* cell based assays is that growth factors are frequently studied in isolation. There is a rich plethora of growth factors and cytokines present in the lung. Consequently, no growth factor can act in isolation in the complex tissue microenvironment. The down stream effects initialized by one growth factor can directly or indirectly affect the expression, release or activity of other growth factors and cytokines. Although not ideal, the work undertaken in this study was designed to determine the effects of TGFβ2 and EGF on fibroblast behaviour both in isolation and together.

As the TGFβ2 and EGF interactions appear to be so intricate, it becomes apparent that the effects of TGFβ2 should be studied in combination with other growth factors. There are a few reports of TGFβ acting synergistically with other growth factors in the literature. For example, ET-1, platelet-derived growth factor (PDGF)-BB and TGFβ1 were reportedly only capable of increasing DNA synthesis and collagen production in normal and asthmatic fibroblasts (368) when added together. TGFβ has also been reported to act synergistically with IL-13 to promote the expression and release of eotaxin in bronchial fibroblasts (141). This finding has interesting implications in asthma as eotaxin is a potent eosinophil chemoattractant.

7.8.5. Fibroblast, myofibroblast and smooth muscle cells?

There is considerable controversy as to the definition of a fibroblast, a myofibroblast and a smooth muscle cell. For the purposes of this thesis, the induction of αSMA expression has been used as a marker of differentiation. This is the most widely used marker of differentiation. Unfortunately total confidence in

separating the cell types can only be achieved with the use of an electron microscope.

Cells grown out from biopsy tissue were characterised for smooth muscle markers prior to use in assays. No cultures stained positive for smooth muscle markers. It has been postulated that TGFβ can induce fibroblasts to differentiate into myofibroblasts and further into smooth muscle cells after prolonged stimulation. As there are no reports in the literature of fibroblasts becoming smooth muscle cells after only 5 days, fibroblasts treated with TGFβ2 for 5 days were not reanalysed for smooth muscle markers. As the flow cytometry data suggested that there were small numbers of αSMA positive cells in the SFM controls, it is acknowledged that the starting fibroblast cultures may have been contaminated with a few myofibroblasts. Unfortunately there are no cell surface markers that are characteristic of myofibroblasts that could be used to sort the starting cultures using a cell sorter. Consequently very little else could have been done to ensure the purity of the fibroblasts used throughout this thesis.

7.9. In vitro extrapolations

The data from this thesis shows that normal fibroblasts, under the appropriate conditions, can release more amphiregulin than asthmatic fibroblasts. Although this is an important finding, it is necessary to try and extrapolate this finding to the *in vivo* situation. In the altered microenvironment of the asthmatic lung, it has been reported that there are more G-protein coupled receptor ligands, proteoglycans and TGFβ2.

Asthmatic fibroblasts release less amphiregulin than normals. Amphiregulin release may be further suppressed by the presence of TGFβ2 in the asthmatic airways. The reduced release of amphiregulin in combination with thickening of the *lamina reticularis*, may effectively prevent the epithelial cells from utilising the growth factor. This may contribute to the lack of epithelial repair seen in asthma.

It has been proposed that there is a re-establishment of bi-directional communication between the epithelium and mesenchyme in asthmatic remodelled airways, akin to events during embryogenesis. Based on the findings of this thesis concerning the production of the epithelial growth factor, amphiregulin, by fibroblasts and the subsequent utilization of amphiregulin by asthmatic fibroblasts, I believe that this hypothesis can be extended. The ability of asthmatic fibroblasts to use amphiregulin as an autocrine growth factor may lead to suppression of paracrine activity. In preventing the epithelial cells from using amphiregulin, the epithelial cells are in effect "starved" of mesenchymal derived growth factors in response to damage. The deterioration of bi-directional communication may be augmented by the thickening of the *lamina reticularis* in asthma. The thickened *lamina reticularis* may reduce the trafficking of soluble mediators from mesenchyme to the epithelium. If the growth factors can not reach their proposed destination, then levels will increase locally. This is especially relevant in the case of amphiregulin and HB-EGF, both of which bind to heparan sulphates proteoglycans present in the ECM. The increased local accumulation in the submucosa could lead to increased numbers of fibroblasts and smooth muscle cells. This in turn may explain a number of observations which have lead to a belief that the epithelium is more susceptible to damage in asthma and that upon damage the epithelium takes much longer to repair resulting in a prolonged "repair phenotype" which accentuates the inflammatory cycle.

A decrease in signalling within the EMTU may also alter TGFβ signalling. I propose that early remodelling events are initiated by the release of TGFβ2 from damaged epithelial cells. However, as the disease progresses, the link between the epithelium and the underlying mesenchyme becomes impaired. Work in this thesis has not identified any major differences between normals and asthmatics with regard to TGFβ1 release from fibroblasts. Therefore, release of TGFβ from the influx of inflammatory cells into the mesenchyme may represent the main source of TGFβ in the later stage of disease, when remodelling is more established. In summary, I propose a modified EMTU model to explain the remodelling events in asthma. From early organogenesis to late adulthood, the epithelium and mesenchyme retain some degree of communication. Upon the onset of disease,

asthmatic airways undergo remodelling which causes a physical hindrance to the flow of mediators through the basement membrane, ultimately resulting in a bronchial epithelium with an increased susceptibility to damage and a diminished supply of growth factors to initiate repair (fig. 7.1).

This proposed sequence of events is very similar to that observed during Age-related macular degeneration (AMD) a disease which leads to remodelling in the eye. AMD is the commonest cause of blindness in the elderly of developed countries. Retinal pigment epithelium (RPE) lines the inner wall of the macula, the region responsible for high acuity vision. The RPE lies on a layer of connective tissue known as the Bruch's membrane (fig 7.2) which is composed of two basement membranes, for the RPE and choriocapillaris, two collagen layers and an inner elastic layer. Below the Bruch's membrane is a network of capillaries, the choriocapillaris, which supply nutrients to the epithelium.

There are two types of AMD, a wet and dry form. In both cases there is thickening of the Bruch's membrane due to excess deposition of ECM proteins and the accumulation of fluorescent cellular debris known as 'drusen'. Wet AMD, a more severe form of the disease, is characterised by neovascularisation, resulting in haemorrhage and scar formation. It has been proposed that the thickening of the membrane effectively limits the flow of nutrients from the blood, causing the epithelial cells to become atrophic (fig 7.2) (369). There are striking morphological similarities between the AMD model, with the thickened Bruch's membrane, and asthma, with thickening of the *lamina reticularis*. The thickened *lamina reticularis* may therefore be key to interpreting the epithelial and mesenchymal changes that occur in asthmatic airways. The epithelium, starved of mesenchymal growth factors and nutrients from the blood, becomes more susceptible to damage caused by oxidant stress and environmental pollutants. Bucchieri *et al* (24) have reported that asthmatic bronchial epithelial cells are more susceptible to apoptosis in response to oxidant stress than normal epithelial cells. This may explain the sloughing of epithelial cells in bronchial epithelium, resulting in denuded areas and detection of creola bodies in the BAL fluid of asthmatics.

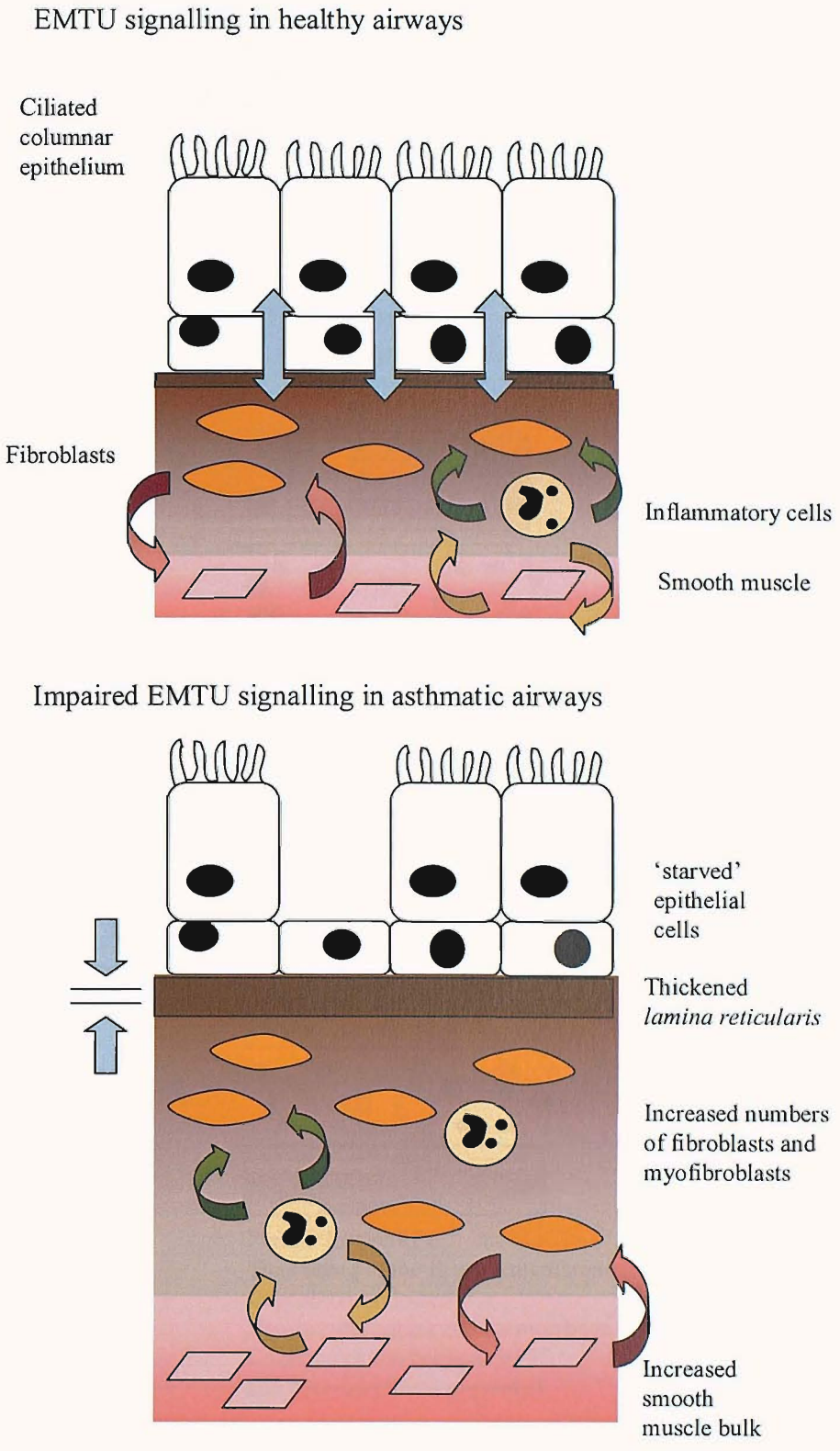

Figure 7.1: Impaired Epithelial-Mesenchymal Trophic Unit (EMTU) signalling in asthma

In the model proposed, there is a decline in signalling from the mesenchyme to the epithelium. The increased thickness of the *lamina reticularis* may prevent or suppress bidirectional communication between the epithelium and the mesenchyme resulting in an epithelium more susceptible to damage and less able to repair due to physical isolation from the mesenchyme.

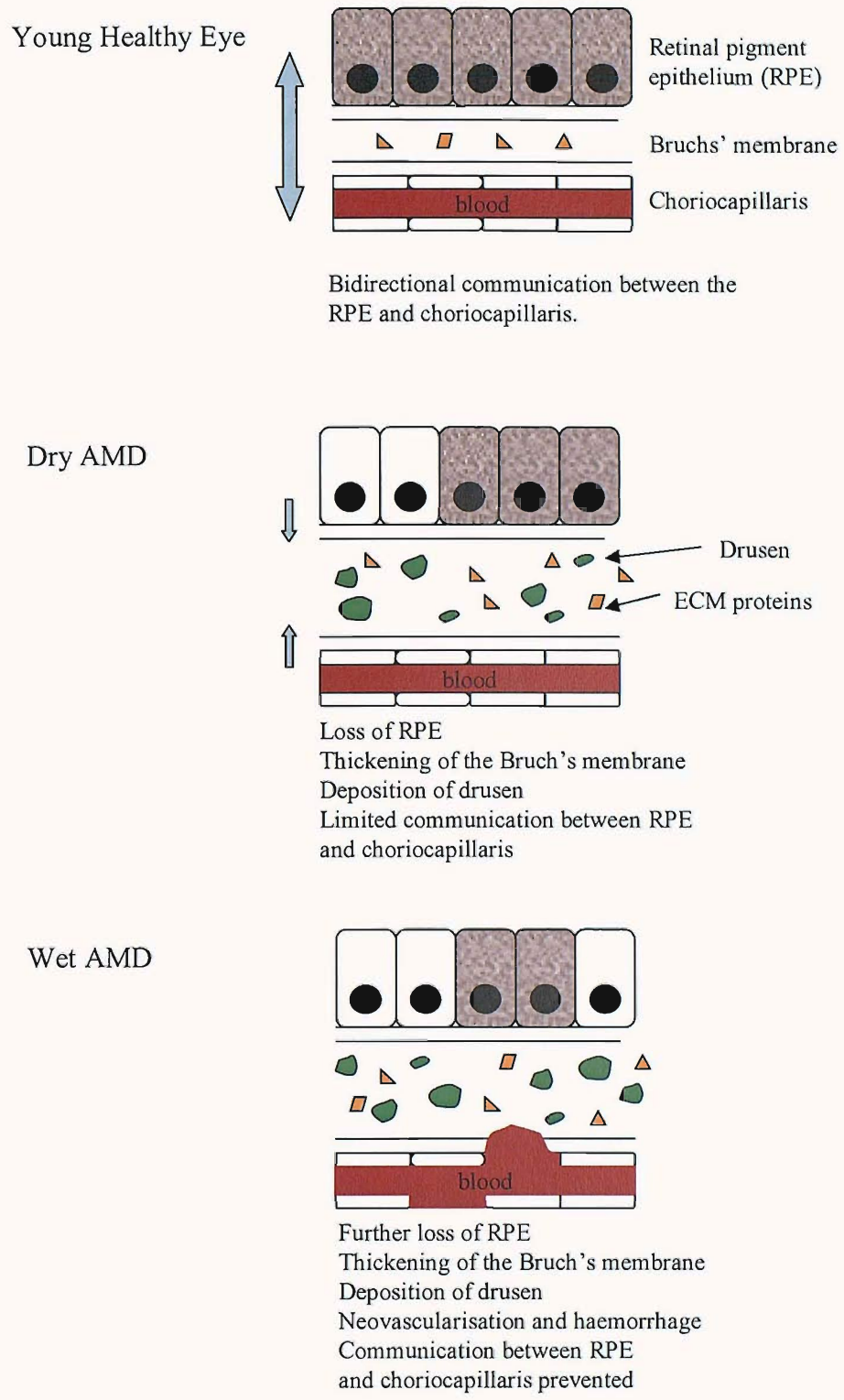

Figure 7.2: Proposed decline in epithelial-mesenchymal signalling in asthma mirrors events in age-related macular degeneration

A schematic representation of the changes in the macula as a result of age-related macular degeneration (AMD). It is proposed that events following the thickening of the Bruch's membrane closely reflect Changes in the asthmatic airways in response to thickening of the *lamina reticularis*. The thickened membrane is postulated to prevent effective bidirectional communication between the epithelium and mesenchyme.

A mutation in fibulin 5 (370) has been implicated in AMD. Fibulins are believed to regulate the assembly of tropoelastin into elastic fibres by linking elastin with integrins on the cell surface. Asthma, like AMD, is a disease of altered elastin expression. In AMD, the misfolded fibulin 5 may account for the detachment of the epithelial monolayer due to reduced interactions between integrins and the elastic layer in the Bruch's membrane (as reviewed by Johnson and Anderson (371)). A mutation in fibulin, or altered expression levels may account for the reduction in elastin levels demonstrated in the bronchial submucosa of asthmatics (47). Furthermore, fibulin 5 is located on chromosome 14q31 (372). 14q has been associated with mutations in total serum IgE and the alpha/delta locus on the TCR (373), providing an interesting link with asthma and the risk of atopy.

Sorsby's fundus dystrophy (SFD) is a disease that closely resembles AMD. It is a dominantly inherited disease characterised by deposition of drusen in the Bruch's membrane, accumulation of ECM proteins and choroidal neovascularisation. It is caused by mutations in the TIMP-3 gene (374), which lead to blindness in middle age. Elevated TIMP-3 levels have been demonstrated in the RPE in SFD (375), with TIMP-3 protein localised to the Bruch's membrane (376) (377). The increased TIMP-3 levels are believed to prevent ECM degradation by inhibiting MP activity. Interestingly, TIMP-3 is reported to act as a binding protein for fibulin 3 (378), missense mutations of which have been linked to Malattia Leventinese (379), a disease very similar to AMD. In the current study, TIMP-3 expression levels were increased upon TGFβ2 stimulation in asthmatic fibroblasts (chapter 6). Increased TIMP-3 levels in asthmatic airways, similar to that observed in SFD, may also contribute to *lamina reticularis* thickening and consequently a breakdown in epithelial-mesenchymal signalling.

7.10. Future *in vivo* studies

In order to fully understand these observations and to determine whether the differences noted in the thesis really are apparent in the asthmatic airways, it is necessary to perform *in vivo* studies. The easiest approach would be to carry out immunostaining on embedded biopsy sections from normal and asthmatic subjects.

Future work would therefore involve examination of EGF, amphiregulin and HB-EGF levels in embedded tissue. This technique would detect differences in the amount of protein present as well as providing information on its location within the tissue, e.g. whether the protein is expressed in the epithelium or in the submucosa. Co-localisation studies could also highlight correlations in expression patterns. This technique may go some way to determine whether the *lamina reticularis* represents a physical barrier to the movement of growth factors. Indeed, the staining may reveal more intense amphiregulin staining below the *lamina reticularis*. However, if the asthmatic fibroblasts can utilise this growth factor as suggested by the findings in this thesis then the area below the *lamina reticularis* may have a higher concentration of fibroblasts.

It would also be interesting to further understand the role of amphiregulin and HB-EGF in asthmatic fibroblasts. The effect of both EGFR ligands on TGFβ2 induced αSMA expression could be performed by flow cytometry. To complete the study it is essential that protein expression of EGFR should also be determined between the normals and asthmatics. Again, surface receptor expression could be measured using flow cytometry.

7.11. EGFR as a therapeutic target

It was hypothesised that an altered EGF signalling pathway may be responsible for the increased number of fibroblasts reported in remodelled asthmatic airways. However, the work presented in the current study showed that asthmatic fibroblasts *in vitro* do not appear to release excess amounts of EGFR ligands. The amphiregulin induced proliferation of asthmatic fibroblasts was an interesting finding, however the increase in fibroblast number, although significant, was small in comparison to the mitogenic activity EGF or HB-EGF. The increase in EGFR mRNA in asthma was very interesting as it mirrors the situation in the asthmatic epithelium where EGFR protein expression is present at a higher level. For example, Puddicombe *et al* reported that EGFR expression in the bronchial epithelium increased in accordance with disease severity. In a further study, Hamilton *et al* (274), demonstrated that phosphotyrosine levels in the epithelium,

corresponding to activation of EGFR, were higher in moderate severe asthmatics than the healthy controls.

This study has shown that fibroblasts can respond to EGF to promote proliferation. Reports from the literature suggest that although mesenchymal cells release EGF, the main source appears to be inflammatory cells and platelets (380), suggesting that fibroblast proliferation may be driven by exogenous sources of ligand. A therapy which works by suppressing signalling via the EGFR would therefore seem very attractive. EGFR antagonists such as tyrphostins, which block the ATP binding site in EGFR, may be beneficial in preventing mesenchymal cell proliferation and goblet cell differentiation. For example, Tsang *et al* (381) demonstrated that tyrphostins can attenuate thrombin induced guinea pig airway smooth muscle proliferation. Tyrphostins have also been shown to prevent OVA induced contraction, mast cell degranulation (382) and antigen induced eosinophil infiltration (383). Furthermore, Vargaftig and Singer (384) demonstrated that AG1478, a tyrphostin, could inhibit hyperresponsiveness, inflammation, and lung remodelling induced by ovalbumin in a murine model. However, an asthma therapy that relies on non-specific suppression of EGFR may prevent epithelial proliferation, prolonging the chronic 'repair cycle' concomitant to the process of remodelling in the lung.

7.12. TGFβ as a therapeutic target

The results from this thesis have confirmed the fibrotic activity of TGFβ2 in bronchial fibroblasts, suggesting that a reduction in mesenchymal TGFβ2 levels may limit remodelling in the asthmatic lung. The thesis has also confirmed the mitogenic role of EGFR ligands. Therefore a dual therapy that could diminish both EGF and TGFβ2 induced responses would be the most attractive option.

TGFβ2 is a pleiotropic growth factor, capable of altering the behaviour of most cell types. For example, TGFβ has anti-inflammatory effects, presenting a potential dilemma for TGFβ suppression. The importance of TGFβ *in vivo* has been demonstrated using knock-out mice. TGFβ1, 2 and 3 knock-out mice either die *in*

utero or shortly after birth (385-387). Hence, a reduction in TGFβ levels as opposed to complete suppression may act to attenuate fibrosis whilst still present at a level to maintain homeostatic functions of the growth factor.

The efficacy of anti-TGFβ antibodies in the clinic has been demonstrated by Cambridge Antibody Technology. An anti-TGFβ2 antibody has reached phase III clinical trials (lerdelimumab, CAT-152) as an anti-scarring agent, particularly effective following eye surgery. Trabeculectomy is the commonest type of surgical operation performed on patients with glaucoma. CAT-152 has been found to significantly reduce the scarring that can occur as a result of the trabeculectomy, three and six months after surgery (387). *In vitro*, CAT-152 has been shown to inhibit TGFβ2 induced events such as Smad translocation, expression of transdifferentiation markers and contraction in a human lens cell line (388). Studies have also examined the anti-fibrotic effects of CAT-152 in other organs. For example, anti-TGFβ2 antibodies have been shown to suppress kidney fibrosis in diabetic rats (389) and cause a reduction in collagen levels in the lung in mice treated with bleomycin (390). An anti-TGFβ1 antibody CAT-192 (metelimumab) is also being developed as a potential treatment for a range of scarring and fibrotic conditions. A Phase I/II trial to evaluate CAT-192 as a potential treatment for diffuse systemic sclerosis began in November 2001. Preliminary data has shown that CAT-192 was generally safe and well tolerated and there were no treatment-related adverse events. These results are encouraging and trials of neutralising TGFβ antibodies in asthma may prove very interesting.

It is important to appreciate that a suppression of mesenchymal TGFβ levels may also attenuate the airway inflammation directly. Additionally, due to the interlinked processes of remodelling and inflammation, where growth factors from myofibroblasts can accentuate the chronic inflammation, a reduction of fibrosis may have a beneficial secondary effect on inflammation. However, while reducing fibrosis may prevent fixed airflow obstruction, a reversal of fibrosis may act to increase the hyperresponsiveness of the airways. Pare *et al* (19) suggested that the increased stiffness of the airways may act as a protective measure to prevent sporadic bronchoconstriction in response to non-specific stimuli. Therefore, in

combating the fibrosis by suppressing TGFβ we may potentially augment the 'twitchiness' of the airways.

7.13. Other anti-fibrotic agents

It would be a naive to suppose that TGFβ is the only fibrotic mediator in the airways or even that in the milieu of growth factors present in the lung that it was solely responsible for fibrosis associated with remodelled asthmatic airways. The actions of TGFβ are controlled not only by the expression levels of this growth factor in the lung and the ability of cells to respond to this signal but also on the balance of fibrotic versus anti-fibrotic factors produced as a result of wound healing.

It is interesting to surmise how halting the actions of signalling molecules down stream from TGFβ may offer a more disease-specific target. Connective tissue growth factor (CTGF) is a potential target to combat the remodelling seen in asthma. CTGF is considered to be an immediate early gene for TGFβ (312). CTGF antisense constructs and neutralising antibodies have been reported to block the effect of TGFβ on fibroblast collagen production and proliferation (391) (392) (393). CTGF is also profibrogenic *per se*, for example it can stimulate collagen I, fibronectin and α5 integrin expression in NRK cells (394) as well as inducing lysyl oxidase and collagen in human gingival cells (395). A study by Kucich *et al* (311) suggested the possible use of PKC inhibitors in suppressing CTGF levels as PKC was found to phosphorylate Smad-3 directly, inhibiting its ability to bind DNA and enhance transcription. As yet, no studies have examined the effect of CTGF on inflammatory cells.

Prostaglandin E2 (PGE2) has a counter regulatory role to suppress the fibrogenic actions of TGFβ. PGE2 can inhibit the differentiation (136), proliferation (396;397) and migration of fibrobasts (398). It also prevents ECM accumulation by inhibiting collagen synthesis and promoting the degradation of collagen (399). Altered PGE2 levels have been associated with fibrotic conditions and airway injury. Fibroblasts isolated from subjects with idiopathic pulmonary fibrosis (IPF)

have a reduced expression of COX-2 and a diminished capacity to synthesize PGE2 (148). Furthermore, Pierzchalska (400) reported deficient PGE2 production by fibroblasts from asthmatic patients after stimulation with LPS, IL-1β and TNFα *in vivo*. Inhalation of PGE2 by aspirin intolerant asthmatic subjects (401) and exercise-induced asthmatics (402) has been shown to increase FEV1 in response to challenge. Furthermore, the early success of PGE2 has been supported by its ability to attenuate allergen-induced airway inflammation and hyperresponsiveness (402) in mild asthmatics, possibly by decreasing PGD2 levels (403).

Finally, although the current study has highlighted the differentiating and proliferative effects of TGFβ and EGF, apoptosis has not been studied due to time restraints. In the normal situation, myofibroblasts are believed to apoptose at the cessation of wound repair. It is interesting to surmise whether the increased number of fibroblasts in asthmatic airways is solely due to an increased proliferative rate, or whether the fibroblasts are more resistant to apoptosis. This idea is supported by a recent report by Moulin *et al* (404) who demonstrated that myofibroblasts isolated from hypertrophic scars had a lower rate of apoptosis compared to normal wound fibroblasts, possibly due to a high expression of the anti-apoptotic factor bcl-2. In response to serum starvation, wound fibroblasts showed an increased apoptotic rate, although neither hypertrophic wound fibroblasts or normal dermal fibroblasts were affected. Another way to continue this study would be to study the protective effects of TGFβ on cell survival, as suggested by Zhang *et al* (130). The potency of TGFβ may not lie only with the differentiating effects, but also in the suppression of apoptosis.

7.14. Concluding comments

TGFβ is a potent fibrogenic growth factor which is over expressed in the asthmatic lung. The growth factor appears to play an essential role in mediating the fibrosis associated with the remodelled asthmatic airways. EGF, instead of antagonising the effects of TGFβ2, initially promoted proliferation of fibroblasts and upon prolonged exposure, permitted differentiation. Suppression of TGFβ2 and EGF signalling may represent a valuable therapeutic target in asthma. It should be

remembered that no growth factor is entirely "good or bad" and that complete ablation could cause a whole host of new problems as evidenced by knock-out mice. However, restoration of the balance between different growth factors may be of therapeutic benefit, for example through the use of neutralising antibodies.

In understanding the way asthmatic cells differ to growth factor stimulation in terms of differentiation and proliferation, it may be possible to design drugs to therapeutically prevent, suppress or even reverse the process of remodelling. Indeed, a reversal of remodelling may also re-establish communication between the epithelium and the mesenchyme. Hopefully the fuller comprehension of how signalling pathways interact in the asthmatic lung, can be manipulated to provide novel therapeutic targets to combat the remodelling and maybe attenuate the chronic inflammation, so intimately linked to the altered structure of the lungs.

CHAPTER 8

References

CHAPTER 8

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