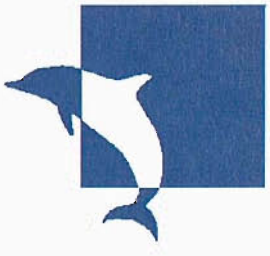
UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCE
Department of Respiratory Cell and Molecular Biology.
Division of Allergy and Inflammation Research
School of Medicine

The expression and regulation of ADAM33, a novel asthma susceptibility gene, during myofibroblast differentiation.

James Wicks



Thesis submitted for the degree of Doctor of Philosophy
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To Becca

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCE

SCHOOL OF MEDICINE

Doctor of Philosophy

THE EXPRESSION AND REGULATION OF ADAM33, A NOVEL ASTHMA SUSCEPTIBILITY GENE, DURING MYOFIBROBLAST DIFFERENTIATION.

by James Wicks

Asthma is a disease affecting millions of individuals worldwide and is characterised both by infiltration of inflammatory cells in the airways and by structural changes to the airway wall, including an increase in the deposition of sub-basement membrane proteins, as well as an increase in smooth muscle mass. The increased mass of smooth muscle is believed to contribute to the hyper-responsive nature of asthmatic bronchi to innocuous stimuli. A potential source of these muscle cells is the pool of fibroblasts that reside below the basement membrane in the airway wall.

Transforming Growth Factor β (TGFβ), a cytokine known to occur at higher levels in asthmatic airways, is known to promote differentiation of fibroblasts into myofibroblasts; this is associated with increased expression of smooth muscle related genes.

To test the hypothesis that TGFβ may promote the ability of fibroblasts to differentiate into smooth muscle cells in asthma, the expression of five smooth muscle associated genes was examined. Heavy Chain Myosin, Calponin and Desmin were all shown to be upregulated to a greater extent ($p<0.05$) in asthmatic ($n=7$) primary airway fibroblast cultures than in healthy control cells ($n=6$). α Smooth Muscle Actin and γ Actin were upregulated maximally at a lower dose of TGFβ in asthmatic cells compared with healthy cells ($p<0.05$). Alongside the upregulation of mRNA, an increase in protein for four of these smooth muscle marker genes was detected in TGFβ treated cells by immunohistochemistry. Desmin protein however, was not detected at the protein level and hence a *bona fide* phenotype switch from fibroblast to smooth muscle cell was not achieved.

Single nucleotide polyporphisms within A Disintegrin And Metalloptrotease 33 (ADAM33) have been shown to be associated with asthma susceptibility (and particularly the phenotype of bronchial hyper-responsiveness) and a decrease in lung function. ADAM33 is not expressed in inflammatory cells but is expressed in fibroblasts and smooth muscle cells within the lung.

To test the hypothesis that ADAM33 is involved in fibroblast differentiation, the effect of TGFβ on ADAM33 expression was investigated. In association with the switch towards a myofibroblast phenotype, global ADAM33 mRNA expression as well as that of individual ADAM33 splice variants was down-regulated in a dose dependent manner by TGFβ. To measure ADAM33 protein expression, antibodies were generated by the immunisation of four rabbits and two chickens with ADAM33 specific peptide sequences. These antibodies were shown to be active against their respective immunising peptides but failed to detect full length ADAM33. However, a commercially available anti-ADAM33 antibody preparation was found to specifically detect ADAM33. ADAM33 protein expression was shown to be down-regulated upon TGFβ treatment as detected by immunofluorescent staining. In addition products of ADAM33 degradation were shown to accumulate upon TGFβ treatment in primary cells as well as a recombinant model, as detected by western blot. This TGFβ initiated degradation was shown to be protease dependent as it was abolished by treatment with protease inhibitors.

In conclusion, this thesis provides novel data regarding myofibroblast differentiation in asthmatic cells versus healthy controls, as well as illustrating for the first time the regulation of ADAM33 expression by an asthma associated cytokine.

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Declaration of Authorship

I James Wicks declare that the thesis entitled THE EXPRESSION AND REGULATION OF ADAM33, A NOVEL ASTHMA SUSCEPTIBILITY GENE, DURING MYOFIBROBLAST DIFFERENTIATION and the work presented in it are my own.

I confirm that

- This work was done wholly or mainly while in candidature for a research degree at this university;
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been published as:

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Signed.....

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Finally I must pay particular thanks to my wife Becca. Her patience and constant support has been an inspiration during my time in the laboratory and whilst writing this thesis.

List of Abbreviations used in this thesis

αSMA	α smooth muscle actin
A2	Phospholipase A2
AAD	amino-acitomycin
ACTB	beta actin
ADAM	A Disintegrin And Metaloproteinase
APC	Anigen presenting cells
ATP	Adenosine triphosphate
B2M	Beta-2-microglobulin
BALF	Bronchoalveolar Lavage Fluid
BHR	Bronchial Hyperresponsiveness
BSA	Bovine Serum Albumin
cDNA	complementary Deoxyribose Nucleic Acid
COS	African Green monkey kidney cell
CT	Threshold cycle
cyt	Cytoplasmic
Da	Dalton
dis	Disintegrin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
DPP	dipeptidyl peptidase
EAR	Early allergic response
ECM	Extra Cellular Matrix
ECP	Eosinophil catioinic protein
EDN	Eosinophil derived neurotoxin
EDTA	ethylenediaminetetracetic acid
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immunosorbent Assay
EMTU	Epithelial-Mesenchymal Trophic Unit
EPO	Eosinophil peroxidase
ET-1	Endothelin 1
FACs	Fluorescence-activated cell sorter
FAM	6-carboxyfluorescein
FBS	Foetal Bovine Serum
FcεRI	High affinity IgE receptor
FEV	Forced expiratory volume
FGF	Fibroblast Growth Factor
FITC	Fluorescein-5-isothiocyanate
FN	Fibronectin
FSC	Forward Scatter
GA-100	Gentomyosin Amphtercin-100
GAPDH	Glyceraldehyde-3-phosphate
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G protein coupled receptor
GPRA	G protein coupled receptor for asthma suceptibility
HB-EGF	heparin-binding EGF-like growth factor
HBSS	Hanks balanced salt solution
HCM	Heavy Chain Myosin
HEK	Human Embryonic Kidney

HMBS	hydroxymethyl bilane synthase
HPRT1	Hypoxanthine phosphoribosyl-transferase 1
HRP	Horse-Radish Peroxidase
IFN-γ	Interferon g
Ig	Immunoglobulin
IGF	Insulin growth factor
IL-	Interleukine
KLH	Keyhole Limpet Hemocyanin
LAP	Latency associated peptide
LAR	Late allergic response
LTBP	Latent TGFβ binding protein
MBP	Major Basic Protein
MEM	Minimum essential medium
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage inflammotory protein 1 alpha
MMP	Matrix Metalloproteinase
MP	Metalloprotease
NAC	National Asthma Campaign
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
PGD	Prostoglandin
RANTES	Regulated upon activation in normal T cells expressed and secreted
RNA	Ribose Nucleic Acid
RPL13A	ribosomal protein L13a
RT	Reverse Transcription
RT	Real Time/ Room temperature/ Reverse transcription
SDHA	Succinate dehydrogenase
SDS	Sodium Dodecyl (lauryl) Sulfate
SNP	Single nucleotide polymorphism
SRF	Serum Response Factor
SSC	Side Scatter
STAT	Signal transducer and activator of transcription
TAMRA	6-carboxy-N,N,N',N-tetramethylrhodamine
TBP	TATA box binding protein
TEMED	Tetramethylethylenediamine
TGFβ	Transforming Growth Factor b
TH	T-Helper
TIMPS	Tissue Inhibitors of Metalloproteinases
TNFα	Tumour Necrosis Factor α
TRAP	TGFβ receptor associated protein
TS	Thrombospondin-like
UBC	Ubiquitin C
UTR	Untranslated region
VEGF	Vascular Endothelial Growth Factor

Introduction

Why Study Asthma?

Asthma is a significant cause of morbidity and mortality in the World today accounting for 180,00 deaths world wide per year. The prevalence of the disease is on the increase by around 50% per decade and now affects approximately 150 million people world wide including 1 in 20 adults and 1 in 10 children. The economic impact of the disease is enormous. The 2002 National Asthma Campaign asthma audit(NAC, 2002) cited a £1.2 billion loss in productivity due to asthma, as well as a £161 million loss in social security costs. Furthermore, asthma treatment costs the taxpayer £850 million each year including £254 million specifically for the treatment of the disease in children. More significantly, an asthma attack can be a terrifying ordeal that millions of sufferers experience each year which can result in a tragic loss of life.

A Historical Perspective

In 1859 Salter was the first to describe asthma; he described it as "a disease of reversible airway obstruction". By 1960 the definition was similar but with the inclusion of "bronchial hyperresponsiveness". By the time the National Heart, Lung and Blood Institute, defined asthma in 1997 the definition has evolved to become:

"a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils and T lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or in the early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partially reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli."(National Heart, 1995)

This rapid expansion of the definition of asthma illustrates clearly the progress in the understanding of asthma that has been made in recent years. This is far from a comprehensive definition and it will inevitably evolve further over the coming years as we further our understanding of the disease.

The increase in the understanding of asthma is due, in part, to the progress made in the techniques used to investigate the disease. Originally, investigations into asthma were restricted to the measurement of airway restriction and inflammatory cell numbers in the peripheral blood. Much of the early pathophysiological investigation relied upon the use of post mortem autopsies. However, this technique is limited, as at gives no clues as to the pathophysiology of the disease during times other than directly after an attack significant enough to cause death. Nevertheless, this technique did identify several features specific to asthma: the blockage of the bronchial lumina with a cocktail of mucus, serum proteins and cellular debris, epithelial sloughing, apparent thickening of the basement membrane, edema and leukocyte (mainly eosinophil) infiltration of the submucosa, hyperplasia of the mucous glands and hypertrophy of bronchial smooth muscle(Dunnill, 1971). It was not until the advent of the fiberoptic bronchoscope that the progression of the pathology of asthma could be followed. Fibreoptic bronchoscopy is now a commonly used technique in the field of respiratory disease research. It allows the collection of biopsies, brushed epithelial cells and Bronchoalveolar Lavage Fluid (BALF) containing luminal secretions.

Immunohistochemical analysis of asthmatic biopsies gave rise to detailed descriptions of the asthmatic submucosa being heavily infiltrated with inflammatory cells, in particular Mast cells, Eosinophils and T-lymphoctes of a Th-2 inflammatory profile.

Inflammation in asthma

The inflammatory response to allergic stimuli in asthma is generally considered to be a Th-2 type response. That is a response in which the predominant inflammatory cytokines are of the Th-2 type: IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte macrophage colony stimulating factor (GM-CSF)(Holgate, 1999). The inflammatory cells generally associated with this inflammatory profile are eosinophils, mast cells and T-lymphocytes.

Eosinophils

Previously it was considered that eosinophils might play a protective role in the asthmatic airway, as they were shown to have the ability to degrade mast cell derived proinflammatory mediators. However it has since been clearly demonstated that they also release numerous other proinflammatory cytokines of their own.

Eosinophils express low affinity IgE receptors. It is not surprising therefore that their numbers have been shown to increase after allergic insult for up to 24 hours(Hansel and Walker, 1992). The size of the increase in numbers has also been shown to correlate with the severity of the allergic response. This recruitment of eosinophils is believed to be due to cytokine (in particular Th-2 cell-derived IL-5) mediated upregulation of adhesion molecules. These adhesion molecules are essential for eosinophil migration and localisation (Hansel and Walker, 1992). Several chemotactic cytokines such as eotaxin, RANTES (regulated upon activation in normal T cells expressed and secreted) and MIP-1α (macrophage inflammatory protein 1alpha) are also responsible for eosinophil recruitment(Resnick and Weller, 1993).

The eosinophil is believed to be the major cell involved in the immune response to parasitic invasion. They are known to release four major basic proteins (eosinophilic

major basic protein MBP, eosinophil cationic protein ECP, eosinophil peroxidase EPO and eosinophil derived neurotoxin EDN) which are toxic to parasitic larvae. However these arginine rich proteins are also toxic to some mammalian cells(Hansel and Walker, 1992). For example MBP has been shown to have toxic effects on guinea pig and human bronchial tissue at doses significantly less than levels present in normal asthmatic sputum(Hansel and Walker, 1992). This may account for the sloughing of epithelial cells commonly observed in asthma. Eosinophils can also release cysteinyl leukotrienes that can cause bronchoconstriction and mucus hypersecretion(Dinh Xuan et al., 1989) as well as numerous other proinflammatory cytokines.

The large increase in eosinophils observed in the asthmatic airway compared to normal control individuals(Brightling et al., 2003) has made the eosinophil a popular target for therapeutic intervention. Anti-IL-5 antibodies, when administered, have been shown to be successful in reducing circulating eosinophil numbers. However this therapy was unsuccessful in reducing asthmatic symptoms (Kay et al., 2004, Leckie et al., 2000). The presence of increased eosinophil numbers has lead to a comparison between asthma and eosinophilic bronchitis. Eosinophilic bronchitis is a disease with similar immunopathology to asthma. However the absence of variable airflow obstruction and airway hyperresponsiveness distinguishes the disease from asthma (Brightling et al., 2003). Brightling *et al* (Brightling et al., 2002) have recently reported that the presence of mast cells within the smooth muscle to be a distinguishing feature of asthma compared to eosinophilic bronchitis and healthy controls.

Mast Cells

Mast Cells are most commonly activated by an IgE dependent mechanism. They express high affinity IgE receptors (FcεRI) on their surface. Allergens can interact with these receptors via crosslinking with IgE already bound, thus causing a release of mediators that trigger the hypersensitive reaction seen in asthma. i.e. The IgE

triggered mediator release is responsible for the immediate bronchoconstriction during an allergen induced asthma attack.

Other mediators released from mast cells include prostaglandin 2 (PGD_2), tryptase and cysteinyl leukotrienes. PGD_2 is a spasmogen so is therefore likely to increase bronchoconstriction. It is also a vasodilator and increase vascular permeability, therefore aiding the influx of other circulating cells (Beasley et al., 1988). Tryptase has been shown to increase smooth muscle responsiveness in dogs(Sekizawa et al., 1989) and also has the ability to degrade native bronchodilators. Virtually 100% of mast cells in the bronchial epithelium and submucosa contain tryptase as the major neutral protease. This differs from mast cells found elsewhere in the body, which contain tryptase and chymase. Cysteinyl leukotrienes increase vascular leakage and are up to 1000 times more potent than histamine at causing smooth muscle contraction. Leukotrienes C_4 D_4 and E_4 are also released upon mast cell degranulation and cause prolonged bronchoconstriction, increased vascular permeability and increased mucus secretion. Platelet activating factor (PAF) released from mast cells causes platelets to aggregate, which in turn release more histamine. Cytokines are also released by degranulating mast cells. These include IL-1, TNFα and IL-6, (Gordon and Galli, 1990). These are associated with further bronchoconstriction, edema and mucus secretion.

As mentioned, it has recently been reported that the presence of significant mast cell numbers in the airway smooth muscle is a distinguishing feature of asthma pathology (Brightling et al., 2002). This group highlight the growing evidence that airway remodelling and consequent hyperresponsiveness are regulated independently of airway inflammation (Brightling et al., 2003). They propose a mechanism where by mast cells within the airway smooth muscle contribute to smooth muscle hyperplasia, differentation, and possibly migration as well as constriction. This proposal raises interesting questions as to how mast cells buried deep within smooth muscle bundles might be activated.

T-lymphocytes

T-lymphocytes or T-cells are the orchestrators of the cellular response to pathogen. Helper T-cells are sub-classified according to the repertoire of cytokines and mediators they release. Th1 cells release IL2, IFNγ and TNFα amongst others that activate the cellular and humoral response to intracellular pathogens. The dominant cytokine profile in asthma however is one of a Th2 type: IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF. Such cytokines mediate the body's response to large extracellular pathogens such as parasites. This Th2 cell type is the dominant T-helper cell species *in utero* and at birth. Evidence exists to suggest that exposure to intracellular pathogens in early life is essential for the polarisation of the immune system to a Th1 profile and that a too clean an upbringing may encourage the development of allergy(Herrick and Bottomly, 2003).

The maintainance/shift towards a Th-2 type CD4+ population,as seen in asthma, is believed to be controlled by a number of factors including the local cytokine environment, the level of antigen and the presence and activity of antigen presenting cells (APC). It should be noted that the Th-1/Th-2 differentiation in human cells is not entirely distinct, and some exceptions to these rules are observed. For instance many studies document an increase in IFNγ in asthmatic airway tissue even though it is typically a Th-1 cytokine (Muraille and Leo, 1998). These exceptions aside, the Th-2 cytokines contribute to inflammation in asthma in a number of ways. IL-4 and IL-5 up regulate the expression of adhesion molecules on endothelial cells thus aiding the migration of other inflammatory cells in to the airways. IL-3, IL-4, IL-5, IL-13 and GM-CSF act as chemoattractants for inflammatory cells, whilst IL-3, IL-4 and IL-5 can aid mast cell and eosinophil differentiation and survival (Holgate, 1997). Recent animal studies have shown IL-4 and IL-13 acting by similar mechanisms to trigger asthma-like pathology in animal airways. For example characteristic features of asthma such as mucus hyper-secretion and goblet cell metaplasia were induced

by treatment with IL-4(Dabbagh et al., 1999) or IL-13(Zhu et al., 1999). In addition when the IL-4/IL-13 signalling transcription factor STAT-6 gene was deleted in mice, a number of asthma associated symptoms ablated including antigen-induced airway hyperresponsiveness(Akimoto et al., 1998).

The allergic response in asthma

In the majority of cases, the influx of inflammatory cells to the asthmatic airway discussed here is the result of a cascade of reactions resulting from the inhalation of allergen by an atopic individual. The process of sensitisation to an allergen is now a well understood process (Fig 1.1). Inhaled allergen is captured by IgE receptors on dendritic cells within the bronchial epithelium. The dendritic cell then processes the allergen and displays the molecule on the cell surface in an HLADR2 cleft.

Via this apparatus the antigen is presented to a naïve Th-precursor (ThP) cell within a microenvironment rich in cytokines (inc IL-10 and IL-4) promoting selective expansion of Th2 polarised cells. The Th2 memory cell then presents the antigen via the T-cell receptor to the MHC class II on a B-lymphocyte. Chaperone protein complexes (CD86-CD28 and CD40-CD154) and the release of IL-4 from the Th2 cell promote a class switch in the B-cell to the release of allergen specific IgE. It is this IgE that binds FcεRI on the surface of local mast cells thus priming them for degranulation on any further contact with allergen.

The release of proinflammtory mediators from a degranulating mast cell (discussed above) is the trigger for the early allergic response (EAR) which can manifest within seconds to minutes of allergen exposure. This displays itself clinically as itch or a "wheel and flare" when in the skin. Similar irritation occurs in the lung but with the

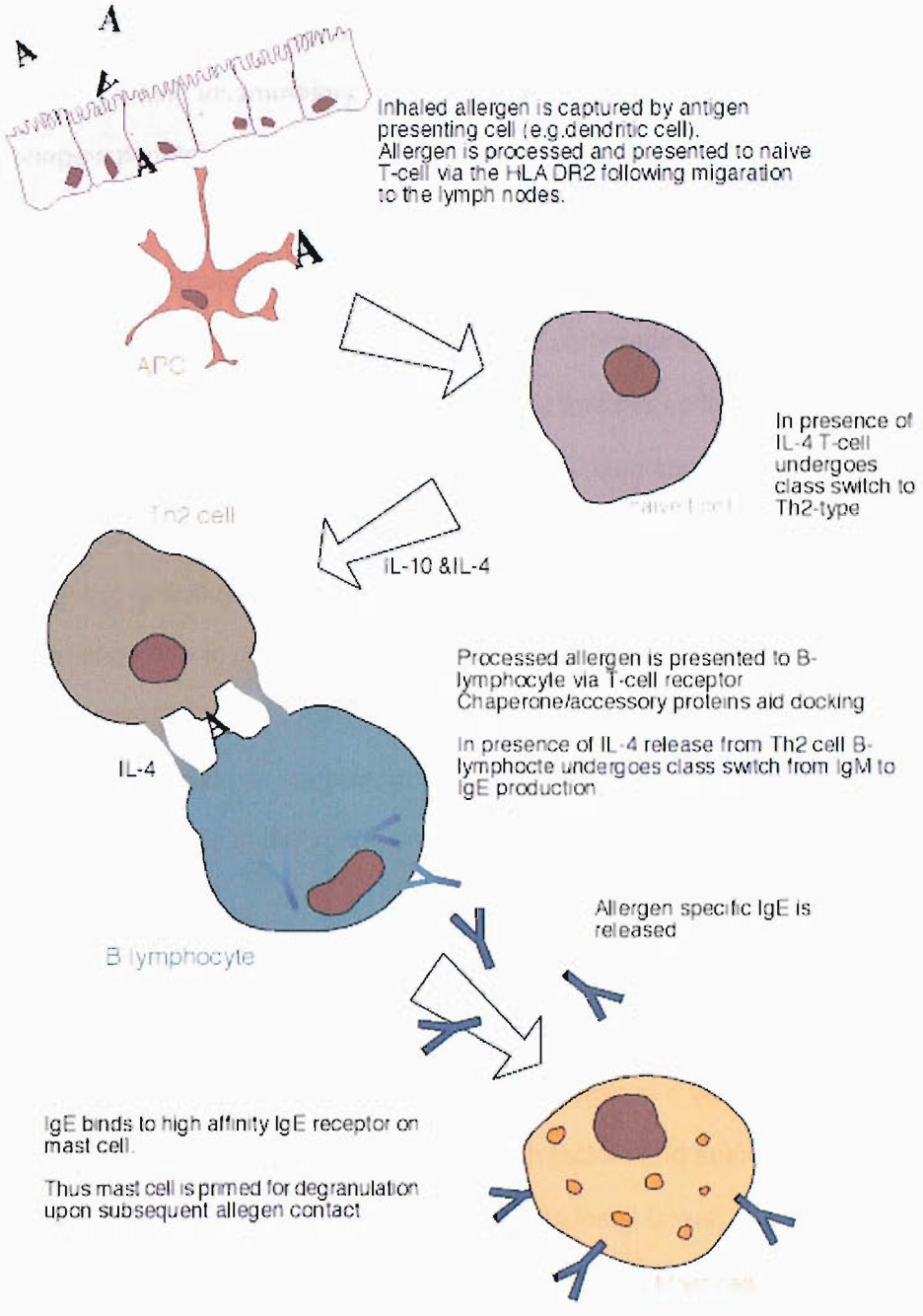


Fig 1.1 Schematic representation of cellular involvement in allergic sensitisation.

addition of the hyperresponsive bronchial constriction and mucus release that blocks the airway causing at best wheeze or at worst suffocation. This EAR is followed several hours later by the late allergic response (LAR) upon the migration in to the airways of circulating inflammatory cells. This LAR can encompass contributions from macrophages, platelets, CD8+ T-cells, eosinophils and neutrophils.

The dramatic inflammatory activity within the asthmatic lung is considered to be activated, promoted and amplified by the stressed asthmatic epithelium. Considerable evidence is available now to suggest that the epithelium in asthmatic patients is intrinsically abnormal. For example it has been shown that the asthmatic epithelium is more susceptible to oxidant-induced apoptosis (Bucchieri et al., 2002), damage by ozone and nitric oxide (Bayram et al., 2002), and releases more IL-8 and GM-CSF in response to diesel particles than from healthy controls (Bayram et al., 1998).

The asthmatic epithelium is considered to be more than just a passive barrier, and in fact plays an active role in asthma(Hamilton et al., 2001).

The epithelial contribution to inflammation

Cells of the epithelium have been shown to interact directly with the cells of the immune system, and mesenchymal cells, via the release of a variety of mediators. The cocktail of proinflammatory cytokines, growth factors and autocoid mediators released by the asthmatic epithelium in response to insult is well documented(Bayram et al., 1998, Bayram et al., 2002, Bucchieri et al., 2002). TNFα, IL-6, IL-11, IL-16, IL-1ß and IL-8 all act as chemoattractants for T-lymphocytes, eosinophils and neutrophils. IL-5, IL-13 and GM-CSF have the same "Th-2 type" affects discussed previously. Fibroproliferative and profibrogenic factors including transforming growth factor beta 1 and 2 (TGFβ), fibroblast growth factor (FGF-2), endothelin-1 (ET-1), insulin growth factor (IGF-1), platelet derived growth factors

(PDGF), and stem cell factor (SCF) are all also released by the damaged epithelium(Hamilton et al., 2001). Other proinflammatory mediators released include oxidative arachadonic acid metabolites (PGE_2, 15-hyroxyeicosatetraenoic acid [HETE], 12,15-diHETE and 8,15-diHETE), lipoxins, nitric oxide, endothelin-1 (ET-1), fibronectin and reactive oxygen (O^-) (Holgate et al., 2000).

In addition to the release of these soluble mediators epithelial stress leads to the activation of transcription factors nuclear factor κB (NF-κB)(Hart et al., 1998), activator proteins(Demoly et al., 1992) and signal transducer and activation of transcription-1 (STAT-1)(Sampath et al., 1999) and by the increased expression of heat shock proteins(Bertorelli et al., 1998) and the cyclin-dependent kinase inhibitor, p21(Puddicombe et al., 2003). Upregulation of such factors is typical of an epithelium activated to form structural changes.

These examples illustrate how the stressed asthmatic epithelium when activated has potential to create an environment ideal for the promotion and amplification of inflammation and fibrogenesis.

Treatments for asthma

The contribution of the inflammatory response to asthma symptoms and pathology has made it an obvious target for therapeutic intervention. Treatments have been developed to suppress the inflammatory response in asthma for therapeutic benefit. Some success has been achieved in this area with the use of corticosteroids (Lundberg et al., 2004),leukotriene antagonists(Leru, 2003) and mast cell stabilisers(Spooner et al., 2003). Such compounds prove a useful tool in controlling asthma in a compliant patient. However, upon cessation of orally administered inflammatory suppressants, asthma returns, thus raising the question as to the significance of inflammation as a primary cause of asthma. Suppressing inflammation with drugs such as corticosteroids manages the symptoms of the disease but goes no further in the curing of the condition. The other major therapeutic agent used in

the management of asthma is the use of β2 adrenergic receptor agonists, either short acting and/or more recently long acting (Jackson and Lipworth, 2004, Roberts et al., 1999).Such compounds cause the relaxation of bronchial smooth muscle during an asthma attack thus relieving the airway constriction that causes breathing difficulties. Although these drugs are an exceptionally useful tool in managing the symptoms of asthma, in a non-compliant patient, or in the case of a patient with severe asthma, such measures are insufficient. Further understanding of the commencement and progression of the disease is required so that therapies to prevent or cure asthma can be developed.

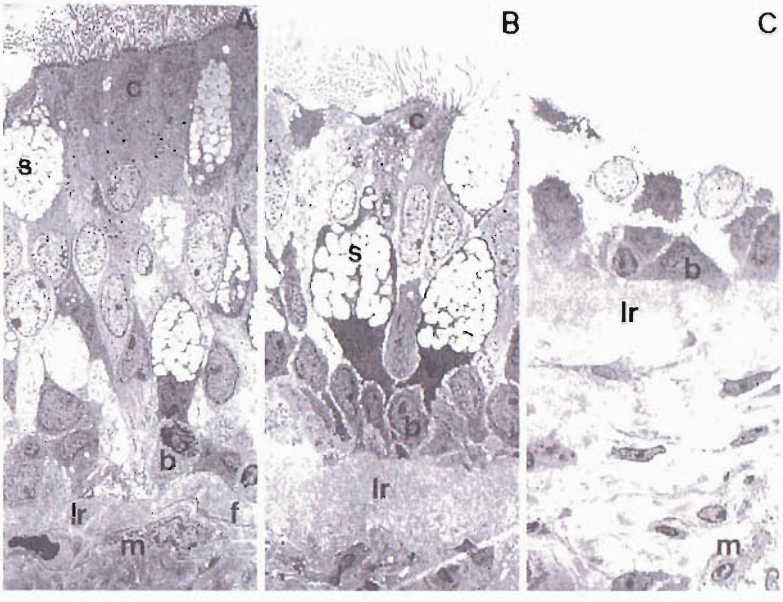
Airway remodelling

The viewpoint that inflammation, and more recently inflammation as a result of epithelial stress, is the cause of asthma has meant that in recent years airway remodelling, the other major histologic feature of asthma has been overlooked.

Airway remodelling is a general term used to describe the structural changes to the airway wall that occur in asthma. These structural changes can lead to an irreversible decline in lung function. An increase in smooth muscle-bulk is often the most obvious change in airway histology and is likely to be one of the most important in terms of bronchial hyperresponsiveness (BHR). BHR is a term used to describe the reversible narrowing of the airways in response to innocuous stimuli. Other structural changes include a thickening of the sub-basement membrane, the *lamina reticularis*, goblet cell hyperplasia, epithelial sloughing, as well as proliferation of airway blood vessels and nerves(Holgate et al., 2000)(Fig 1.2).

Until recently it was assumed that cytokines and damaging proteins (e.g. Basic proteins) produced by inflammatory cells was the cause of airway remodelling. However, recent evidence is causing some to discuss the viability of this traditional linear model of asthma pathogenesis. For example, genetic studies have shown that

a.



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

b.

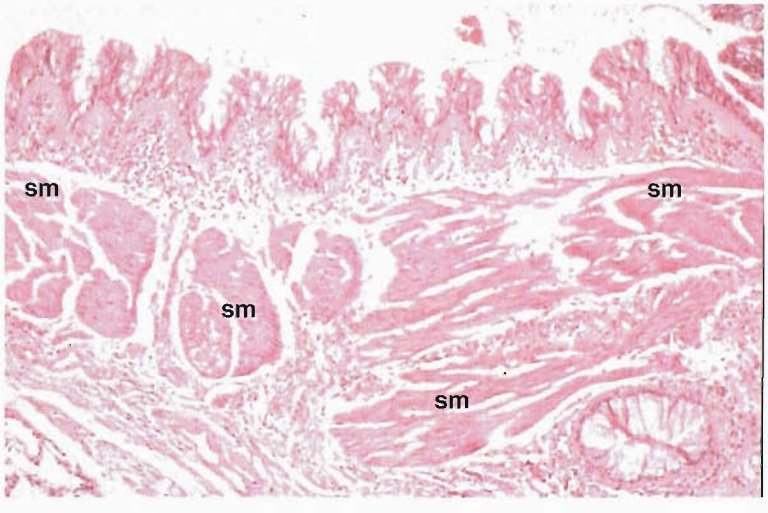


Fig 1.2 a. Structural changes to the epithelium and basement membrane in asthma
Disruption of the epithelium is often observed in asthma. Sloughing of epithelial cells can leave the basal cells are exposed (C). (B) shows increased goblet cell number in the epithelial layer. Thickening of the *lamina reticularis* is visible in both transmission electronmicrographs of the asthmatic airway. Mag x1600. (reprint by kind permission from Holgate *et al*, 2000).
b Basal cell, **c** columnar epithelial cells, **f** (myo)fibroblast, **lr** lamina reticularis, **m** mast cell, **s** secretory goblet cells, **sm** smooth muscle.

Fig 1.2 b. Airway wall of a severe asthmatic showing large amount of smooth muscle.

bronchial hyperresponsiveness and inflammation in asthma have different patterns of inheritance(Skadhauge et al., 1999). Whilst other studies have found evidence that airway remodelling occurs in early life, and has been observed in the airways of children up to 4 years before the clinical diagnosis of asthma(Holgate, 2000).
A study by the Childhood Asthma Management Program (CAMP) found that treatment with inhaled corticosteroids led to improved airway function upon administration of a bronchodilator in 5-11-year-olds. However this initial beneficial effect was lost over the following three years(2000). These findings suggest that airway remodelling continued during the treatment period despite significant suppression of the inflammatory events associated with asthma.
In another study, anti-IL-5 antibodies were administered systemically to asthma patients(Leckie et al., 2000). This resulted in a marked reduction in the number of circulating eosinophils but did not affect the severity of the disease. Therefore, while eosinophil levels are often elevated in asthmatic subjects, particularly those who also suffer from allergies, they may not contribute significantly to BHR.

Bearing this evidence in mind a new model for asthma pathogenesis has been suggested in which airway remodelling and inflammation occur in parallel, and that remodelling is equally relevant to the cause of asthma as is inflammation(Holgate, 2000).

Recent evidence suggests a contributing factor to airway remodelling is the activation of fibroblasts that lie directly under the *lamina reticularis*. The number of activated fibroblasts (termed myofibroblasts) has been shown to correlate positively with levels of subepithelial fibrosis(Brewster et al., 1990). Thus it has been suggested that the myofibroblast is responsible for the subepithelial fibrosis characteristic of asthma. Myofibroblasts are synthetically active and secrete a range of ECM proteins and growth factors including endothelin 1 (ET-1) and vascular endothelial growth factor (VEGF) which are potent smooth muscle and vascular endothelial cell mitogens

respectively(Richter et al., 2001). Thus the myofibroblast may contribute to the increase in smooth muscle bulk observed in asthma. Assuming this crucial role of the (myo)fibroblast in the pathogenesis of airway remodelling makes it a particularly interesting target for research.

The nature of the (myo)fibroblast

Microscopic analysis of the myofibroblast structure shows an elongated form containing intracellular filaments, abundant rough endoplasmic reticulum and irregularly shaped nuclei(Gizycki et al., 1997).

The myofibroblast phenotype is essentially an intermediate between that of the fibroblast and the smooth muscle cell.

Whilst there are no known specific markers for fibroblasts, myofibroblasts are classically characterised by the presence of filaments of α smooth muscle actin (αSMA). However the definition of myofibroblast is more complex than this. There are several situations *in vivo* where myofibroblasts do not express αSMA(Hinz et al., 2001, Tomasek et al., 2002). They do however express stress fibres containing non-muscle actins and myosins. Therefore two sub-classes of myofibroblast phenotype have been proposed. That of proto-myofibroblast (i.e. expression of stress fibres containing cytoplasmic actins.) and differentiated myofibroblast (i.e. expression of stress fibres containing αSMA.)(Tomasek et al., 2002). In addition myofibroblasts can express other smooth muscle proteins such as heavy chain myosin (HC myosin) and Calponin.

Possibly the most reliable feature to distinguish between fibroblasts and myofibroblasts is the presence of super-mature focal adhesion complexes, termed fibronexus junctions, which are detectable by electron microscopy in myofibroblast populations. Fibronexus junctions, as the name suggests, are structures at which a number of fibronectin fibrils link with intracellular actin stress fibres. These

fibronexus junctions are located at the cell surface and are linked to the ECM via integrins.

The state of differentiation of the myofibroblast relates to the size of their focal adhesion complexes. Proto-myofibroblasts express immature focal adhesion sites ($<2\mu m^2$) associated with ED-A fibronectin (an alternatively spliced form of fibronectin), vinculin, integrin $\alpha5\beta1$ and paxillin. Differentiated myofibroblasts express super-mature focal adhesion complexes ($6\text{-}30\mu m^2$).

Smooth muscle cells express additional muscle associated proteins such as smooth muscle γ-actin(Qian et al., 1996), muscarinic m3 receptor(Billington and Penn, 2002), desmin(Sappino et al., 1990) and smoothelin(van der Loop et al., 1996) (Fig 1.3)

Myofibroblasts play a major role in wound repair. Myofibroblasts propagate inflammation in a wound scenario via the release of growth factors, cytokines and other soluble mediators. They also play a crucial role in wound repair by the release growth factors, collagen, fibronectin and proteoglycans to repair the extracellular matrix (ECM). Subsequent myofibroblast contraction then reduces the volume of ECM and closes the wound. It is believed also that the tractional forces generated by the migrating fibroblast reorganise the ECM to aid wound closure and repair(Grinnell, 1994).

The role of the myofibroblast in fibrotic diseases is well documented. Pulmonary fibrosis is an excellent example of a fibrotic disease in which the fibroblast plays an essential role (Phan, 2002), as are hepatic and pancreatic fibrosis (Bachem et al., 1998).

In an unusual condition called palma facsia, irreversible myofibroblast contraction in the connective tissue of the back of the hand becomes so severe that the sufferer loses the use of their digits. Another disease in which the myofibroblast plays a significant role in diseasse pathogenesis is scleroderma, a chronic, auto-immune

disease of the connective tissue generally classified as one of the rheumatic diseases. Myofibroblasts have been cited as the source of abnormal collagen production resulting in thickening of the skin, swelling of the hands and feet, pain and stiffness of the joints, joint contractures as well as numerous other systematic problems which can, in severe cases, result in mortality.

Myofibroblast differentiation: The markers of differentiation

As discussed, the myofibroblast phenotype is essentially an intermediate between that of the fibroblast and the smooth muscle cell. Consequently, to understand the subject of myofibroblast differentiation it is important to understand the contractile apparatus within the cells of interest, as the proteins involved are often the best markers of differentiation (Fig 1.3). The smooth muscle contractile apparatus consists of two major polymerised molecules; actin (αSMA) and myosin. Both actin and myosin exist as monomers but are polymerised to form filaments within a contractile cell. Myosin molecules are effectively the motors driving contraction. "Heads" upon each myosin molecule can attach to adjacent actin molecules. A single molecule of ATP attached to the myosin head is hydrolysed resulting in large conformational change, thus causing the myosin molecule to detach and reattach approximately 5nm along the actin polymer. Upon binding the inorganic phosphate molecule formed during hydrolysis of the ATP molecule is detached. This triggers another shape change in the myosin molecule sufficient enough to generate movement between the two filaments. To this extent smooth muscle contraction is mechanistically very similar to skeletal muscle contraction. It is at the level of regulation of this actin/myosin interaction that smooth muscle contraction differs from it's skeletal

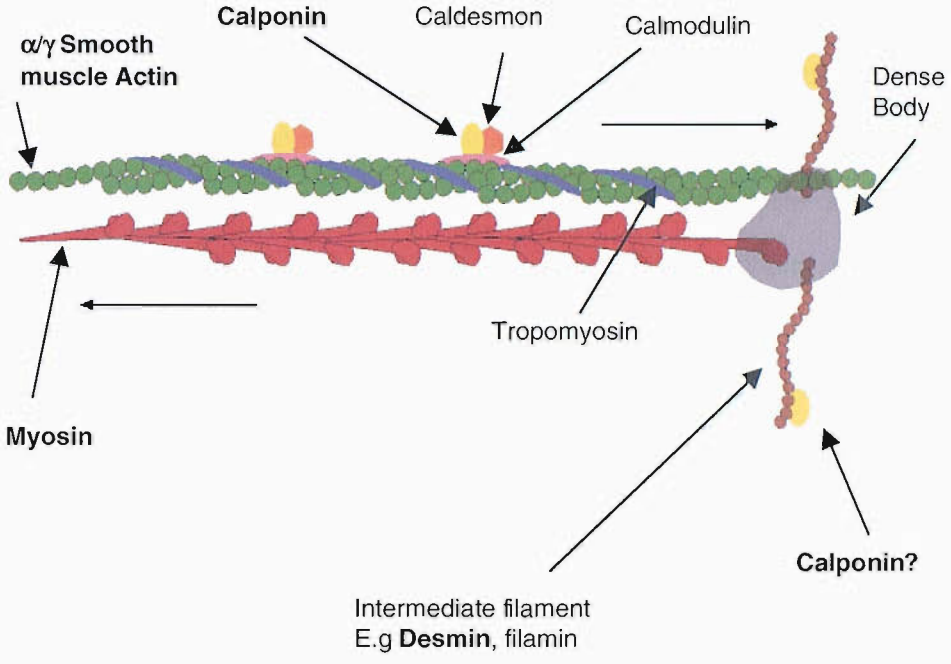


Fig 1.3 Smooth muscle contractile apparatus. Schematic representation of the smooth muscle cell contractile apparatus. Elements measured within this thesis are highlighted in bold.

counterpart. Calponin and caldesmon are two proteins found in the smooth muscle cell, which are assumed to play a role in regulating this process. Both calponin and caldesmon can bind to actin and *in vitro* can both inhibit myosin ATPase activity thereby inhibiting contraction. Calponin is also found distributed throughout the cytoplasm particularly localised with desmin (Mabuchi et al., 1997), an intermediate filament. It is thought then that calponin may play an additional role in maintaining the contractile phenotype of the smooth muscle cell(North et al., 1994). Calmodulin is another essential regulatory protein in the smooth muscle contractile apparatus. It can be activated by the binding of Ca^{2+} or by phosphorylation. Upon activation Calmodulin will inhibit the anti-ATPase activity of calponin and caldesmon thus activating contraction. Therefore nervous stimulation resulting in Ca^{2+} release from the sarcoplasmic reticulum can trigger contraction, but so can a number of other stimuli (adrenaline, prostoglandins, serotonin etc.) by phosphorylation of caldesmon via a GPCR.

Fibroblast, myofibroblast or smooth muscle cell?

The origin of the myofibroblast *in vivo* is not entirely understood. In granulation tissue the most likely progenitor cells are the local fibroblasts, as myofibroblasts are seen to "revert" back to a fibroblast phenotype after wound closure(Darby et al., 1990).

One other possibility is that myofibroblasts originate from circulating fibrocytes. Fibrocytes are circulating cells that express fibroblast products (e.g. collagen 1), but with the unique characteristic of expressing hemopoietic stem cell antigen CD34. In culture, fibrocytes will develop properties both functionally and structurally similar to those of a myofibroblast(Chesney and Bucala, 2000). Schmidt et al(Schmidt et al., 2003) identified CD34-positive/collagen1/αSMA positive cells localised to areas of collagen deposition below the epithelium of patients suffering from allergic asthma.

The number of such cells increased significantly as quickly as four hours after allergen challenge. By tracking labelled fibrocytes in a mouse model of asthma, this team were also able to show fibrocytes localising sub-epithelialy in the airways of allergen challenged mice. These fibrocytes then appeared to differentiate into myofibroblasts. Furthermore this group found that the addition of the profibrogenic cytokines ET-1 and TGFβ_1 to cultured human fibrocytes resulted in a significant increase in the number of αSMA positive cells in the cultures.

Studies of smooth muscle cells in culture show smooth muscle cells acquiring morphological characteristics of the myofibroblast after three days in culture(Chamley et al., 1977).These non-contractile smooth muscle cells are termed synthetic smooth muscle cells. Conversly Animal work on obstructed rabbit bladder has shown that myofibroblasts can, under certain conditions, express αSMA, HC myosin and desmin thus suggesting that it is possible for myofibroblasts to differentiate to smooth muscle cells(Buoro et al., 1993). Recent *in vitro* evidence substantiates this finding. Micro-array analysis of genes induced by TGFβ_1 in cultured human lung foetal fibroblasts identified several smooth muscle associated genes including smoothelin, considered previously to be a smooth muscle specific gene(van der Loop et al., 1996), that were upregulated in response to TGFβ_1. Their observations were confirmed at the protein level in adult human airway fibroblasts (Chambers et al., 2003). Another animal study examined the source of new vascular smooth muscle cells seen in the hypertensive lung. Using microscopic techniques interstitial fibroblasts were identified as the precursor source of these cells(Jones and Jacobson, 2000).

These examples illustrate the plastic nature of the myofibroblast phenotype, which appears to shift towards a fibroblastic or smooth muscular phenotype according to the microenvironment (Fig 1.4). This variable phenotype can make identifying cell type at a given time particularly difficult. This highly plastic phenotype has recently

become the subject of intense research in the field of asthma as well as several other diseases.

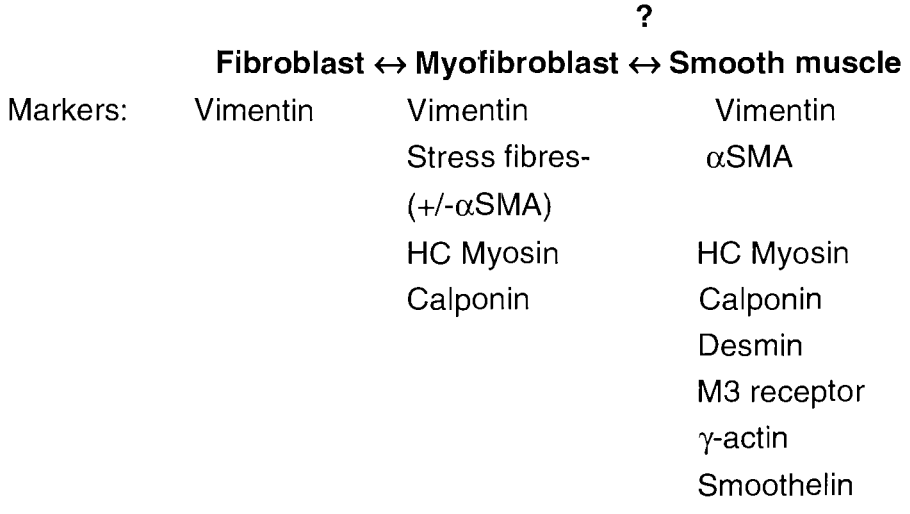


Fig 1.4 Markers of the fibroblast, myofibroblast and smooth muscle phenotypes.

Changes to the microenvironment of the (myo)fibroblast can cause alterations to the phenotype observed. Several studies have identified factors that effect fibroblast phenotype, and can be reliably used to manipulate fibroblast phenotype *in vitro.* For example a considerable volume of literature regarding smooth muscle phenotype in particular highlights the importance of ECM in mesenchymal cell differentiation. Collagen I and IV, fibronectin, vitronectin and in particular laminin have been shown to maintain the contractile phenotype of the smooth muscle cell *in vitro*(Hayward et al., 1995, Zhang et al., 1999). An independent investigation by Beqaj *et al* proposes a mechanism by which laminin-2 could regulate smooth muscle differentiation. They observed that prior to smooth muscle differentiation RhoA, a small GTPase signalling protein, inhibits smooth muscle gene expression, although following differentiation RhoA maintained the smooth muscle phenotype. RhoA levels were seen to drop rapidly when cells were grown on laminin-2, and thus smooth muscle differentiation was more prevalent on cultures grown on laminin(Beqaj et al., 2002). Although these data refer to smooth muscle cells in particular, it is reasonable to hypothesise that

ECM components such as these will be of similar importance to the differentiating myofibroblast phenotype. For example, the ECM component vitronectin and vitronectin integrins have already been shown to down-regulate myofibroblast transformation(Scaffidi et al., 2001).

Mechanical influences also seem important in regulation of (myo)fibroblast phenotype. For example it has been demonstrated that myofibroblast features appear earlier in splinted wounds, where the fibroblast population is under mechanical tension, than in control wounds(Hinz et al., 2001). This observation has been confirmed in *in vitro* studies where fibroblasts were grown in collagen lattices. Control lattices were allowed to float freely in tissue culture medium. Others were tethered, so as the myofibroblast phenotype developed and compaction of the collagen fibres occurred, tension built due to the tethering of the lattice. Under these conditions the proto-myofibroblast phenotype developed and was maintained. If the lattice was released from its points of attachment the proto-myofibroblast markers were quickly lost(Mochitate et al., 1991, Tomasek et al., 1992). Similar work using mesenchymal cells rather than fibroblasts may give insight as to how tension effects cell phenotype. Yang and his colleagues grew undifferentiated mesenchymal cells on a collagen coated silicon membrane, which was stretched once the cells had reached the desired confluency. Sustained stretch induced the expression of smooth muscle proteins and accelerated smooth muscle myogenesis(Yang et al., 2000). The same team of researchers established that alternative splicing of Serum Response Factor (SRF) was the cause of this stretch induced myogenesis. SRF is a transcription factor noted to be critical for the induction of muscle-specific gene expression. An alternative splice variant, SRFΔ5, was found to down-regulate bronchial myogenesis whilst the full transcript was a promoter of myogenesis. Interestingly another group observed that when a population of synthetic smooth muscle cells was starved of serum for seven days, approximately 1/6 of the cells returned to a contractile phenotype. They attribute this differential response of

subpopulations of cells to serum deprivation to differences in SRF cellular location. i.e. the effects were only apparent when SRF was located in the nucleus rather than cytoplasmically(Camoretti-Mercado et al., 2000). The continuous expansion and contraction of the airways makes the influence of mechanical stimuli on fibroblast phenotype of particular importance in respect to asthma.

In addition to the importance of ECM components and a mechanical stimulus, a number of soluble mediators have been identified that mediate fibroblastic phenotype. For example the work of Gailet *et al* demonstrated that mast cell histamine upregulated αSMA expression in fibroblasts from human foreskin(Gailit et al., 2001). However they also observed an even more potent effect of mast cell tryptase in upregulating αSMA expression in the same fibroblast population. Tryptase inhibitors blocked this effect. Other studies have suggested that IL-4 can induce αSMA expression in fibroblasts, although the work of Richter *et al* (Richter et al., 2001) failed to repeat this observation. The most potent mediator of fibroblast/myofibroblast transformation identified to date appears to be TGFβ.

Transforming Growth Factor beta

It has been demonstrated in several species that TGFβ is a potent inducer of myofibroblast differentiation(Kurosaka et al., 1998, Desmouliere et al., 1993, Ronnov-Jessen and Petersen, 1993). TGFβ expression is elevated in asthmatic bronchoalveolar lavage fluid (BALF) and is increased upon allergen challenge(Redington et al., 1997). TGFβ mRNA and protein expression is also increased in asthmatic bronchial biopsies compared to healthy controls. This was particularly evident in the inflammatory cells, in particular the eosinophils, located below the basement membrane(Vignola et al., 1997). Cultured asthmatic fibroblasts and macrophages release more TGFβ than normal controls(Vignola et al., 1997).

The TGFβ super family consists of over 30 members including Bone Morphogenic proteins and Activins as well as the three human members of the TGFβ family, $TGF\beta_1$, $TGF\beta_2$, and $TGF\beta_3$. All three have similar differentiation effects on fibroblasts *in vitro,* although interestingly $TGF\beta_3$ has the opposite effect *in vivo*(Serini and Gabbiani, 1999). This observation is believed to be due to other changes in the microenvironment of the *in vivo* situation.

TGFβ is synthesised as a large 390-412 amino acid (plus a 20 amino acid signal sequence) precursor (Fig 1.5). Endopeptidases cleave this precursor to yield a 112 amino acid mature TGFβ molecule. These molecules form a homodimer which are most commonly linked to a 75 kDa latency associated peptide (LAP). Numerous other scavengers of TGFβ including type IV collagen, FN and elastin can bind TGFβ and act as local reservoirs. LAP is commonly covalently linked to a 135kDa protein termed Latent TGFβ binding protein (LTBP). This three-protein complex is known as a large latency complex (LLP). This complex has the ability to bind to various ECM components. Activation of TGFβ from this complex is achievable by numerous mediators including plasmin, furin, thrombospondin1, low dose radiation, low pH and reactive oxygen species (Blanchette et al., 2001).

TGFβ acts via a family of TGFβ receptors, namely TGFβR 1-6 (Fig 1.5), although only the roles TGFβR 1-3 are fully understood in respect to down stream signalling. Both betaglycan and endoglin are considered TGFβR3 receptors. They bind active TGFβ and present it to TGFβR2. Once ligand-bound, TGFβR2 recruits and phosphorylates the TGFβR1. This phosphorylation causes the release of TGFβR-associated protein 1 (TRAP-1) thus activating the receptor complex. This receptor complex is then responsible for downstream signalling via a cascade of Smad proteins (Wurthner et al., 2001).

The 8 Smads exist as 3 sub families. Namely the receptor activated or R-Smads (Smads 1,2,3,5 and 8), the common mediator or Co-smad (Smad 4) and the

Inhibitory or I-smads (Smads 6 and 7). The activated TGFβR1 causes R-smad phosphorylation. Smads 2 and 3 are the TGFβ activated smads. Smads 1,5 and 8 are BMP activated. The co-Smad then forms a heteromeric complex with the R-Smads causing them to dissociate from the TGFβR1. This Smad complex then translocates to the nucleus where it assembles with DNA-binding co-factors and activates transcription.

Beyond this cascade of proteins, the mechanism by which TGFβ acts to cause myofibroblast differentiation and the classical induction of αSMA, is not well understood. At the promoter level, it is known that a short sequence within the 150 base pairs of the 5' upstream sequence, known as the TGFβ control element (TCE), is essential in TGFβ-1 induced αSMA expression. Mutations or deletions of this element significantly reduce TGFβ1 induced αSMA expression in cultured rat lung fibroblasts(Roy et al., 2001).

TGFβ possesses the ability modulate the synthesis of extracellular matrix components such as fibronectin (FN) and collagen (Ignotz and Massague, 1986). FN in particular has been implicated with a role in myofibroblast differentiation. FN is a 440-kDa protein that exists as a soluble plasma form (pFN) and an insoluble cellular form (cFN) which is secreted as ECM fibrils. Each FN sub-unit is composed of repeating units with binding sites for other ECM components. ED-A is one of these sub-units, and has proved to be of particular interest within the field of myofibroblast differentiation. ED-A deposition proceeds αSMA expression both *in vitro* and *in vivo* following TGFβ1 stimulation (Serini et al., 1998). Blocking of the ED-A domain with monoclonal antibodies of treatment with soluble recombinant ED-A domain inhibits TGFβ1 induced αSMA expression. Crucially, plating fibroblasts on to ED-A FN does not elicit αSMA alone. It would seem then that the induction of αSMA by TGFβ requires permissive outside-in signalling derived from ED-A FN.

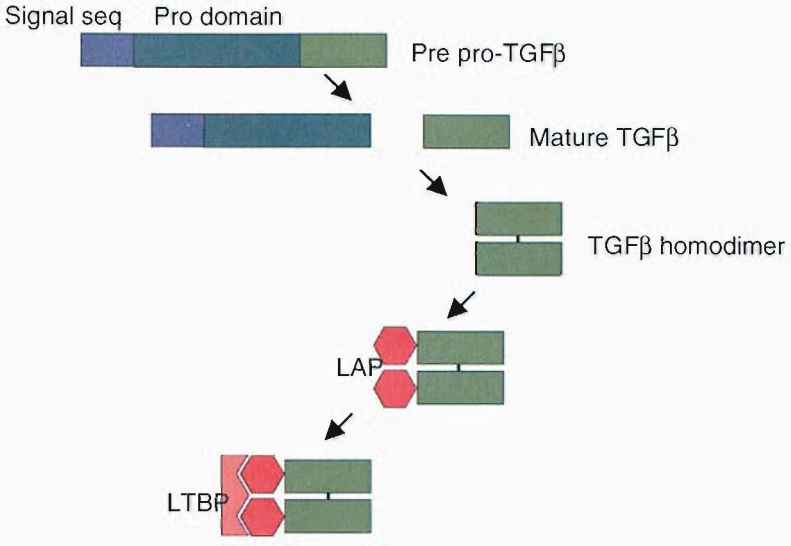


Fig 1.4 a. Proccessing of TGFβ. Pre pro-TGFβ is cleaved by endopeptidase to yield mature TGFβ. TGFβ homodimer binds Latency-associated peptide (LAP) which in turn binds Latent TGFβ binding protein LTBP. LTBP binds to the ECM.

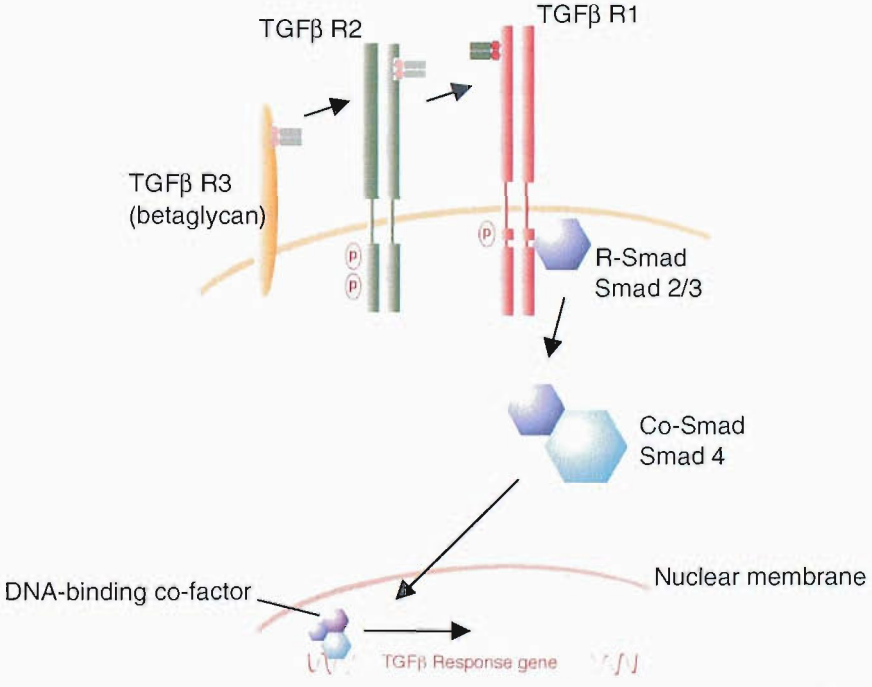


Fig 1.5 b. $TGF\beta_2$ signalling. TGFβ R3 presents $TGF\beta_2$ to TGFβ R2 which in turn recruits TGFβ R1 into a heteromeric complex . TGFβ R2 mediates phosphorylation of TGFβ R1. TGFβ R1 causes phosphorlation of R-smads. Co-smad binds R-Samds dissociating them from TGFβ R1.Smad complex translocates to nucleus, assembles with DNA-binding proteins and activates transcription of TGFβ response genes.

It has been known for some time that TGFβ plays a crucial role in normal wound healing by stimulating the formation of ECM components (Mattey et al., 1997). Also TGFβ modulates an inhibition in synthesis and release of metalloproteinases such as collagenase (MMP1), and indirectly controls an accompanying stimulation of tissue inhibitors of metalloproteinases (TIMPs) (Edwards et al., 1987), thus effectively increasing ECM deposition. Other effects of TGFβ include suppression of T- and B-lymphocyte proliferation, and inhibition of IL-1, IFN-γ and TNFα production by macrophages (Mattey et al., 1997).

The Epithelial-Mesenchymal Trophic Unit

The airway epithelium is the primary site of tissue damage in asthma due to the inhalation of allergens, oxidants and other injurious agents. *In vitro* studies have shown that injury to cultured epithelial monolayers causes the release of fibroprotective and profibrogenic growth factors, including $TGF\beta_2$, fibroblast growth factor (FGF-2), insulin growth factor (IGF-1), platelet derived growth factor (PDGF), endothelin-1 (ET-1). Therefore it has been hypothesised that "remodelling signals from the epithelium are propagated through the submucosa via the subepithelial myofibroblasts"(Holgate, 2000). This communication between the epithelium and mesenchymal cells is reminiscent of the modelling of the airways during embryonic development(Warburton et al., 2000). For example it has been shown in animal models that the differentiation of mesenchymal to smooth muscle cells during rat bladder embryogenesis is reliant on signals from the epithelium (Liu et al., 2000).This type of paracrine signalling is known as a trophic unit. Consequently, it has been suggested by Holgate and colleagues(Holgate et al., 2000) that the "epithelial-mesenchymal trophic unit (EMTU) is reactivated in asthma, and drives the remodelling of the asthmatic airways."

Fibroblast survival/ apoptosis within the EMTU

When discussing the asthmatic airway in terms of a reactivated "trophic unit" it is important to consider cell survival as well as their differentiation and survival. Apoptosis is the highly regulated mechanism by which cells die during embryonic organ development. Interestingly, and perhaps crucially, $TGF\beta_1$ has been documented as a central mediator in regulating lung fibroblast apoptosis.

Zhang and his colleagues (Zhang et al., 1997) have shown that, in rat lung fibroblast cultures, IL-1β causes a decrease in αSMA expression and a subsequent dose dependent loss of contractility in collagen gel contraction. In making this observation it was noted that a significant loss of cell number occurred. Further evaluation proved IL-1β to be causing a dose-dependent increase in apoptosis. These observations have also been observed in smooth muscle cell populations(Trinkle et al., 1992, Beasley et al., 1989).

In a continuation of this work Zhang (Zhang and Phan, 1999) showed that $TGF\beta_1$ can inhibit this IL-1β induced apoptosis via at least two mechanisms. $TGF\beta_1$ suppressed the expression of inducible NO synthase and thus suppressed NO production. $TGF\beta_1$ was also shown to decrease the expression of the anti-apoptotic protein bcl-2, but did not affect the expression of another pro-apoptotic protein bax.

Another investigation into fibroblast apoptosis provided a different perspective on fibroblast survival. Niland and his colleagues(Zhang and Phan, 1999, Niland et al., 2001) grew dermal fibroblasts in three-dimensional collagen lattices and noted that when the lattices were untethered significant apoptosis occurred, whilst in tethered, stressed lattices apoptosis was absent. It was assumed therefore that a contraction-dependent mechanism of cell survival versus apoptosis was at work. Adhesion-blocking antibodies against particular integrins caused a loss of apoptosis in the relaxed collagen gels. This evidence highlights the importance of integrin signalling in fibroblast survival or apoptosis.

A genetic component of asthma?

Despite substantial evidence that environmental factors such as allergen exposure are risk factors in the development of asthma, there is considerable evidence pointing to a genetic component to the disease. These include family and twin studies(1997a, Los et al., 2001). There is no evidence for a single gene for asthma susceptibility, and thus asthma is considered to be a complex genetic disease. Historically, identification of asthma-associated genes has been by a "candidate gene" approach, whereby polymorphisms within a known gene are identified and comparison between allele frequency in diseased populations is compared to controls. The employment of this technique has lead successfully to the identification of a number of asthma-associated genes. For example, a polymorphism within the gene encoding the β-chain of the high affinity IgE receptor (FcεRI-β) was linked to asthma (Shirakawa et al., 1996) and bronchial hyperresonsivness, (van Herwerden et al., 1995) as well as other allergy associated symptoms(Hill et al., 1995, Marsh et al., 1994). The β-chain of FcεRI is not essential for function but augments the expression of the molecule at the cell surface(Shirakawa et al., 1996). Hence work to establish if polymorphisms within the coding region for the gene alter levels of expression would be revealing indeed.

A cluster of cytokine genes occurs on chromosome five. Polymorphisms have been recognised in several of these including IL-4 (Marsh et al., 1994), IL-13 (Meyers et al., 1994) IL-9 (1997b) and CD14 (Baldini et al., 1999), which are linked to the modulation of the allergic response. Other work has associated polymorphisms within the proinflammatory cytokine TNFα (Moffatt and Cookson, 1997). This observation is perhaps particularly interesting, as the association of the gene with asthma was independent of markers of atopy, thus suggesting that asthma is not entirely driven by allergy.

The genes encoding IFNγ (Ober et al., 1998) and the MHC-classII (Daniels et al., 1996, Ober et al., 1998) (Wjst et al., 1999) have also been linked to asthma in a number of other studies.

Evidence that coding variations in the β-adrenergic receptor are significant in asthma and/or an individuals response to β-adrenergic agonists exists, although some inconsistencies exist between observations (Green et al., 1994) (Hall, 1996) (Weglarz et al., 2003, Taylor and Kennedy, 2002).

Other studies linking coding changes to a likelihood to respond to treatment have been carried out. For example a polymorphism within the promoter of 5-lipoxygenase has been suggestive as a predictive factor in an individual's likelihood to respond to leukotriene antagonists(Fowler et al., 2002). Other workers have shown a leukotriene C4 synthase promoter coding change to be significant in an individuals risk of aspirin-induced asthma.

All of these findings were all made using a "candidate gene" approach. The alternative approach for locating disease-related genes, that of positional cloning, is expensive but carries the advantage of identifying genes with as yet unknown links to a disease. Recent work has led to the identification of genes that are associated with asthma but have as yet unknown function, by employing this more elaborate technique of positional cloning. The first of these genes was A Disintegrin And Metalloprotease 33 (ADAM33)(Van Eerdewegh et al., 2002). The expression and biology of this molecule is to be a major component of this thesis. Further discussion of this finding is to be found below.

The second positionally cloned asthma gene was located on chromosome 13q14. This region of chromosome 13 as been linked to asthma in several screens because of its linkage to high serum IgE levels. Linkage disequilibrium analysis lead to the identification of PDH finger protein 11(PHF11)(Zhang et al., 2003) PHF11 is likely to play a role in transcriptional regulation and is expressed particularly in immune-

related tissues. Similarly to PHF11, dipeptidyl peptidase 10 (DPP10), (Qi et al., 2003), another recently identified asthma susceptibility gene, was located adjacent to a gene cluster previously associated with atopy (Karjalainen et al., 2002, Pessi et al., 2003).

The most recent gene to have been linked with asthma susceptibility is a G-protein coupled receptor subsequently named G-protein-coupled receptor for asthma susceptibility (GPRA) (Laitinen et al., 2004).The group identified a distinct distribution of protein isoforms between groups of asthmatics and healthy controls. An association with high IgE levels matched the association of GPRA with asthma. In a murine model of ovalbumin-induced inflammation the murine orthalogue of GPRA was also upregulated. These data imply a role for GPRA in the pathogensis of asthma and allergy. Studies have shown GPRA to contain numerous single nucleotide polymorphism's (SNPs), and several alternatively spliced transcripts were detected by northern blot analysis(Laitinen et al., 2004). The two major transcripts differ by their 3' exons, which encode proteins of 371 and 377 amino acids, and are named GPRA A and GPRA B respectively. Immunohistochemical staining showed these two isoforms to have different patterns of expression. Isoform A was predominantly expressed in smooth muscle cells of bronchus, gut and skin sections. Whilst isoform B was predominantly expressed in the epithelial cells of the same sections. In a disease comparison, isoform B was shown to be more greatly expressed in the asthmatic bronchus sections than the healthy control samples(Laitinen et al., 2004).

As mentioned, ADAM33 was identified as an asthma susceptibility gene in a recent publication by Van Eerdewegh and a collaboration of UK and US based researchers (Van Eerdewegh et al., 2002). ADAM33 was implicated in particular to asthma and bronchial hyperresponsiveness (BHR). As the National Heart Lung and Blood Institute definition implies, BHR is fundamental to the abnormal asthmatic airway.

Van Eerdewegh and his colleagues carried out genetic-linkage analysis on 460 pairs of affected siblings from the UK and the US. The linkage-analysis lead to the identification of a locus on chromosome 20p13. Statistical significance of this linkage-analysis increased when a phenotype of asthma and BHR was used, despite reducing the sample size by approximately half. Similar analysis including either serum total IgE levels or allergen specific IgE levels weakened the statistical significance of the finding. This logically suggests the presence of a gene more closely linked to airway hyperresponsiveness rather than inflammation. Further transmission disequilibrium analysis of the region led to the identification of the ADAM33 gene. It is believed that polymorphism's within the ADAM33 gene may account for a significant percentage of asthma susceptibility. Numerous single nucleotide polymorphism's (SNPs) within the gene have been identified (Chae et al., 2003, Van Eerdewegh et al., 2002). A discussion of the potential significance of these is to be found below.

Further evidence for the role of ADAM33 in BHR includes the finding that ADAM33 is expressed only in the airway fibroblasts and smooth muscle cells within the lungs, and not bronchial epithelial cells, T-cells or inflammatory cells. In addition polymorphisms within ADAM33 have been shown to predict impaired early life lung function. For example four SNPs (F+1, M+1, T1 and T2) have been shown to be associated with lover FEV1 at age 5(Simpson et al., 2005). Another study has shown the rare S2 SNP to be associated with reduced FEV1 in a cohort of 200 asthma patients(Jongepier et al., 2004). These findings further implicate the significance of ADAM33 in the remodelling abnormalities associated with asthma.

Following Van Eerdewegh's publication the association of ADAM33 with asthma susceptibility has been repeated in 4 further ethnically diverse populations(Howard et al., 2003), that of African American, US white, US hispanic and Dutch white. Signifiant association with at least one SNP and asthma were observed in each population (Fig 1.7).

Furthermore a region on mouse chromosome 2 has been linked to BHR. This region overlies an orthologue of ADAM33 with approximately 70% homology with the human equivalent(De Sanctis et al., 1995). The association of ADAM33 with asthma may however be gene pool specific as no association between ADAM33 SNPs and asthma was found in Peurto Rican or Mexican populations(Lind et al., 2003).

The ADAM Family

The ADAMs are a family of transmembranous proteins belonging to the zinc protease super-family. To date 40 ADAM genes have been described(White and Wolfsberg), 19 of which are expressed in humans. Other ADAMs are expressed in *C.elegans, Drosphila* and *Xenopus.* The family are distinguished by their unique multi-domain structure. This consists of a pro-, metalloprotease, disintegrin, cysteine-rich, EGF-like and transmembranous domain as well as a cytoplasmic tail. It is the presence of the metalloprotease as well as disintegrin-domain that gives the ADAMs their name; A Disintegrin And Metalloprotease. The ADAMs show some homology to other members of the adamalysins sub-family such as the snake venom metalloproteases (similar in structure but with no cysteine-rich, transmembranous domains or cytoplasmic tail), and the ADAM-TS family (similar but with a number of thrombospondin-like (TS) motifs downstream of the cysteine rich domain)(Seals and Courtneidge, 2003).

Current data suggests that ADAMs are synthesised in the rough-endoplasmic reticulum and are then processed (by removal of the pro-domain) in the golgi apparatus(Roghani et al., 1999, Lum et al., 1998). The cellular location of the molecule then varies according to the ADAM in question. Some studies indicate that the majority of ADAM protein resides in the golgi apparatus as indicated by the co-localisation with golgi markers(Roghani et al., 1999, Lum et al., 1998), although catalytically active ADAM protein can be found at the cell surface(Lammich et al., 1999, Roghani et al., 1999). ADAM12 exists as a full-length form and a truncated,

secreted form. It has been shown that the secreted form traffics to the cell surface whilst the full-length form is retained in the trans-golgi network(Hougaard et al., 2000) The modular design of the ADAM proteins means a large number of potential functions. The pro-domain has been shown to regulate the activity of the metalloprotease site via a cysteine switch. A cysteine residue in the pro-domain binds preferentially with the zinc atom at the active site thereby blocking the active site of the molecule(Becker et al., 1995). ADAM17 sheddase activity for example, can be inhibited in live cell experiments by the addition of cysteine switch peptides that compete for the ADAM17 active site(Roghani et al., 1999). Evidence exists that some ADAMs undergo autocatalytic activation. For example if cells are transfected with a mutated form of ADAM28 with an inactive metalloprotease site only the precursor form of the protein is detectable(Howard et al., 2000). The other possible function of the pro-domain is to confer correct folding of the ADAM protein. When ADAM17 is expressed without the pro-domain a mis-folded inactive form of the molecule is generated(Milla et al., 1999).

The logical function of the metalloprotease domain is in the cleavage of substrate. This can include the shedding of cytokines and/or cytokine receptors. ADAM17 for example has been shown to convert TNFα to its active form(Black et al., 1997) as well as being responsible for the proteolysis of the TNFα receptor from the cell surface(Solomon et al., 1999). ADAMs have also been implicated in the shedding of growth factors. Evidence exists for ADAM9(Izumi et al., 1998), ADAM12(Asakura et al., 2002) and ADAM17(Merlos-Suarez et al., 2001) playing a role in the shedding of HB-EGF. ADAMs may also play a role in ECM degradation. In *in vitro* models, ADAM10(Millichip et al., 1998) and ADAM15(Martin et al., 2002) have both been shown to cleave type IV collagen. These findings have lead to speculation that ADAM mediated ECM degradation may aid cell migration. The catalytic activity of ADAMs *in vivo* may be regulated by the presence of Tissue Inhibitors of Metalloproteases (TIMPs). There are four of these naturally occurring regulators of metalloproteases that occur in vertebrates(Brew et al., 2000). These molecules

function by the N-terminus physically blockading the active site of an MMP. The C-terminus is believed to confer specificity. These proteins are potent inhibitors of metalloproteases but have been shown to have some cross reactivity with ADAMs. ADAM17(Amour et al., 2000) and ADAM12(Loechel et al., 2000) have been shown to be inhibited by the presence of TIMP-3, whilst TIMP-1 and TIMP-3 can inhibit ADAM10(Amour et al., 2000). Not all ADAMs are inhibited by TIMPs however. A study in to the proteolytic activity of ADAMs 8 and 9 failed to find any inhibitory effect by any of the four TIMPs (Amour et al., 2000).

The disintegrin domain of an ADAM is so called due to it's homology with a region in snake venom metalloprotease. These regions disrupt the integrin mediated binding of platelets hence preventing platelet aggregation at a wound site. The resultant haemorrhaging is the means by which these snakes kill their prey. The disintegrin domains of snake venom metalloproteases operate by mimicking the matrix proteins like fibronectin for integrin receptors. For example a protruding structure known as the disintegrin loop on ADAM15 contains an RGD consensus sequences that mimics fibronectin(Nath et al., 2000). The function of the disintegrin domain in ADAMs is likely to be in cell adhesion. The best documented example of this is the mediation of sperm/ egg fusion. ADAM2 (fertilinβ), ADAM3 (cyritestin), ADAM5, ADAM16 and ADAM18 are all expressed on sperm cells and are believed to be involved in sperm/egg adhesion via integrins on the egg surface prior to fusion and fertilisation(Evans, 2001).

The function of the Cysteine-rich and EGF-like domains in ADAMs documented thus far has suggested a role in adhesion also. The cysteine-rich domain of ADAM12 has been shown to promote adhesion of both myoblasts and fibroblasts(Zolkiewska, 1999). Whilst treatment of cells with antibodies to the cysteine-rich domain of ADAM13 has been shown to reduce binding to β1-containing integrin receptors(Gaultier et al., 2002).

The best documented ADAM cytoplasmic tail is that belonging to ADAM12. The complexity of the potential interactions of this domain illustrates well the potential for

multiple function of this domain. ADAM12 has 10 putative SH3-binding domains as well as two potential sites for tyrosine phosphorylation(Suzuki et al., 2000). Src has been reported to bind to the tail of ADAM12 via one of the SH3-binding domains as well as two related kinases Yes, and Grb2(Kang et al., 2000, Suzuki et al., 2000). The cytoplasmic tail of ADAM12 has also been shown to associate with and activated PI-3 kinase. The presence of a cytoplasmic tail adds greatly to the potential functionality of the ADAM proteins. Such a domain may regulate signals from inside the cell to the extracellular domains. It may regulate signals from outside of the cell to intracellular pathways, or may regulate the cellular location of the protein.

A role for ADAM33?

Little is known about ADAM33 as a recently reported member of the ADAM gene family. Hence it is logical to compare other known ADAM functions to begin to understand the potential biological roles for ADAM33. As discussed, the structure of the ADAM's is complex (Fig 1.6) thus conferring a broad spectra of potential functions.

Such a complex-structural family of genes have a wide variety of potential functions. Both the disintegrin and cysteine-rich domains have potential adhesion activity. The EGF-like domain may be involved in membrane fusion, whilst the metalloprotease domain has the potential for proteolytic activity. Inhibition of proteolytic activity is likely to be a relatively simple target for therapeutic intervention if such activity is shown to be significant to an individuals susceptibility to asthma. Hence, at this stage the majority of the work on this novel molecule has been focused towards metalloprotease domain(Orth et al., 2004, Prosise et al., 2004, Zou et al., 2003). However further understanding of the basic biology of ADAM33 is necessary to validate the usefulness of blocking the catalytic activity of the molecule.

Thus far the crystal structure of the catalytic domain has been deduced (Orth et al., 2004) and the domain has been shown to be catalytically active using a number of synthetic peptides in a model system (Zou et al., 2003). The overall structure of the ADAM33 metalloprotease domain is that of an elliptical shape measuring 26Å x 38Å x 52Å (Fig 5.8)(Orth et al., 2004). This consists of compact central structure made up of five beta-sheets and four α-helices wrapped by the remaining 57 residues of the C-terminus. Six cysteine residues forming three disulphide bridges maintain the structure. This catalytic domain has been shown to be active in a recombinant model. ADAM33 metalloprotease domain was expressed in *Drosphilia* S2 cells and purified(Prosise et al., 2004). A number of synthetic peptides were then assayed with the purified metalloprotease in peptide cleavage assays. Four synthetic peptides were successfully cleaved by ADAM33: β-amyloid precursor protein (APP), kit-ligand 1 (KL-1), TNF-related activation-induced cytokine (TRANCE), and insulin B chain. Mutation of the active site by replacing glutamate 346 with an alanine (E346A) residue inactivated the catalytic activity. Of these only kit-ligand 1 has any known potential physiological role in asthma as kit-signalling is known to effect mast cell, maturation, proliferation, adhesion and degranulation(Kubo-Akashi et al., 2004). In a follow-up to this study, a range of APP mutants were synthetically produced and tested against the recombinant ADAM33 metalloprotease domain. In addition the optimal laboratory conditions for assaying ADAM33 were established. These tools will prove useful in studying the catalytic activity of ADAM33. However, none of the substrates cleaved by ADAM33 in these *in vitro* studies are likely to be the native ligand for ADAM33, as cleavage efficiency of naturally occurring ligands was relatively low in all cases. Interestingly ADAM33 catalytic activity was moderately inhibited by TIMP-3 and TIMP-4, weakly by TIMP-2 but not at all by TIMP-1(Zou et al., 2003).

Despite the finding that ADAM33 metalloprotease domain is potentially activate, other work in our own laboratory has shown the metalloprotease domain to be selectively spliced out of >95% of ADAM33 transcripts in primary airway

fibroblasts(Powell et al., 2004). This finding may indicate that the catalytic activity of ADAM33 is of not the major function of ADAM33 in these cells at least, or conversely may highlight the need for stringent regulation of a highly potent enzyme. Despite either conclusion, the fact that <5% of transcripts contain the metalloprotease domain would indicate that the other domains will play some kind of functional role.

ADAM33 is most closely related to the ADAM12, ADAM15, ADAM17 and ADAM19 subfamily of ADAMs, all of which have been shown to posses proteolytic activity. ADAM12 for example has been shown to activate HB-EGF by cleavage(Asakura et al., 2002). It may be the case then that ADAM33 could cleave pro-fibrogenic or proliferative growth factors essential within the EMTU.

It is possible that the role of ADAM33 is to do with the developing embryo and be less influential in adult life. ADAM15 for example has been implicated as crucial to the development of the nervous system. Deletion mutants of ADAM15 in mice exhibit a phenotype indicative of a loss in Delta/Notch signalling: The system involved in the development of the nervous system(Hartmann et al., 2002). Similar findings have been made in *Drosphillia* (Rooke et al., 1996). ADAM13 has been shown to play a role in embryogenesis by migration of cells during *Xenopus* development. Injection of embryos with protease-inactive ADAM13 lead to significant disruption of embryonic development(Alfandari et al., 2001). Explanted tissue from these embryos showed a marked reduction in an ability to disperse compared to control tissue. The authors of this study suggest a role for ADAM13 in adhering cells to their surrounding matrix and allowing the removal of matrix to allow the migration of cells further. If ADAM33 were to play role in embryogenesis then SNP's within the ADAM33 gene may cause abnormalities that manifest prenatally thus predisposing an individual to asthma.

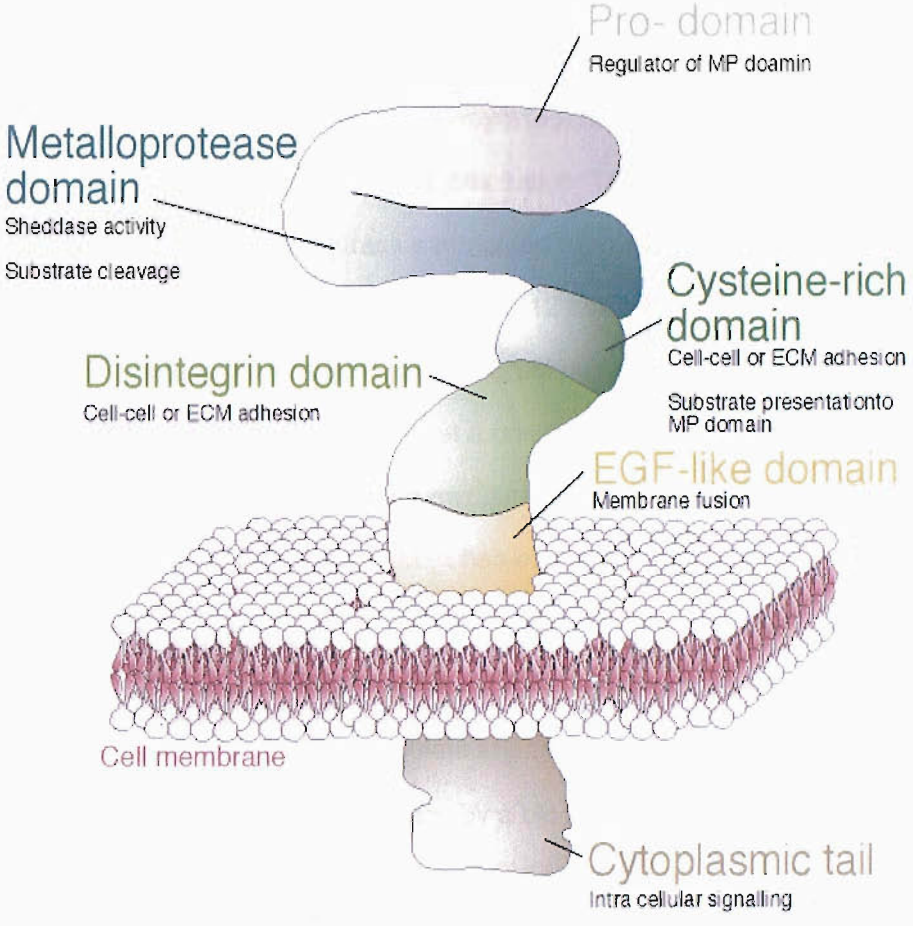


Fig 1.6 Schematic diagram of ADAM33 structure illustrating potential role of functional domains.

ADAM12 has also been shown to be crucial in the functioning of myoblasts (Yagami-Hiromasa et al., 1995). These cells differentiate and fuse to form multinucleated myotubes during the deposition of skeletal muscle. C2C12 myoblasts provide an excellent *in vitro* model of myoblast differentiation and fusion. Cultures of these cells will differentiate to form myotubes upon removal of serum from culture medium(Yagami-Hiromasa et al., 1995). Antisense ADAM12 constructs inhibit this process suggesting a role for the protein in myoblast fusion. However over-expression of full-length ADAM12 also inhibits fusion. This paradox has been explained however. Myoblasts express a truncated form of ADAM12 minus the pro- and metalloprotease domains. Treatment of C2C12 cells with the truncated form of ADAM12 gave rise to a two-threefold stimulation of myoblast fusion(Yagami-Hiromasa et al., 1995). These data suggest a positive role for ADAM12 disintegrin, cysteine-rich, and/or EGF-like domain in myoblast fusion, and a negative role for the metalloprotease domain. Subsequent work has examined the molecular partners required for ADAM12 to promote this myoblast differentiation and fusion. Specifically the interplay of ADAM12 and $\alpha 9\beta 1$ integrin was shown to be central to the process. ADAM12 and $\alpha 9$ were shown to accumulate in parallel at the time of fusion. Inhibition of the interplay between the two molecules by a blocking antibody to $\alpha 9\beta 1$ or ADAM12 antisense oligonucleotides inhibited myoblast fusion by 47-48% or by up to 62% in a combined strategy. The authors therefore propose a model whereby ADAM12 and $\alpha 9\beta 1$are operative in the deposition of nascent skeletal muscle. Parallels between the differentiation of myoblasts to form functional skeletal muscle and the possible differentiation of fibroblasts to form smooth muscle are obvious. This parallel is more striking given a recent study in to the recognition of integrins by the disintegrin domains of a number of ADAMs. Of all the integrins tested ADAM33 exclusively supported only $\alpha 9\beta 1$ dependent adhesion(Bridges et al., 2004).

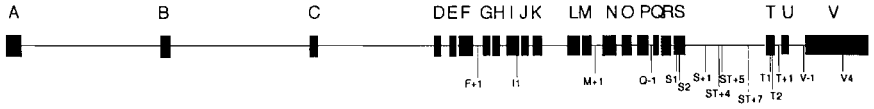
To date approaching 100 SNP's have been identified within the ADAM33 gene. Of these 15 have been shown to be linked to asthma in at least one study(Werner et al.,

2004), the majority of which lie towards the 3' end of the molecule (Fig 1.7). Haplotype analysis of pairs of SNPs increases the association of the SNPs with asthma in some cases by several orders of magnitude. Within the UK population studied for example, the most significant pair reached a P value of 0.000003(Van Eerdewegh et al., 2002).

The cytoplasmic tail of ADAM33 is relatively short compared it's nearest homologues but is rich in prolines and contains a putative SH3 binding site. The T_2 SNP is located within this site and therefore has potential to affect function. There is also casein kinase F/II phosphorylation site and a MAPK consensus sequence, which maybe key to ADAM33 function. The T_1 SNP (M→T) resides in this region also, but does not create a new phosphorylation site.

Nine of the asthma associated SNPs are located intronically. It has been speculated therefore that the disease significance of such polymorphisms may be to do with affecting gene stability and/or splicing of the molecule. To investigate the potential of SNPs to regulate splicing the alternative splicing of ADAM33 has been studied in detail. A spliced form of ADAM33 lacking exon 17 was cloned from a human testis cDNA template(Yoshinaka et al., 2002) and a similar variant has subsequently shown to exist in the mouse(Umland et al., 2004). This form has been designated the β form of ADAM33. More recently a number of novel splice variants of ADAM33 have been cloned from human airways fibroblasts (Powell et al., 2004). As mentioned, of particular note from this study was the finding that the metalloprotease domain of ADAM33 was selectively spliced out of >95% of all ADAM33 transcripts. This study also found the majority of total ADAM33 transcripts to be retained in the nucleus. This is either indicative of a large reservoir of ADAM33 transcript available to respond rapidly to stimulation, or that the majority of transcripts are fated for destruction in the nucleus. A percentage of each of the transcripts quantified was found to be exported to the cytoplasm however, and the existence of multiple protein isoforms of ADAM33 was confirmed by western blot analysis. The relative expression ratios of most of the

a.



b.

SNP	Location	ς UK case control	ς US case control	Ψ German case control	* Dutch case control	* African-American case control	* US case control 2	* US Hispanic case control
F+1	Intron	0.0295						
I1	MP domain		0.0105					
M+1	Intron		0.0116					
Q-1	Intron	0.0419						
S1	TM domain	0.026						
S2	TM domain	0.0041				0.03		0.04
ST+4	Intron	0.0191						
ST+7	Intron			0.0078			0.017	
T1	Cyt tail		0.003				0.03	
T2	Cyt tail		0.0188				0.02	0.04
T+1	Intron		0.0334					
V-1	Intron	0.0105						
V4	3' UTR	0.0328			0.0012			

Fig 1.7 a.Genomic structure of ADAM33 showing the location of asthma associated SNPs. Exons are shown as boxes and are designated by letters A-V.

b. p values attributed to asthma significance of SNPs in a number of case-controlled studies. (ς *Van Eerdwegh et al 2002*, Ψ *Werner et al 2004*, * *Howard et al* 2003)

ADAM33 splice variants was found to be similar in the cytoplasm as the nucleus, and hence whole cell mRNA can be used to measure ADAM33 splice variant expression in subsequent studies. No disease related patterns of ADAM33 splice variant detection were detected and subsequently no data inferring a role for particular SNPs in the regulation of ADAM33 splicing has been forthcoming. A recent study examined the association of SNPs within ADAM33 and the phenotype of accelerated decline in lung function that occurs in a subgroup of severe asthmatics(Lange et al., 1998). Results indicted a significant association between the S2, T1 and T2 SNPs and an accelerated decline in lung function(Jongepier et al., 2004). This is the first evidence implementing ADAM33 SNPs with the progression of asthma and adds weight to the literature in support of ADAM33 playing a role in airway remodelling. The authors postulate a role for ADAM33 whereby normal ADAM33 function is to maintain a normal level of fibroblast activity. Whilst in a diseased individual ADAM33 function is lost and a subsequent increase in subepithelial fibrosis occurs.

A summary

Asthma is a common disease that effects the quality of life of millions of people; particular in the western industrialised world(NAC, 2002). The disease is characterised by structural changes to the airways known as "airway remodelling"(Davies et al., 2003). One of these histological changes seen in the asthmatic airways is an increase in smooth muscle mass(Holgate, 2000) that has been implicated as central to the hyperresponsive narrowing of the airways that occurs during an asthma attack. The cellular and molecular mechanisms that drive the remodelling of the airways in asthma are not well understood. Recent findings have found remodelling changes to occur early in the progression of the disease(Holgate, 2000, 2000), and that such structural changes occur in parallel with the development of an altered inflammatory response rather than as a result

of(Holgate et al., 2000). Myofibroblasts within the asthmatic lung are believed to be central to the remodelling changes that occur in asthma. They are for example, responsible for the laying down of interstitial collagen under the basement membrane seen in asthmatic bronchial studies(Brewster et al., 1990). Myofibroblasts have been shown to undergo a phenotypic transition to become smooth muscle cells in other tissues(Jones and Jacobson, 2000) and experimental models(Buoro et al., 1993) and may act as precursors of smooth muscle cells in asthma. Therefore a major component of this study will be to examine the extent to which primary airway fibroblasts can undergo a phenotypic transition towards a smooth muscle phenotype upon stimulation with the asthma associated cytokine transforming growth factor β. ADAM33 is a recently described "asthma susceptibility gene" that has been linked to an individuals susceptibility to develop bronchial hyperresponsiveness(Van Eerdewegh et al., 2002) and a predisposition towards a decline in lung function(Jongepier et al., 2004). ADAM33 is expressed exclusively within fibroblasts and smooth muscle cells (and neuronal cells) within the airways(Van Eerdewegh et al., 2002). The biology of a closely related ADAM, ADAM12, has been shown to be central to the differentiation and fusion of myoblasts in to mature skeletal muscle(Lafuste et al., 2004, Yagami-Hiromasa et al., 1995). Therefore this thesis will examine the expression of ADAM33 in fibroblasts, with particular analysis of the expression of the molecule during the differentiation from fibroblast to myofibroblast.

The project aims

The overall aims of this project are:

- To examine the phenotypic plasticity of myofibroblasts and investigate their ability to act as precursors of smooth muscle cells.

- To investigate the expression ADAM33 in myofibroblast differentiation.

Materials and Methods

Cell Culture

All media and supplements were purchased from Invitrogen unless otherwise specified, although a full list of reagents and their suppliers can be found in the appendix. Cells were grown in a humidified Heraeus incubator at 37°C, 5% CO_2. Fibroblasts were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% v/v heat inactivated foetal bovine serum, 50 IU/ml penicillin, 50μg/ml streptomycin, 2mM glutamine, 100X non-essential amino-acids and 100mM sodium pyruvate, unless otherwise stated.

Ethical approval

Bronchial biopsies were obtained by a qualified physician using a fiberoptic bronchoscope in accordance with standard published guidelines(Djukanovic et al., 1991), following ethical approval from the Southampton and southwest Hampshire Joint Ethics Committee.

Primary Fibroblasts

To investigate the phenotypic plasticity of primary airway fibroblasts, cells were cultured from bronchial biopsies from 6 normal subjects (4:2 M: F, mean age 21 (range: 20-21) years) with an FEV1 of 97.25±5.2% predicted and 7 asthmatic subjects (4:3 M:F, mean age 21 (range: 20-26) with an FEV1 of 76.4±7.1 % predicted. All asthmatic subjects were using β2-agonists as required, but not inhaled corticosteroids

Prior to use, all media and trypsin were pre-warmed to room temperature.

Primary human bronchial fibroblasts cells were grown from biopsies obtained by fiberoptic bronchoscopy. Biopsies from each subject were placed in a small petri dish with 3ml of supplemented DMEM. The biopsy was chopped into approximately 0.5mm pieces and anchored into grooves upon the petri dish surface freshly scored with a sterile scalpel blade. The tissues were cultured for approximately one week

during which time fibroblasts migrated from the tissue and proliferated on the base of the petri-dish.

The fibroblast cultures were then grown in the same supplemented DMEM (changed every other day) until confluent, then passaged 1:5. Cultures were used for assays up to passage 9.

To passage confluent cultures, the cell monolayer was washed 2x 1min with Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}. 10ml of 1xTrypsin ethylenediaminetetracetic acid (EDTA) solution diluted in HBSS without Ca^{2+} and Mg^{2+} was then washed over the cells and removed. The cultures were then incubated at 37°C for 1-3 mins. The flask was then sharply tapped and the detached cells collected in supplemented DMEM.

Prior to use cultures were screened for the presence of αSMA by immunofluorescent staining (See *Materials and Methods; immunofluorescent staining*) to eliminate the possibility of smooth muscle contamination.

Collagen coating of flasks

Within experimental assays, unless otherwise stated, all tissue culture-ware was coated with a solution of bovine collagen. Vitrogen 100(Nutacon UK), a purified solution of bovine collagen (95-98% type 1, 2-5% type 3) was diluted 1:100 in sterile H_2O. T25 flasks were coated with 3ml of the Collagen/ H_2O solution then left for 1-2 hours before the solution was removed.

Treatment of fibroblasts with TGFβ_2

The focus of this thesis is to examine the effects of treatment of primary airways fibroblasts with TGFβ_2. A standard protocol for treatment of these cells was used throughout.

Fibroblasts of passage 3-9 were grown to 85%-95% confluence in collagen coated T25 flasks. The medium was then changed to Ultraculture (serum free medium) supplemented with 50IU/ml penicillin, 50μg/ml streptomycin and 2mM glutamine. After 24 hours the media was replaced by fresh ultraculture without or with TGFβ_2 (SIGMA) at 0.004nM, 0.04nM or 0.4nM).

MRC5 foetal fibroblasts.

Later work in this thesis uses the MRC5 foetal fibroblast cell line (ECACC No.97112601) as a model of the primary airway fibroblast.

MRC5 foetal fibroblast cultures were grown in uncoated culture flasks in the same supplemented MEM (minimum essential medium), changed every other day, until confluent, and then passaged 1:5. Cultures were used for assays up to passage 19.

HEK293 full length ADAM33 transfected stable cell line.

HEK293 cells (ECACC No. 85120602) stably transfected with ADAM33-full length inserted into pcDNA3 were a generous gift from Professor Gill Murphy (Collaborative group, Cambridge University).

ADAM33 transfected HEK293 cells were grown in supplemented DMEM with the addition of G418 to a final concentration of 0.5mg/ml (Sigma). Medium was replaced every two days until confluent. Cells were then passaged 1:5. Control untransfected HEK293 cells (as mock-transfected cells were not available) were grown in the same DMEM without the addition of G418.

Transfections

To over-express ADAM33 in a mesenchymal cell line, both primary airway fibroblasts and MRC5 fibroblasts were transfected with full-length ADAM33 clones driven by a viral promoter. Full length ADAM33-pCDNA3 was a kind gift from Professor Gill Murphy (Collaborative group, Cambridge University) and was originally cloned by Glaxo Smithkline. Full length-A33-GFP was constructed by PCR amplification and sub-cloning from the full length expressing clone ADAM33-pCDNA3 into pEGFP-n1 (Invitrogen) using Hind III and BamH1 restriction sites and the primers;

Forward TCGTAATAAGCTTCCACCATGGGCTGGAGGCCCCGGAGA

Reverse TGGATCCGCCCAGAGGCAGGATCTTGGCATCT. This work was done in our laboratory my Dr H Yoshisue.

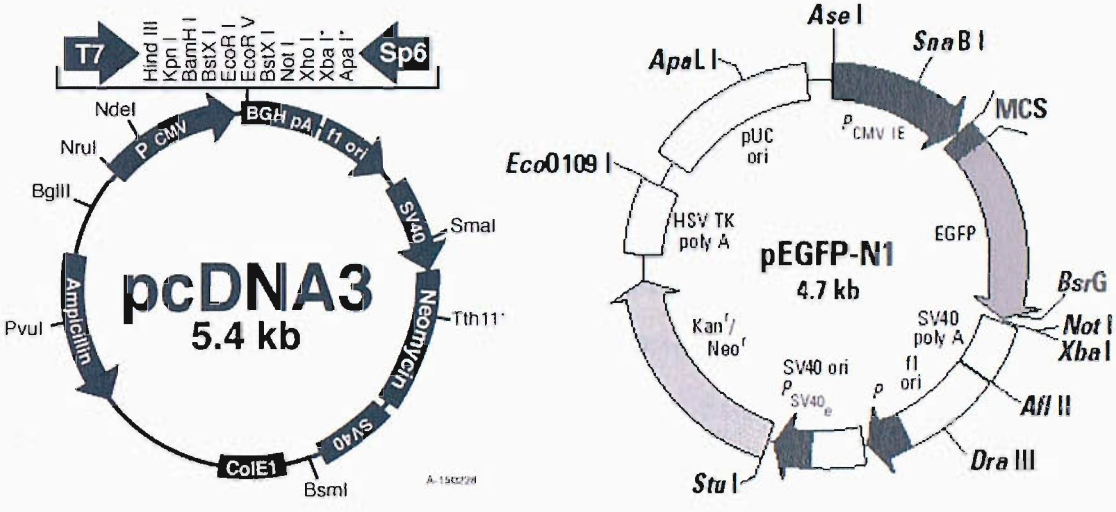


Fig 2.1 Schematic representation of expression vectors used in this thesis.

Primary fibroblasts or MRC5 fibroblasts were plated at a density of $8x10^4$ cells/ml in 2ml of supplemented DMEM or MEM respectively in a 6-well dish the day prior to transfection. 24hours later the cells were 50-60% confluent.

Transfection conditions were optimised according to guidelines outlined in the Effectene protocol. 0.5µg of DNA pEGFP-n1vector alone (Clontech),ADAM33-full

length inserted into pEGFP-n1vector or ADAM33-full length inserted in to pcDNA3 vector (Invitrogen) was added to 100µl of Buffer EC followed by 3.2µl of enhancer reagent. The solution was vortexed for 1 sec and incubated for 5 minutes RT. 10ml of Effectene reagent was then added before the solution was vortexed for 10 secs. During a subsequent 5 minute incubation at room temperature, lipid micells form encapsulating DNA. The cell monolayer was then washed twice with serum free medium and 1600µl of serum free DMEM or MEM was added to the cell monolayer. A further 600µl of serum free DMEM or MEM was then added to the Effectene-DNA mix and the solution added drop-wise to the cells. After 4hours the medium was replaced with serum containing medium. Transfection efficiency was evaluated 24-48hours later.

Evaluation of transfection efficiency by flow cytometry.

To provide a measure of transfection efficiency, cells were transfected to express green fluorescent protein using pEGFP-n1. This allows a simple count of transfection efficiency by flow cytometry.

Cells transfected with pEGFP-n1 were trypsinised from their growth surface, spun for 2mins at 1000g, and resuspended in FACs wash buffer(see appendix). The cells were then washed twice by spinning at 1000g for 2mins, before removal of supernatant and resuspension in FACs wash buffer.

The percentage of fluorescent cells was then assessed by flow cytometry. Flow cytometry analysis was carried out using a FACSCalibur (Becton Dickinson. San Jose CA, US). Threshold was set to exclude any debris. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was created and the population of viable cells gated. From this gated population a dot plot of fluorescence via channel 1 green(FL-1) against fluorescence via an irrelevant channel (FL-2) was plotted . The percentage of fluorescent cells was determined from the shifted population.

Selection of G418 resistant MRC5 clones.

To select only successfully transfected MRC5 fibroblasts from the transfection cultures, cells were treated with the antibiotic G418. A G418 resistance gene was present in all vectors used.

48 hours post transfection, media was replaced with the identical media with the addition of G418 to a final concentration of 1mg/ml. Selection medium was replaced every 48 hours for 30 days. On establishment of a G418 resistant cell line, the G418 concentration was reduced to 0.5mg/ml for maintenance of the line.

Coating of flasks with differing extra cellular matrix components

To examine the effect of the extracellular matrix upon fibroblast differentiation induced by TGFβ_2, cells were assayed as described but on alternatively coated tissue culture surfaces.

Vitrogen (collagen) (Nutacon UK), Fibronectin (Sigma) and Laminin (Sigma) stocks were diluted in sterile H_2O to a working concentration of 5µg/ml. The surfaces of T25 flasks were then with covered with 3ml of either solution or with sterile H_2O for 2 hours. The solution was then removed and the flasks air-dried before plating with cells.

Low passage fibroblasts (passage 3-9) were grown to 95% confluence in each flask before changing the medium to Ultraculture supplemented with 50IU/ml penicillin, 50µg/ml streptomycin and 2mM glutamine. After 24 hours the medium was replaced by fresh Ultraculture without or with TGFβ (SIGMA) at 1ng/ml (0.04nM). Samples were collected into Trizol at time zero (i.e. following 24 hours of serum deprivation) and 24 hours post TGFβ treatment. RNA was then extracted, reverse transcription carried out and Real Time qPCR was used to measure ADAM33 expression as described below.

Treatment of cells with protease inhibitors.

To examine the effect of protease inhibition on the degradation of ADAM33, cells were treated with either a cocktail of protease inhibitors or the ubiquitin protease inhibitor MG132.

Primary fibroblasts were grown to 85-95% confluence on collagen coated 6 well dishes. DMEM was then replaced with DMEM containing protease inhibitor (see table 2.1) or a DMSO as a control. Following a 2hour incubation the media was again replaced with ordinary supplemented DMEM. Higher concentrations or longer exposure to protease inhibitor were shown to be toxic as indicated by visible cell death. Cells were then TGFβ treated as previously described.

Protease inhibitor	Source	Stock concentration	Working dilution
Protease inhibitor cocktail for cell culture	Sigma	Unknown	1:600
MG132	Sigma	10mg/ml	1:800

Table 2.1 Protease inhibitors used

RNA extraction, analysis and Reverse Transcription

RNA expression is indicative of gene transcription levels at a particular moment in time. Therefore RNA was extracted from the primary airway fibroblasts cultures for analysis at each time point of interest following TGFβ treatment.

Collection of cell lysate for RNA extraction

At 0, 4, 8, 24 and 48 hours the conditioned medium was removed from a control and a TGFβ treated flask of cells. 750μl of TRIZOL (Invitrogen) was added, enough to cover the cell monolayer. Trizol reagent is a solution that allows single step extraction of RNA. The flask was then incubated for 5 minutes at room temperature. After agitating and pipetting up and down the solution was removed and stored at -20°C for no more than 4 weeks prior to RNA extraction.

RNA extraction

Trizol samples were thawed and 150μl of chloroform added. Each tube was shaken vigorously by hand for 15 seconds and then incubated at room temperature for 10 minutes. The tubes were spun at 12,000g for 15 minutes at 4°C. The top aqueous layer containing the RNA was removed, being careful not to disturb the interface between the two phases, and transferred into a sterile microfuge tube containing 500μl isopropanol. The tubes were vortexed and incubated overnight at -20°C. The samples were spun at 12,000g for 40 minutes at 4°C. The supernatant was removed leaving a small white pellet of RNA. The pellet was washed with 1ml 75% ethanol, gently vortexed, and then spun at 7,500g for 5 minutes at 4°C. The ethanol was removed and the tube was centrifuged again before removing the final traces of liquid. The pellet was air dried for approximately 5 minutes.

DNase treatment

To remove contaminating genomic DNA, the extracted RNA samples were treated with Dnase according to the manufacturers instructions.

1µl DNase1, 2µl 10x DNase buffer (Ambion, Austin, USA) and 17µl dH_2O was added to each tube containing a pellet of RNA and gently mixed. The reaction was incubated at 37°C for 1 hour. Addition of 5 µl of DNase neutralisation slurry (Ambion, Austin, USA) halted the action of the enzyme. The tubes were agitated to suspend the slurry for 5 minutes and then pulse spun. The samples were stored at –80°C.

Agarose gel

The RNA was run on a 1% agarose/Tris-Borate-EDTA-buffer (SIGMA) gel containing 1:10,000 dilution of ethidium bromide to identify any degradation of samples. The Ribosomal RNA 18S and 28S subunits bands on the gel were clearly visible under UV light as two distinct bands where RNA samples were intact. Degraded RNA appeared as a smudge and was eliminated from subsequent analysis .The intensity of the bands compared to standard RNA of a known concentration, allowed the quantification of the samples. To achieve this 1,2,3 and 4µl of Standard 0.2µg/µl RNA of was loaded alongside the samples (Fig 2.2). GeneTools software from Syngene was used to quantify the intensity the bands upon the gel. The intensity of the bands from the standard RNA was plotted against their respective concentrations to create a standard curve of formula y=mx+c. From this standard curve the RNA concentration of the samples of interest was calculated using the formula (y-c)/m=x.

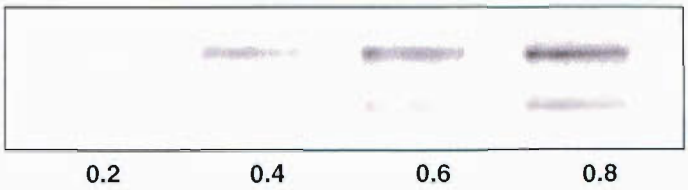


Fig 2.2 example RNA standard curve showing increasing mass of standard RNA in µg. 18S and 28S rRNA sub units are visible as 2 distinct bands.

Reverse Transcriptase (RT) assay

The RNA was reverse-transcribed to create cDNA. The cDNA was the template used for the Taqman PCR reactions.

The reaction was carried out in sterile RNAse/DNAse free thin walled strips of 200µl microtubes.

A mix was made up for all the tubes allowing the following for each tube; 1µl 10mM dNTP (Invitrogen), 1µl random hexamer primers at 3µg/ml (MWG biotech) and 6 µl dH_2O. A volume of RNA template equivalent to 1µg was added to each tube, along with an appropriate volume of the above mix to give a total reaction volume of 10 µl per tube.

The samples were incubated for 5 minutes at 85°C using an Eppendorf Mastercycler Thermocycler. This step caused denaturation of the RNA to ensure it was in a single stranded suspension. As soon as the 5-minute period was complete the sample tubes were plunged in to an ethanol/dry-ice bath. This ensures rapid cooling of the reaction thus causing the random hexamer primers to bind to the template RNA.

A second mix was prepared for all the tubes. The volume for one tube comprised of 0.5µl MMLV RT enzyme (Promega), 4µl 5x RT buffer and 5.5µl dH_2O.

10µl of this mix was added per sample and the final volume of 20µl was incubated for 1 hour at 42°C using the Eppendorf Mastercycler gradient. The enzyme reaction was stopped by 10-minute incubation at 85°C and then the tubes were held at 4°C. The RT tubes were stored at –20°C.

Real time quantitative PCR

The theory

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 a selected region of interest, between the forward and reverse primers, during the annealing stage of PCR (Fig 2.3). 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 detachment from the quencher. Therefore, the faster the fluorescence reaches a set threshold (CT value), the greater are the amounts of cDNA encoding the gene of interest in the reaction mix.

When two probes were used in the TAQman reaction (multiplexing), the emission wavelengths of the two probes had to be far enough apart to allow their individual detection. The FAM (490) and VIC (530) fluorophores were sufficiently different to allow simultaneous detection.

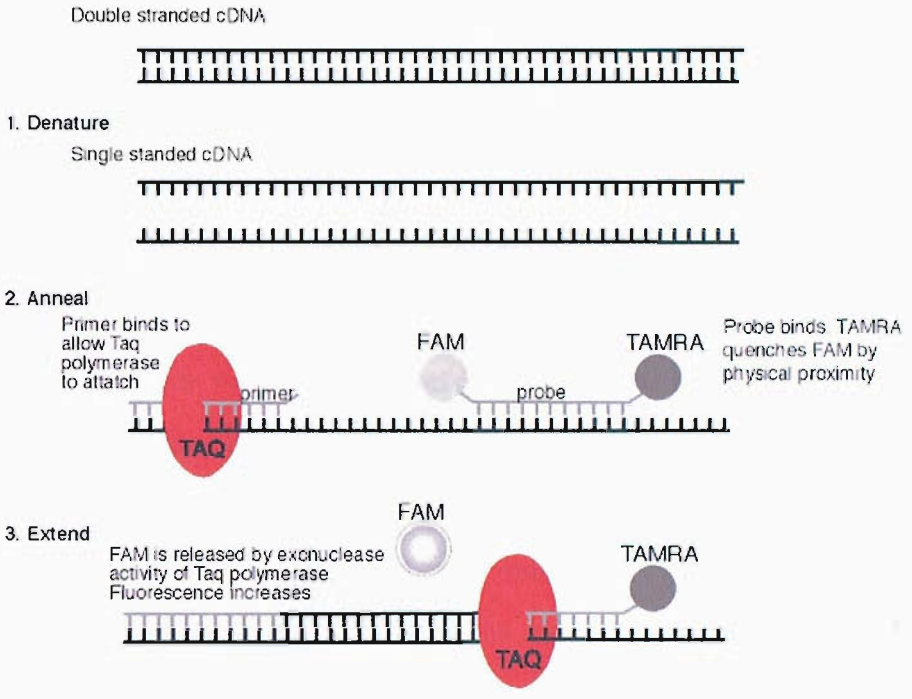


Fig 2 .3 Schematic representation of molecular events involved in TAQman real-time qPCR.

Primers and probes.

The forward and reverse primers were ordered from MWG biotech, and the probes from Oswell, and optimised according to the Perkin-Elmer protocol. Primers and probes were designed by Dr R.Powell using Beacon Designer 2.1 (premier bio-soft) (see table 2.2 and 2.3). Where possible the primers were designed to span two exons on the gene of interest. Therefore exponential amplification of genomic DNA would be highly unlikely to occur. The primers and probes should not therefore recognise any genomic DNA contaminating the reaction, only cDNA transcribed from the mRNA. In the case of the primer and probes sets designed to detect ADAM33 (Table 2.3), targeting of splice variants was achieved by manually designing forward primers that spanned the introns of interest and using a standard reverse primer and probe. The exception to this was in the targeting of splice variants that contain the ADAM33 MP domain. In this case a forward primer in exon G and a reverse primer in exon H were used, with a discontinuous probe spanning the splice site (Fig 2.4).
As a further precaution a control sample was present in every experiment which had not been reverse-transcribed. If any DNA amplification was detected in this control sample the data from the corresponding samples of interest were ignored as this indicates genomic DNA contamination.

In order to validate these assays for use with Δ_{Ct} method, melt curve analysis was performed to determine the specificity of the PCR reaction. A cDNA standard curve for each primer set was generated by measuring amplification of 10-fold serial dilutions of cDNA obtained from pooled samples. Each sample was measured in triplicate. If the priming efficiency is 100% a 10-fold increase in template will give a decrease in C_T value of just over 3. The Log [Template concentration] was plotted against the average C_T value for each dilution. The gradient of this line was used to calculate the primer efficiency using the formula $(10^{(-1/\text{gradient})})-1$, where a value of –3.2 was equal to 100% efficiency.

All primer sets used had very similar amplifications efficiencies (gradients of -3.28 ± 0.15) close to the theoretical maximum.

Target	Primers	Probe
Calponin 1	**F** GGTGAAGCCCCACGA CATT **R** GTTCACCTTGTTTCCTTT CGTCTT	TGCAGTCCACCCTCCTGGCTTTGT
Heavy Chain Myosin	**F** GCCTCCGTGCTACAC AACCT **R** CACGCAGAAGAGGCC AGAG	ACGTATATATTAGCCCTGAGAAGTACCGC TCCCTT
Desmin	**F** GGAGAGGAGAGCCG GATCA **R** GGGCTGGTTTCTCGGA AGTT	TCTCCCCATCCAGACCTACTCTGCCCTT
γActin	**F** CAGGTTATCACCATT GGCAATG **R** ATGAATTCCAGCGGA CTCCAT	CCCTGAGACCCTCTTCCAGCCTTCCTTT
ADAM33 3'UTR	**F** GGCCTCTGCAAACAAACATA ATT **R** GGGCTCAGGAACCACCTAG G	CTTCCTGTTTCTTCCCACCCTGTCTTCTCT
α smooth muscle actin	**F** GACAGCTACGTGGGTG ACGAA **R** TTTTCCATGTCGTCCCA GTTG	TGACCCTGAAGTACCCGATAGAACATGGC T

Table 2.2 Sequences of Primers and probes for smooth muscle markers used. F: Forward, R: Reverse. Primers and probes listed in a 5' to 3' orientation.

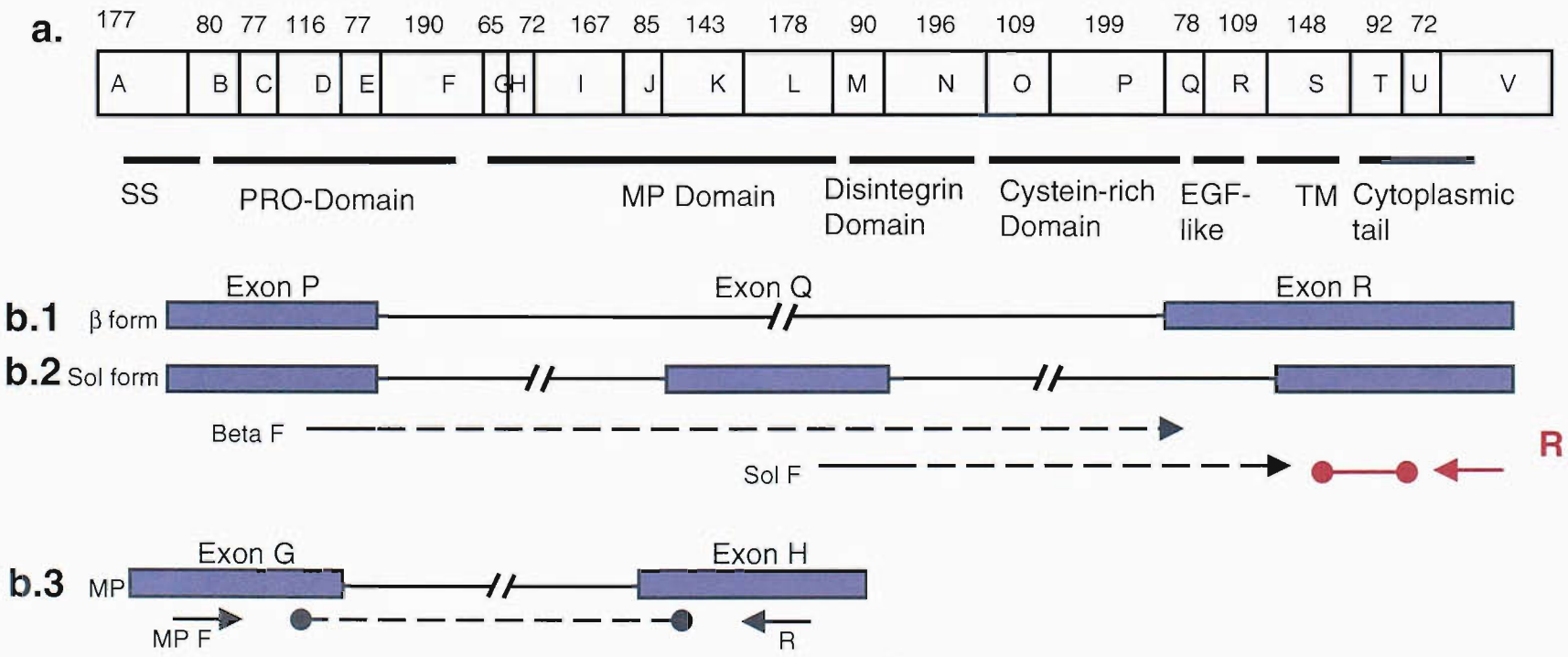


Fig 2.4 a. Schematic representation of ADAM33 showing the 23 exons and relative location of functional domains.

b. Assay designs for detection of ADAM33 splice variants. β form and soluble form detected by common reverse primer and probe (shown in red). β forward contiguous from exon R to Q thus detecting deletion of exon Q (b.1). Sol forward primer is discontinuous spanning 37 base deletion in exon R predicted in soluble form (b.2). MP domain detected by forward primer in exon G, reverse in exon H and discontinuous probe spanning splice site. Global A33 detection was achieved with an assay in exon V.

Adapted with the kind permission of Dr R. Powell.

Target	Primers	Probe
ADAM33 3'UTR	**F** GGCCTCTGCAAACAAACA TAATT **R** GGGCTCAGGAACCACCTA GG	CTTCCTGTTTCTTCCCACCCTGTCTTCTCT
ADAM33 βForm	**F** ACCCAGTGTGGACCTAGA ATGGTTTGCAAT *R* TGTCCATGCTGCCACCAA	CCACCCTTCTGTGACAAGCCAGGCT
ADAM33 metalloprotease domain	**F** GATCCTGGGAACAAAGCG GG **R** TCAGGACTCTGGACATTCA GGT	CCACACCCTGTTCTTGACTCGGCAT
ADAM33 soluble form	**F** CTGCCACATCCACGGGGC TG **R** TGTCCATGCTGCCACCAA	CCACCCTTCTGTGACAAGCCAGGCT

Table 2.3 Sequences of Primers and probes for ADAM33 and ADAM33 splice variant detection. F: Forward, R: Reverse. Primers and probes listed in a 5' to 3' orientation.

Primer sets were also tested by performing a melt curve (a slow ramping 0.1°C/sec from 50°C –90°C) in the presence of SYBR green. SYBR green is an intercalating agent that fluoresces more intensely in the presence of double stranded DNA than in solution. As the temperature increases fluorescence gradually decreases. However as the template DNA melts the rate of change in fluorescence rapidly increases resulting in a peak. (Fig 2.5)

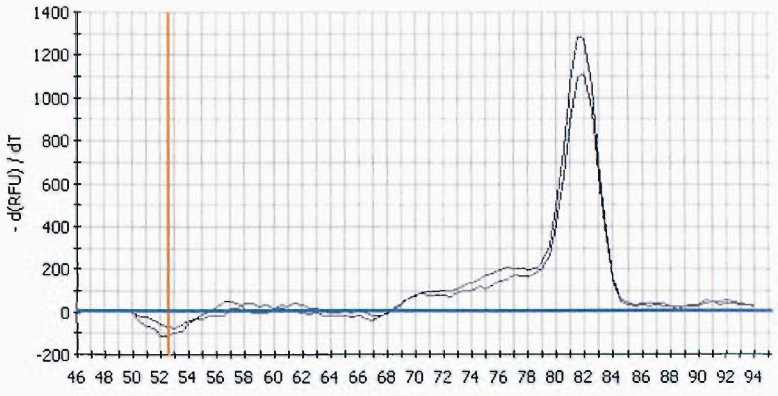


Fig 2.5. A typical melt curve showing a single peak representing the amplified double stranded DNA of the gene of interest. Image courtesy of Dr Rob Powell http://www.primerdesign.co.uk

If primer-dimerisation has occurred a second peak would be present on the melt curve at a lower melting temperature as the smaller species has a lower melting temperature. A primer-dimer curve is wider than that of a specifically amplified target because primers tend to dimerise as a number of small species of slightly different melting temperatures.

Primer/probe mix

15µl of both forward primers and reverse primers were combined. To this were added 310pmols of probe. The primer/probe mix was made up to 100µl with dH_2O to give a final probe concentration of 3.1pmol/µl. 1µl of this stock in a 12.5µ l total volume TAQman reaction gives a final concentration of 250nM probe and 900nM forward and reverse primers.

TAQMan Reaction

Each cDNA sample was diluted 1:10 in dH_2O (RNAase free) to give a concentration of 5ng/µl

5µl (25ng) template was added to 6.5µl Taqman Master mix and 1µl target primer/probe mix.

Taqman Master mix

Mastermix kits were purchased from Eurogentec and mixed according to the manufacturers specifications. Taqman Master mix was stored in 1ml aliquots as a 2x mix at –20°C until use.

cDNA amplification

Taqman reactions were performed on an iCycler IQ sequence detection system under standard cycling conditions: The Taq polymerase was activated on heating 95°C, 10 min, followed by 42 cycles of denaturation 95°C, 15s, and annealing/extension 60°C, 1 min. Quantitation, and real-time detection of the PCR products was followed on an icyclerIQ real time detection system (Bio-rad) .

The iCycler IQ sequence detection system software plots an emission intensity verses time (cycle number) graph. This is known as an amplification plot (Fig 2.6) The software sets a threshold of emission intensity. The cycle number at which an amplification plot crosses this threshold is known as the Ct (Cross Threshold) value.

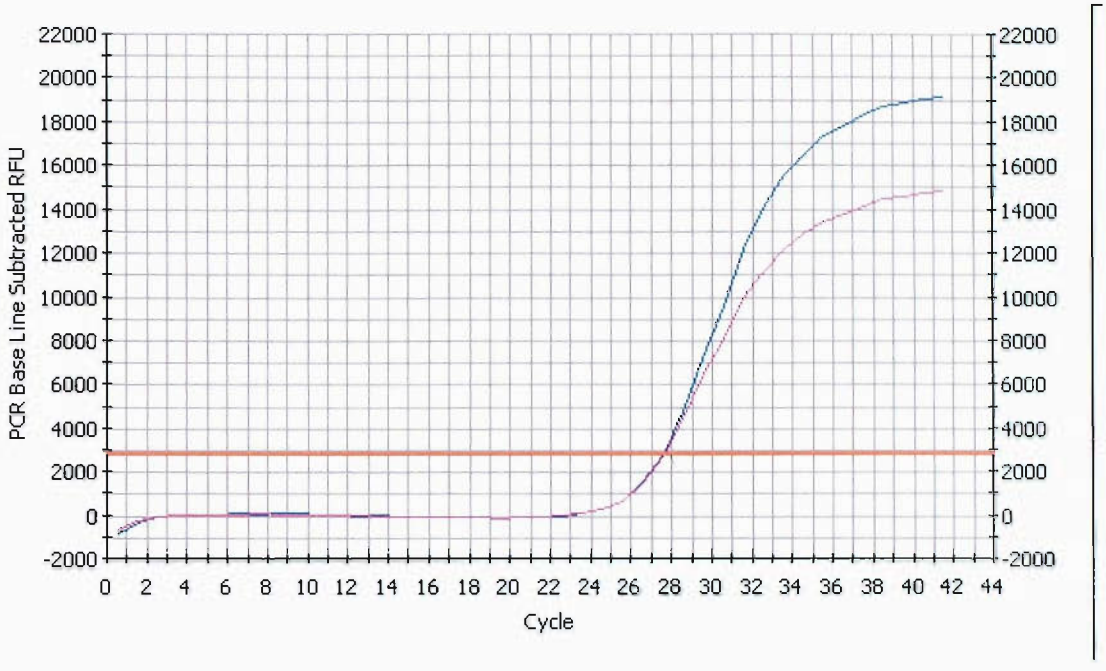


Fig 2.6 A typical real-time qPCR amplification plot showing emission intensity verses time (cycle number). Samples in duplicate are shown in blue and pink and cross threshold at cycle no.27.8. Threshold ~3000 is shown in orange.

Accurate Normalisation

The establishment of the most appropriate genes for accurate normalisation is discussed in great detail in Results chapter 1.

In summary; to establish the most stable genes for normalising, control gene expression was measured in 12 fibroblasts and the same cell lines following differentiation into myofibroblasts using 0.4nM TGFβ as described above.

12 normalising gene control kits (Eurogentech, Belgium) were selected for analysis; 18S ribosomal RNA (18S), 28S ribosomal RNA (28S), beta actin (ACTB), Glyceraldehyde-3-phosphate (GAPDH), Ubiquitin C (UBC), Beta-2-microglobulin (B2M), Phospholipase A2 (A2), ribosomal protein L13a (RPL13A), Succinate dehydrogenase (SDHA), Hypoxanthine phosphoribosyl-transferase 1 (HPRT1), TATA box binding protein (TBP), and hydroxymethyl, bilane synthase (HMBS). Each primer set was validated and met the criteria for analysis by the Δ_{Ct} method, as described above. Genome analysis of these data sets was performed using the

geNorm applet (available from http://medgen31.ugent.be/jvdesomp/genorm/) according to the guidelines and theoretical framework previously described(Vandesompele et al., 2002). For each normalising gene, ΔCt calculations were performed relative to the strongest signal, which was assigned the value 1, and these data used as the input for geNorm. Output files rating gene stability (M) and variation in normalising signal (NF) were collected for fibroblasts, myofibroblasts and the combined data set for both.

Analysis of Taqman PCR data by $\Delta\Delta C_T$ method.

The ΔC_T value is the gene of interest Ct – normalising gene Ct. The normalising genes CT used in this work was the geometric mean of A2 and UBC. The formula is based upon the fact that a decrease in the ΔC_t by 1 unit represents a two-fold increase in the expression of the target gene. Because the chosen normalising genes were more abundant than some of the target genes, the ΔC_T values were very small and difficult to interpret. For this reason an arbitrary point from each data set was chosen to which all other gene expression levels were expressed relatively. This complication of the ΔC_T calculation is known as the ΔΔCT calculation:

$$=2^{-(\Delta CT-(\text{arbitary1 } \Delta CT))}$$

Relative expression levels for all smooth muscle marker genes were calculated using the ΔΔCT method and expressed relative to the average ΔCT of all the untreated time zero controls within the data set.

Data from the geNorm analysis were analysed by one of two methods. Data were either analysed as a relative to the geometric mean of UBC and A2 using a standard ΔCT calculation or normalised to the geometric mean of UBC and A2 and expressed

relative to the signal obtained for the average the untreated time-zero controls using a $\Delta\Delta CT$.

Immunohistochemistry

Immunohistochemistry allows the detection and localisation of protein in whole cells via the use of antibodies against specific proteins. In this work the expression of a number of smooth muscle marker proteins as well as ADAM33 were examined.

Streptavidin biotin peroxidase technique

Fibroblasts were plated on to collagen coated 8-well culture slides at a density of 1.5 x 10^5 cells/ml. They were grown for two days in DMEM to 85-95% confluence. The medium was then changed to Ultraculture for 24 hours, and replaced with Ultraculture ± TGFβ (0.04nM) prior to incubation for 48 hours.

The medium was removed and the slides air-dried overnight before being wrapped in aluminium foil and stored at –20°C for up to 14 days prior to staining.

Cells were fixed in dry acetone for 30 minutes at room temperature, then allowed to air dry for 10 minutes. The slides were washed 3 x 2 minutes in PBS (pH 7.4), drained and a blocking solution of DMEM/10% FBS with an additional 3%BSA was added for 30 minutes. The slides were again washed 3 times in PBS before adding the primary antibody diluted in PBS/3% BSA.

A variety of antibodies against smooth muscle marker proteins were used (Table 2.4);

Antibody	Host	Source	Stock concentration	Working dilution
Anti-αSMA	mouse	Sigma	2.2mg/ml	1/500
Anti-HCM	mouse	Sigma	35mg/ml	1/100
Anti-Calponin	mouse	Sigma	2mg ml	1/500
Anti-Desmin	rabbit	Sigma	42mg/ml	1/20

Table 2.4. Primary antibodies used for detection of "smooth muscle markers".

Primary antibody incubation was for 2 hours. Following incubation at room temperature the slides were washed 3 x 2 minutes in PBS, before the appropriate rabbit or mouse horse-radish peroxidase-conjugated secondary antibody was added for 1 hour at room temperature, again diluted in PBS/3% BSA (Table 2.5).

Antibody	Host	Source	Stock concentration	Working dilution
Anti-mouse HRP	Rabbit	Dako	1.3mg/ml	1/100
Anti-rabbit HRP	Swine	Dako	1.3mg/ml	1/100

Table 2.5. Secondary antibodies used in immunohistochemistry.

The slides were then washed 3 x PBS and developed using DAB substrate/buffer tablet (Sigma) for 10 minutes. Washing with PBS stopped the reaction. The nuclear counterstain Mayer's Haemalum was then applied for 5 minutes and the slides "blued" in running tap water for 5 minutes. Following dehydration through graded alcohol's the slides were air dried, mounted in DPX and a cover slip applied.

Immunofluorescent staining

Cells were fixed in 4% paraformaldehyde for 15mins RT. The slides were then washed 3x2mins in PBS (pH7.4) Cells were then permeablised for 5mins at room temperature in PBS containing 0.1% Triton-X. A blocking incubation of 45mins in PBS containing 2% BSA was carried out. Following 3x2min washes in PBS, directly conjugated 1° antibody (See *Materials and Methods; Direct labelling of 1° antibodies*) diluted in PBS (see table 2.6) was then added and incubated in the dark at RT for 1 hour. After 3x2mins PBS washes a further fixation step of 15min in 4%paraformaldehyde fixed the conjugated antibody and attached fluorofluor to prevent bleaching. Cells were washed again 3x2mins and TO-pro3 blue (Molecular probes, Invitogen, UK) or 7-AAD (7-amino-acitomycin) (Sigma) nuclear-counterstain (see table 2.7) was added for 3 minutes. After final 3x2min PBS wash step, cells were mounted in Vectashield (Vector Laboratories Inc. CA, US.) and cover-slipped for immediate analysis by confocal microscopy on a Leica TCS SP2 (Leica, Mannheim, Germany).

Antibody	Host	Source	Added flurofuor	Stock concentration	Working dilution
Anti-ADAM33 cytoplasmic tail	Rabbit	Triple biologica	Alexa-fluor	1.3mg/ml	1/100
Anti-αSMA FITC	Mouse	Dako	n.a	1.3mg/ml	1/200

Table 2.6 Fluorescently labelled antibodies used in immunofluorescence.

Counterstain	Source	Working dilution
7-AAD	Sigma	12.5µg/ml
TO-pro3	Molecular probes	1/1000

Table 2.7 Nuclear counterstains used in immunofluorescence.

Direct labelling of 1°antibodies

Some ready-labelled primary antibodies were available (e.g. anti-αSMA) whilst the antibodies against ADAM33 require directly labelling "in-house".

1μl of 1mg/ml primary antibody was added to 4μl PBS and 5μl of Zenon alexa-fluor label (Molecular probes, Oregan) and incubated in the dark at RT for 5mins according to the manufacturers instructions. PBS was then added to create the desired final concentration of antibody.

Antibody Production

At the commencement of this work no antibodies against ADAM33 were available. In an attempt to generate antibodies against ADAM33, a strategy was employed involving the immunisation of four rabbits and two chickens with carefully designed peptides from the predicted ADAM33 structure.

Rabbit immunisations

Three rabbits were immunised with an individual KLH (Keyhole limpet hepatocyte) linked peptide (Rabbit A: metalloprotease domain, Rabbit B: disintegrin domain, Rabbit C: cytoplasmic domain). A fourth rabbit was immunised with two of the peptides simultaneously in an attempt to produce a fourth antibody with broader activity against ADAM 33 (Rabbit D: metalloprotease domain and disintegrin domain).

Peptide design

Three domains of the ADAM 33 protein were selected as possible antibody targets: the metalloprotease domain, the disintegrin domain and the cytoplasmic domain. A short peptide sequence from each domain was chosen based upon corresponding regions from ADAM 19 that were successfully used to produce antibodies(Zhao et al., 2001) (Table 2.8). BLAST searching revealed that the selected sequences were unrelated to any protein other than ADAM33.

Peptide name	Domain	Peptide sequence
Pep M	metalloprotease-like	LFLTRHRNLNHTKQR
Pep D	disintegrin-like	CGPGQECRDLCCF
Pep C	cytoplasmic	PLPAVSPDPQADQVQMPRSSCLW

Table 2.8 Peptide sequences used for rabbit immunisations.

KLH-linked and unlinked peptides for characterisation were synthesised by Southampton PolyPeptides Ltd. In all cases a purity of >95% was achieved. The compound identity was confirmed by mass spectrometry.

Immunisations

Qualified staff carried out the handling of all animals in the Southampton University Hospitals biomedical research facility in accordance with Home Office guidelines. A sample of pre-immunisation sera was collected from each of four *New Zealand white* rabbits 2 weeks prior to the initial injection of antigen. The peptides for immunisations were diluted in saline to a concentration of 0.5mg/ml. They were then emulsified into an equal volume of Freund's complete Adjuvant to a final concentration of 0.25mg/ml. Each rabbit received 4 sub-cutaneous injections of 0.5ml each. The rabbits were boosted with the same peptide in incomplete Freund's Adjuvant after 18 days and again after a further 32 days. A further 10 days later a test bleed was taken. 32 days after the second boost another boost was administered. Another test bleed was taken after a nine-day interval. 48 days after the third boost a fourth boost was administered. 10 days later the rabbit immunised against the disintegrin-like domain peptide was terminally bled. The remaining three rabbits received a final fifth boost 35 days after the fourth. After 10 days these rabbits were also terminally bled.

Collection of Sera from bleeds

Blood was allowed to clot overnight at 4°C. Serum was removed and spun at 2,000g to remove any further cellular debris.

Analysis of test bleeds

Enzyme linked immunosorbent assay (ELISA) was used to asses the specificity of IgG in the test bleed sera. Immmunoabsorbent ELISA plates (Nunc) were coated with

100µl/well of 0.1µg/ml synthetic peptide (not KLH-linked) in coupling buffer (see appendix) overnight. Unbound peptide was removed by washing 4 times with wash buffer (see appendix). 200µl/well of blocking buffer was then added to each well and incubated for 1 hour at room temperature. Sera was diluted in assay buffer (see appendix) and 100µl added per well. Following a 2-hour incubation unbound antibody was removed with four wash with wash buffer. A secondary swine-anti-rabbit antibody was diluted 1:1000 in assay buffer and 100µl applied to each well for 1hour. Unbound antibody was removed and the plate washed four times with wash buffer. 100µl of chromagen solution (see appendix) were added to each well. Following a10 minute incubation 50µl/well of 2M H_2SO_4 was added to halt the reaction. Plates were read at 450nm with a 630nm-reference filter on a Multiskan Acsent microplate spectrophotometer (Labsystems, US).

Ammonium Sulphate Precipitation of antibodies from sera

Raw sera from rabbits contain proteins that may affect the performance of the preparation in immunoassays. Hence an ammonium sulphate precipitation step was carried out to partially purify proteins antibodies from the sera. Ammonium sulphate takes up the water molecules around the proteins exposing hydrophobic sites. These hydrophobic groups tend to prefer to be together so the proteins aggregate and thus can be spun out of solution.

A volume of saturated ammonium sulphate equivalent to 0.6 x the serum yield was added drop wise to the rabbit sera at room temperature whilst stirring. Following a further 15 minutes stirring the ammonium sulphate precipitate was collected by centrifugation at 10,000g for 25 minutes at 4°C. The supernatant was removed and the pink precipitate re-suspended in a volume of 0.2M TE8 buffer (see appendix) equal to the original volume of sera. This precipitation procedure was repeated 2 or

3 times until the precipitate formed was white. This white precipitate was re-dissolved in a volume of 0.03M PE7.3 buffer equal to the original volume of sera. This protein solution was dialysed overnight at 4°C against 2 litres of 0.03M PE7.3, replacing the PE.7.3 (see appendix) twice. This partially purified IgG from each rabbit was tested by ELISA in the same way as the test bleeds.

Chicken antibody production

In addition to the four rabbits, two chickens were immunised in an attempt to generate anti-ADAM33 antibodies against the domains of ADAM33 that were less successfully target by the rabbit immunoglobulins generated. In addition antibodies from an additional species would be a useful reagent for "dual-staining" experiments. Chicken antibodies against ADAM33 were produced by Aves Labs. A sequence from the cytoplasmic domain was chosen and another from the metalloprotease domain, with 100% homology with the same domain on Mouse ADAM33. It was hoped that this second sequence would enable the production of an antibody that could be used for both human and mouse work.

Chickens were immunised by Aves Lab Inc. Chickens were immunised with one 0.5-1.0ml injection of antigen solution in Complete Freund's adjuvent, followed by three subsequent injections with antigen solution in a 1:1 mixture of Incomplete and Complete Freund's Adjuvent. Immune antibodies were isolated from eggs laid by the chickens and stored in PBS with 0.02% azide.

Characterisation of Chicken Antibodies

Sera from both chickens were tested for their affinity to their corresponding peptides by means of ELISA in the same way as the rabbit sera with the omission of the pre-immune sera control. An uncoated plate control was used however. The optimum concentration of sera for coating the immmunoabsorbent ELISA plates with peptide and the optimum starting dilution of sera was established by means of a

"checkerboard" ELISA (Fig 2.7). 100µl of peptide diluted in coupling buffer was loaded in to each well on the top row of the 96-well plate at a concentration of 20µg/ml. Serial dilutions of peptide in coupling buffer were used to coat the 96-well plate with peptide concentration decreasing down the y-axis of the plate.
After an overnight incubation unbound peptide was removed by washing four times with ELISA wash buffer. 200µl/well of blocking buffer was then added to each well and incubated for 1 hour at room temperature. 100µl of Serum diluted 1:20 was then loaded in to each well in the 1st column of the 96-well plate and then serially diluted in PBS pH 7.4 along the x-axis of the plate.

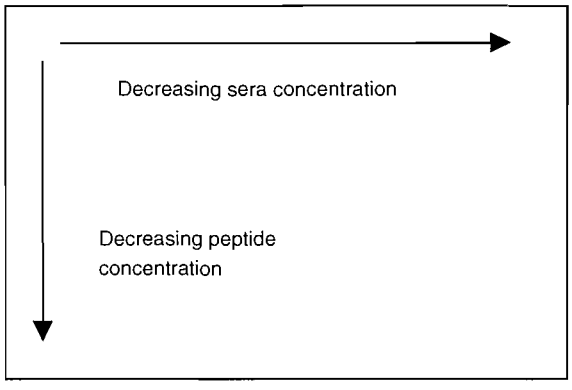


Fig 2.7 Schematic layout of 96 well "checkerboard" ELISA for establishing the optimum concentration of Chicken sera

Following a 2-hour incubation the ELISA protocol continued as described above but using a rabbit-anti-chicken HRP-linked secondary antibody. Plates were read at 450nm with a 630nm-reference filter on a microplate spectrophotometer.
The optimum peptide coating concentration was established to be 1.25µg/ml and a starting concentration of 1:640 and 1:20 was established for sera from chicken A and B respectively.

Analysis of anti-ADAM33 antibodies for immunofluorescence suitability

Rabbit Anti-ADAM33 antibodies generated in-house as well chicken anti-ADAM33 antibodies from Aves laboratories were extensively tested against ADAM33 transfected HEK293 cells for their suitability for detection of ADAM33 by immunofluorescence. Dilutions or sera in the range 1:10 through 1:50,000 were tested with a number of alternative secondary antibodies. Data from these analysis proved negative and are not shown in this thesis.

SDS-Polyacrylamide gel electrophoresis and Western blotting

In addition to immunohistochemistry, SDS-polyacrylamide gel electrophoresis followed by western blotting was used for detailed protein expression analysis of cells. Live cells were lysed in to denaturing buffer, and the protein content separated according to size by SDS-polyacrylamide gel electrophoresis then transferred to a nitrocellulose membrane for probing with protein-specific antibodies.

All buffers and solution preparations are listed in the appendix .

Sample collection

Following appropriate treatment cell grown in a 6-well dish were washed once with 1xPBS pH 7.4. Then 250µl of 1x sample buffer (see appendix) at 95°C was applied to the cells. Agitation with a pipette tip allowed the removal thereof of the complete cell lysate from the dish. The samples were then sonicated for 10 seconds and frozen at –20°C until required.

SDS-Polyacrylamide gel electrophoresis

The Mini Protean II system (Biorad) was used to cast 1mm thick gels for electrophoresis. Plates were cleaned thoroughly with 10% SDS followed by methanol. The 1mm spaced plates were then stood upright in a casting frame. A separation gel stock was then prepared as follows:

To prepare 2x1mm gels, 10ml of gel stock was used, to which 33µl of 10% (w/v) ammonium persulphate and 5µl of TEMED were added. The gel mix was then poured in to the gel set up and overlaid with water-saturated propanol. After 45mins

the polymerised gel was rinsed with distilled water and a stacking gel was prepared (see appendix).

5ml of the stacking gel stock solution was mixed with 16.7µl of 10% (w/v) ammonium persulphate and 3.8µl of TEMED. This was poured on top of the separation gel and a comb inserted. Polymerisation took 30-40mins. The combs were removed and the gel cassettes inserted in to the electrode assembly. The chamber was filled with running buffer (pH 8.3).

10µl of Rainbow Molecular weight marker (Amersham) or 20µl of sample was loaded in to each well. Electrophoresis proceeded at a constant 160V up to a 300mA limit for approximately 50 minutes or until the bromophenol blue dye front approached the edge of the gel cassette. The gel was then ready for either Coomassie Brilliant Blue staining or Western Blotting.

Coomassie Brilliant Blue Staining

Gels were placed in 20mls of Coomassie Brilliant Blue stain (see appendix) in a glass container and agitated on a shaker for 1 hour at 22°C.

Gels were then transferred into destain (see appendix) for 2-3 hours, or until the gel is virtually clear with blue stained protein bands visible. The destain was replaced with fresh destain when it became saturated with dye.

For long term storage, the gel was transferred onto filter paper wetted in dH_2O, covered with cling film and dried onto filter paper using a gel drier (Biorad) and visible protein bands photographed or captured and quantified using the Gene-Genius CCD camera and the GeneTools software.

Western Blotting

The gel obtained from the SDS-PAGE electrophoresis was placed carefully into cold transfer buffer for 15 minutes, to remove salts and to preshrink the gel.

Pre-cut nitrocellulose was cut to the size of the gels and presoaked in water for 5 minutes and transfer buffer for 15 minutes.

The Transblot apparatus (Biorad) consisted of a transfer cassette assembled with a sandwich of layers presoaked in transfer buffer. Firstly, a pad of scotchbrite and a one layer of Whatman 3MM filter paper were placed onto the black side of the cassette followed by the pre-shrunk gel and the membrane. Air bubbles were removed using a test tube rolled over the membrane and the cassette sandwich was completed with one layer of filter paper and scotchbrite pad.

The cassette was inserted into the Transblot tank containing chilled transfer buffer orientated with the gel closest to the negative electrode. Electrophoretic transfer was achieved using a constant voltage of 90V for 3 hrs, with a current limit of 350mA. An internal ice block was placed in the tank whilst the whole apparatus was placed in an icebox, with a magnetic stirrer inside the tank, and placed on a stirrer to distribute the chilled buffer.

Once transferred the blotted membrane was removed and air-dried on a piece of filter paper.

The NC blot was rehydrated for 10 minutes in 10ml of Wash buffer. Non-specific binding sites were blocked by incubation for 30 minutes in 10 mls of blocking buffer. Residual buffer was removed during a 1x 15 minute and 2x 5 minute washes in PBS/Tween before addition of the primary antibody.

The primary antibody was diluted in wash buffer with inhibitors (Roche) and incubated with the membrane at 22°C for 1 hour (Table 2.9).

Antibody	Source	Host	Working dilution
Anti-ADAM33 MP domain	Aves labs	Chicken A	1:25000
Anti-ADAM33 cyt tail	Aves labs	Chicken B	1:2500
Anti-ADAM33 MP domain	In house	Rabbit A	1:1000
Anti-ADAM33 dis domain	In house	Rabbit B	1:1000
Anti-ADAM33 cyt tail	In house	Rabbit C	1:1000
Anti-ADAM33 MP/dis domain	In house	Rabbit D	1:1000
Anti-ADAM33 pro domain	Triple biologica	Rabbit	1:2000
Anti-ADAM33 MP domain	Triple biologica	Rabbit	1:1000
Anti-ADAM33 Cyt tail	Triple biologica	Rabbit	1:2000

Table 2.9. Anti ADAM33 antibodies used in western blotting.
Extensive titration of all antibodies was carried out using dilutions ranging from 1:10 through 1:50,000. Dilutions in this table represent the most effective working dilutions for each antibody and also reflect the concentrations used to generate any figures in this thesis.

After the incubation, antibody was removed and fresh wash buffer used to wash unbound antibody from the blot using 1x 15 minute and 2x 5 minute washes.
The secondary antibody (Table 2.10) was diluted and incubated with the membrane for 1 hour at 22°C, before the final wash sequence, which consisted of 1x 15 minute and 4x 5 minute washes in wash buffer

Antibody	Host	Source	Stock concentration	Working dilution
Anti-chicken HRP	Rabbit	Sigma		1/2500
Anti-rabbit HRP	Swine	Dako	1.3mg/ml	1/2500

Table 2.10. Secondary antibodies used in western blotting.

Specific antibody binding was visualised using enhanced chemiluminescence (ECL PLUS, Amersham) according to manufacturers instructions and luminescence

detected using Hyperfilm ECL (Amersham). Blots were placed onto blotting paper to remove excess wash buffer and were then placed protein side up onto cling film. The membrane was covered with 2ml/blot of ECL-PLUS solution (2mls solution A and 50ul Solution B) for 5 minutes. The membrane was then blotted onto blotting paper and placed protein side down onto another piece of Cling film. This was folded over to form an envelope.

The enveloped membrane was expose to film in a darkroom for 10-90 seconds. The film was then developed by agitation in developer solution (Fotospeed PD5 print developer, Jay House Ltd, UK) for 20-30 seconds followed by a fixation step in fixative solution (Fotospeed FX20 rapid fixer, Jay House Ltd, UK) for 1 minute. Fixative was removed by rinsing in water and the film allowed to dry.

Statistical analysis

The data in this thesis were assessed for normality using the Anderson-Darling test (Empirical cumulative distribution function based test). This indicated that the data did not fit a pattern of Normal distribution. Consequently non-parametric tests of statistical significance were used. Data are expressed as median (Inter Quartile Range). The Wilcoxon signed rank test for paired data was used for within-group comparisons of gene expression (e.g.treated versus untreated controls) unless correction for multiple testing was required. In which case a Friedmanns test was perfomed. Between group data sets were analysed by Kruscal Wallis to identify potential for coincidental occurence of significant findings due to multiple testing. Then subsequently the Mann-Whitney U test was used (e.g. asthma versus normal controls). A probability score of less than 0.05 between data was considered a statistically significant difference.

Results Chapter 1

Results Chapter 1

Background

The fibroblast and its activated form, the myofibroblast, have been shown to play a significant role in the levels of sub-epithelial fibrosis seen in asthma (Brewster et al., 1990). They are also a source of smooth muscle and vascular endothelial cell mitogens, so have been assumed to play a central role in airway remodelling (Richter et al., 2001).

The myofibroblast phenotype is essentially an intermediate between that of a fibroblast and a smooth muscle phenotype. TGFβ, a growth factor expressed at elevated levels in the asthmatic airways (Redington et al., 1997), is well documented as a potent inducer of the myofibroblast phenotype(Desmouliere et al., 1993, Kurosaka et al., 1998, Ronnov-Jessen and Petersen, 1993). Some evidence is available to suggest that the fibroblast may have the ability to transform to a genuine smooth muscle phenotype(Jones and Jacobson, 2000, Buoro et al., 1993, Chambers et al., 2003).

TGFβ regulates the expression levels of a large number of genes during differentiation(Chambers et al., 2003). This is likely to include constitutively expressed housekeeping genes, which are used as controls for gene quantification. Accurate gene quantification within such a heterogeneous cell population with a highly plastic phenotype can be problematical (Vandesompele et al., 2002). For this reason it is particularly important to establish which housekeeping genes are most stably expressed during the process of differentiation to enable accurate gene quantification.

geNorm(Vandesompele et al., 2002) is a freely available (on request) Microsoft Excel applet designed to identify the optimum housekeeping genes for accurate normalisation within a particular experimental assay. This is achieved by comparing the expression ratio of each gene with all other candidate housekeeping genes, and hence generating a stability measure for each gene. This measure relies on the

principle that, regardless of the conditions, the expression ratio of two ideal normalising genes will remain constant in all samples. Hence, any variation in expression ratio between the two is indicative of one or both genes being variably expressed.

The software also indicates the number of measured housekeeping genes required to achieve optimum normalisation.

The aims of this chapter will be to identify the most stably expressed and practically useful control genes during TGFβ_2 induced fibroblast differentiation. Then having established this, to use this normalising assay to accurately investigate the extent to which TGFβ_2 will differentiate cultured primary airway fibroblasts towards a smooth muscle phenotype via the expression of αSMA and other markers of a smooth muscle phenotype. The study is designed to allow a comparison in the response of fibroblasts from asthmatics and healthy controls as well as examining the time course over which TGFβ_2 acts to induce the myofibroblast phenotype.

Results

geNorm analysis of control genes.

12 primary fibroblast lines were either untreated or treated with TGFβ_2 (0.4nM) for 48 hours. By real-time qPCR the expression levels of 12 of the most commonly used "house-keeping" or "control" genes were measured (Table 3.1). These data were then analysed by geNorm analysis(Vandesompele et al., 2002) to reveal the stability of expression of each gene during TGFβ induced myofibroblast differentiation.

The geNorm analysis compares the ratio of the ratio of control genes in different samples to find those that are most stable as previously described(Vandesompele et al., 2002). The 12 normalising genes were rated for stability (M) with the most stable producing the lowest M value (Fig 3.1). Analysis of fibroblasts populations compared to myofibroblasts produced contrasting orders of gene stability. Underlying this was the consistent finding that HPRT1 and RPL13A were the least stable genes in this system. Combining the two data sets produced a third sequence of gene stability ratings indicating the genes that are most stable during the differentiation of fibroblasts to myofibroblasts. In all situations, the four best genes gave M values <0.5 indicating that they are reasonably stable. The top two genes cannot be ranked in order because of the requirement for a gene ratio to calculate gene stability. The pair-wise variation analysis in normalising signal (NF_n) was also calculated for the three data sets using geNorm (Fig 3.2). This measures the degree of variation in normalisation signal that is achieved by using n control genes compared to n+1, with genes added step-wise into the analysis in the order of their gene stability rankings. The stability of the normalisation signal improves up to the addition of the eighth gene and then deteriorates as the four least stable genes are added to the analysis. In all three situations, the top three genes gave values close to the recommended threshold of 0.15 for accurate normalisation.

Gene Name	Abbreviation
18S ribosomal RNA	18S
28S ribosomal RNA	28S
beta actin	ACTB
Glyceraldehyde-3-phosphate	GAPDH
Ubiquitin C	UBC
Beta-2-microglobulin	B2M
Phospholipase A2	A2
ribosomal protein L13a	RPL13A
Succinate dehydrogenase	SDHA
Hypoxanthine phosphoribosyl-transferase 1	HPRT1
TATA box binding protein	TBP
hydroxymethyl bilane synthase	HMBS

Table 3.1 Abbreviations of normalising genes analysed

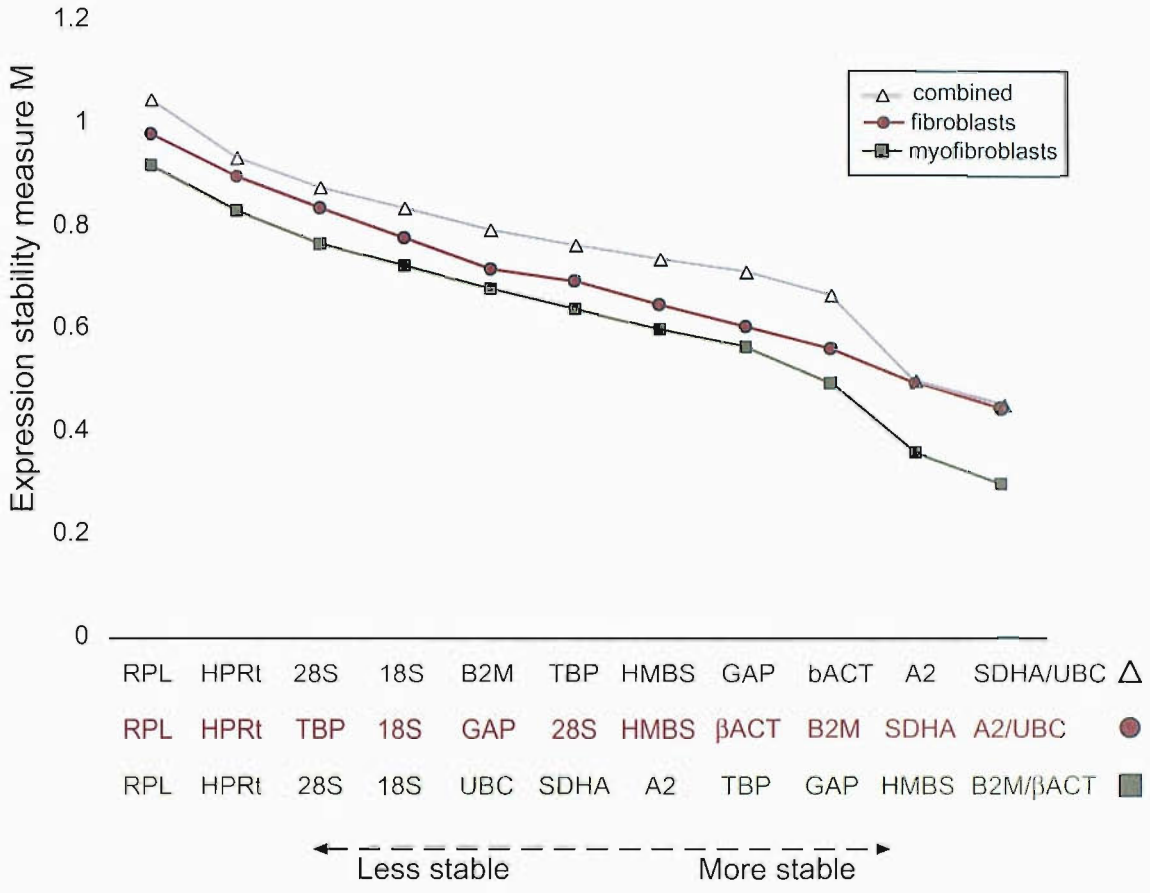


Fig. 3.1 The stability of housekeeping genes was measured in fibroblasts, myofibroblasts and in a combined data set using geNorm software. The M value is calculated based on the changing ratios of genes in the analysis as previously described (ref), such that lower M values indicate more stably expressed genes and an M value <0.5 indicates a relatively stable control gene.

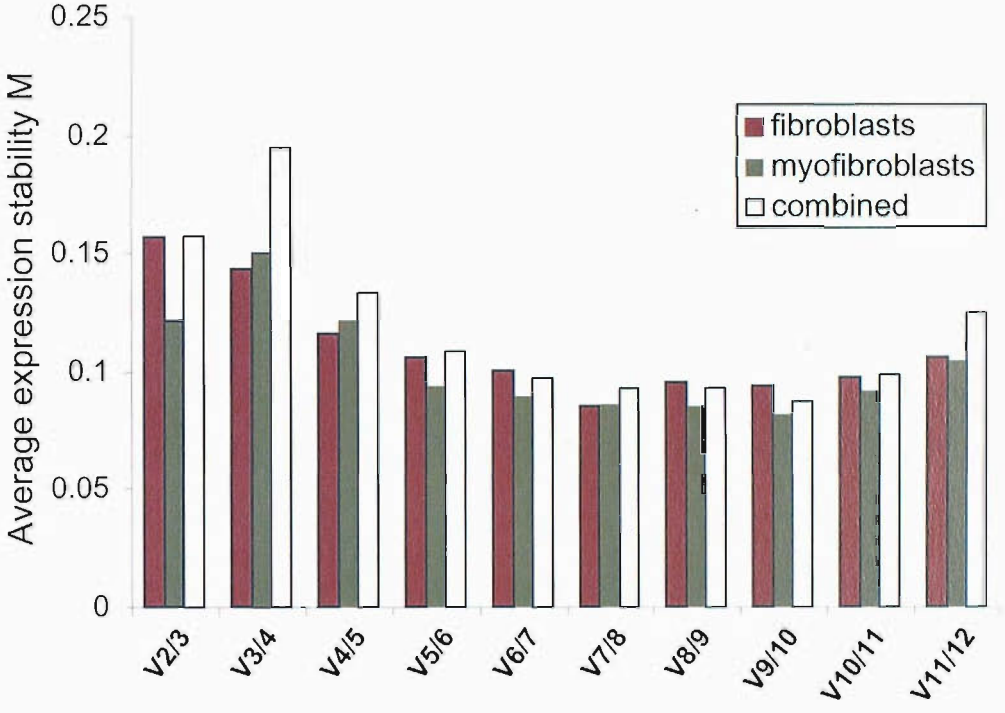


Fig.3.2 Variations in the normalising signal (NF) derived using different numbers of control genes were calculated using geNorm. Control genes are added to the analysis in the order of decreasing stability as determined above and a change in NF with n genes compared to NF using n+1 genes. Changes in NF value less than 0.15 are considered ideal for accurate normalisation.

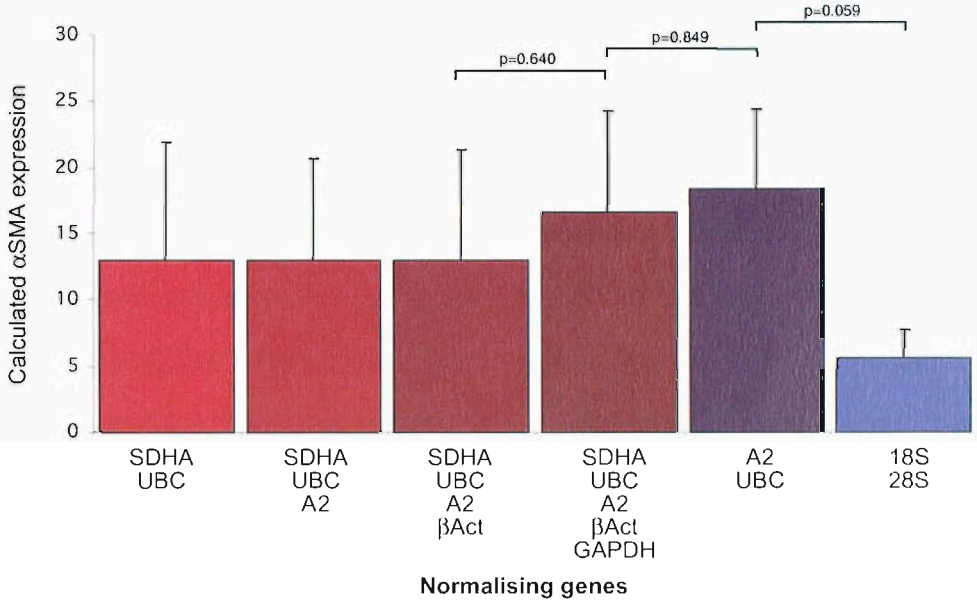


Fig 3.3 To further validate the geNorm analysis, the fold induction of αSMA was measured in four fibroblasts and normalised using different numbers of control genes. Control genes were added to the analysis in decreasing order of stability left to right and the geometric mean of n and n+1 genes used to calculate the normalisation factor. The geometric mean of 18S and 28, which were less stable normalising genes, were also used to calculate the fold induction and gave a contrasting result. The geometric mean of A2 and UBC was include in this analysis since these are two of the best control genes in the combined analysis (fig 3.1) and are also available in a multiplex format. Normalisation with the geometic mean of A2 and UBC gave a similar fold induction to that derived using the geometric mean of the five most stable normalising genes.

αSMA gene induction normalised to differing normalising controls

To demonstrate the validity of the geNorm findings, αSMA induction was measured at 48 hours post treatment with 0.04nM TGFβ relative to time zero with a stepwise inclusion of the top five housekeeping genes according to the geNorm analysis (Fig 3.3). The same data were processed using the geometric mean of two of the less stable genes 18S RNA and 28S RNA. Stepwise inclusion of the top five housekeeping genes gave very little variation in quantification. By contrast the 18S/28S combination still showed an induction of αSMA but the increase was much lower. Although in this small sample, the difference in the calculated αSMA expression was not significantly lower using the less highly ranked normalising genes (p=0.059), the mean expression was considerable less (5-fold induction using 18S/28S versus 17 fold induction using A2/UBC). The geometric mean of A2 and UBC were included in this experiment as this combination of housekeeping genes is available in a multiplex format which is highly desirable when working with limited amounts of clinical material. A2 and UBC have a similar level of expression making them well suited for multiplex detection. The A2 and UBC multiplex gave very similar results to the geometric mean of the best 5 genes and were selected as the normalising control for subsequent studies.

"Smooth muscle cell marker" gene expression at baseline.

In order to determine the extent to which TGFβ is able to induce a smooth-muscle phenotype in primary airway fibroblasts the expression of a number of "smooth muscle cell marker" genes was examined by real-time qPCR. The markers were chosen to include genes known to be upregulated in airway fibroblasts in response to TGFβ (αSMA is the classical marker of the myofibroblast phenotype) as well as markers of a highly differentiated contractile smooth muscle phenotype (e.g Desmin, γActin) .

In order to determine the level of mRNA expression of smooth muscle cell markers in fibroblast cultures, the detection of target genes was quantified using a ΔC_T measure relative to the geometric mean of UBC and A2. Since these genes are known to be highly abundant constitutively expressed house keeping genes, expression relative to these controls gives some indication of the overall expression level of target genes (Fig 3.4). αSMA is very highly expressed at the mRNA level in fibroblasts cultures having a ratio of approximately 1:1 relative to the geometric mean of UBC and A2. Calponin 1 and γActin are also expressed relatively abundantly whilst both HC myosin and Desmin showed very low basal levels of transcription. No difference in the baseline expression levels of any "smooth muscle marker" gene was detected between asthmatic or healthy control cells.

Time course for regulation of "smooth muscle cell marker" gene expression by TGFβ

Expression levels of all five "smooth muscle marker" genes increased significantly from baseline by 24 hours when treated with TGFβ (Fig 3.5-3.9). Further changes in gene expression levels over the next 24-hour period were dependent upon TGFβ dose, disease origin of the cells, and the target gene.

"Smooth muscle cell marker" gene expression TGFβ dose response.

Induction of expression of all five "smooth muscle marker" genes was shown to be TGFβ dose dependent (Fig 3.5-3.9). A dose of 0.04nM TGFβ was sufficient to cause maximal induction of αSMA and γActin expression in asthmatic fibroblast cultures (Fig 3.5b and 3.9b). An increase in dose caused further increases in αSMA and γActin expression however, in healthy control fibroblasts (Fig 3.5a and 3.9a). Expression of HCM, Calp1and Desmin continued to increase with increasing TGFβ dose in all cultures (Fig 3.6-3.8).

The lowest dose of TGFβ did not cause an induction of any of the genes significantly different from the untreated controls although there was a trend for a this low dose to cause an increase in gene expression by 24 hours that had diminished by 48 hours. Immunofluorescent staining of a representative cultures of healthy fibroblasts (Fig 3.12) shows TGFβ treatment to induce αSMA protein in a dose dependent manner.

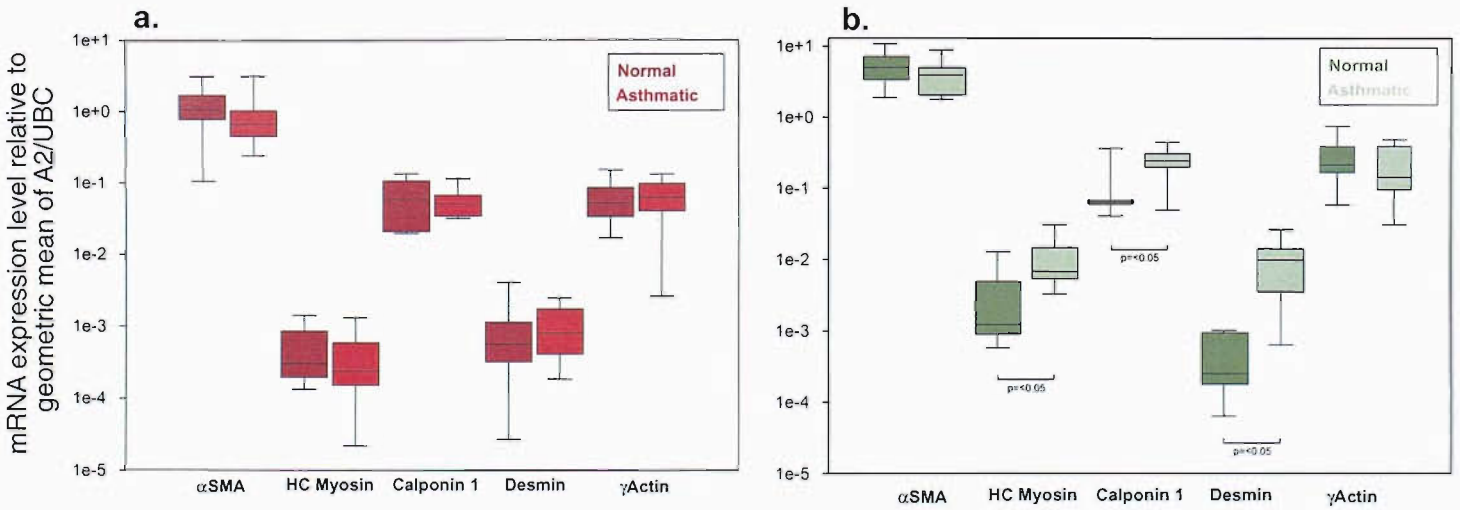


Fig 3.4 (a.) The steady state levels of mRNA expression of smooth muscle cells markers were measured in both asthmatic and healthy control untreated fibroblasts and expressed relative to the geometric mean of the highly abundant and constitute expressed genes A2 and UBC. αSMA was expressed at an equivalent level (ratio of 1:1) whilst HC myosin and desmin mRNA were very rare transcripts. Calponin and γactin were moderately highly expressed approximately 10 fold less that A2/UBC. Data were analysed using a Mann Whitney U test. (b.) The TGFβ-induced mRNA expression of smooth muscle-related transcripts was measured in both asthmatic and healthy control fibroblasts and expressed relative to the geometric mean of the highly abundant and constitute expressed genes A2 and UBC. Data were analysed using a Mann Whitney U test.

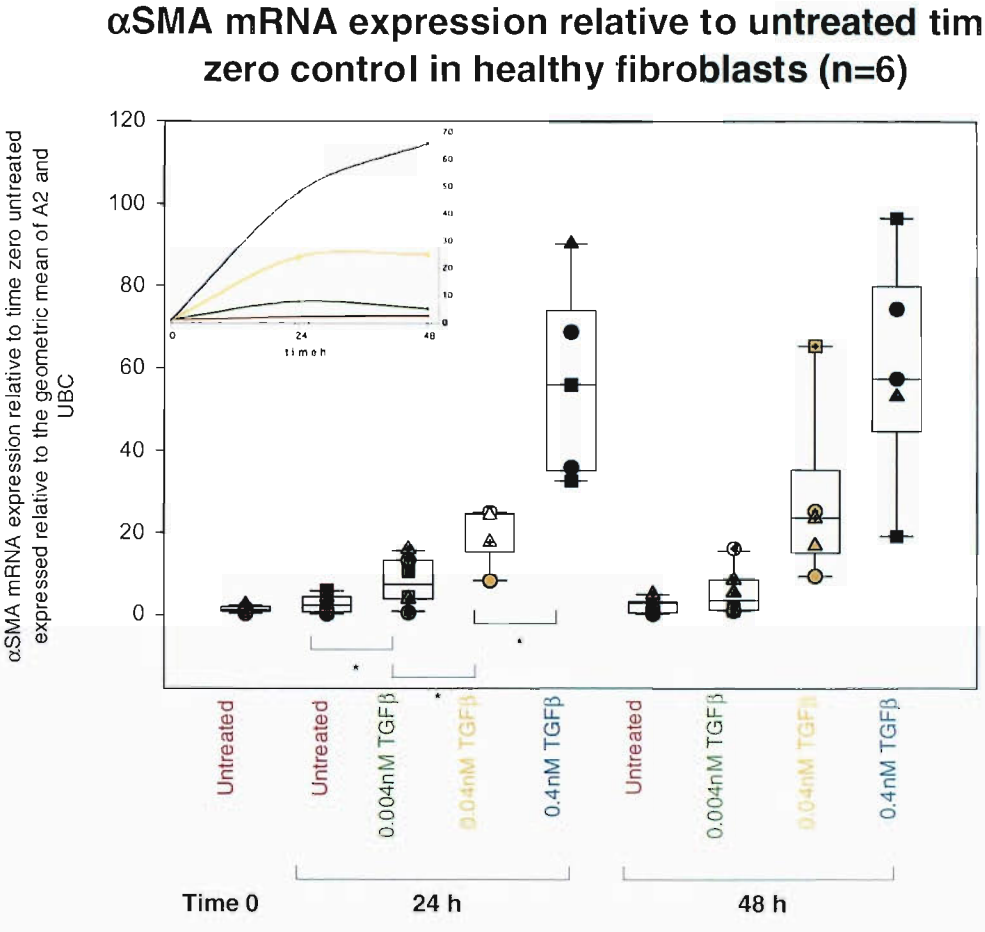


Fig 3.5a αSMA mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median αSMA expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

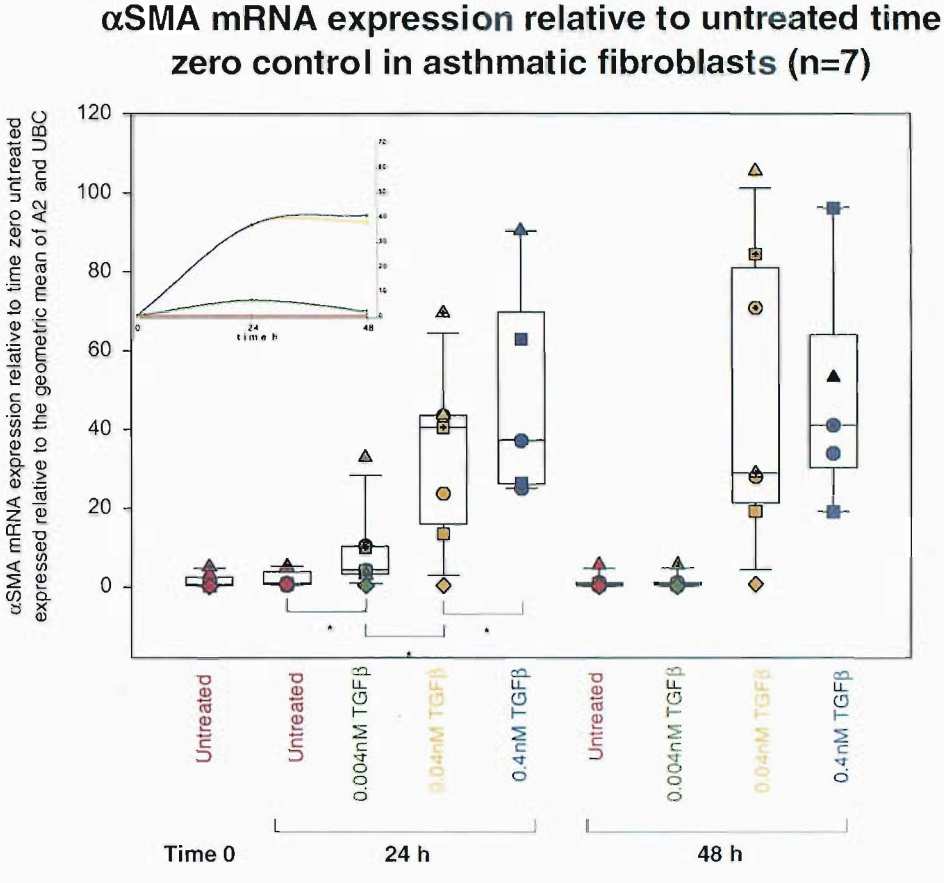


Fig 3.5b αSMA mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median αSMA expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

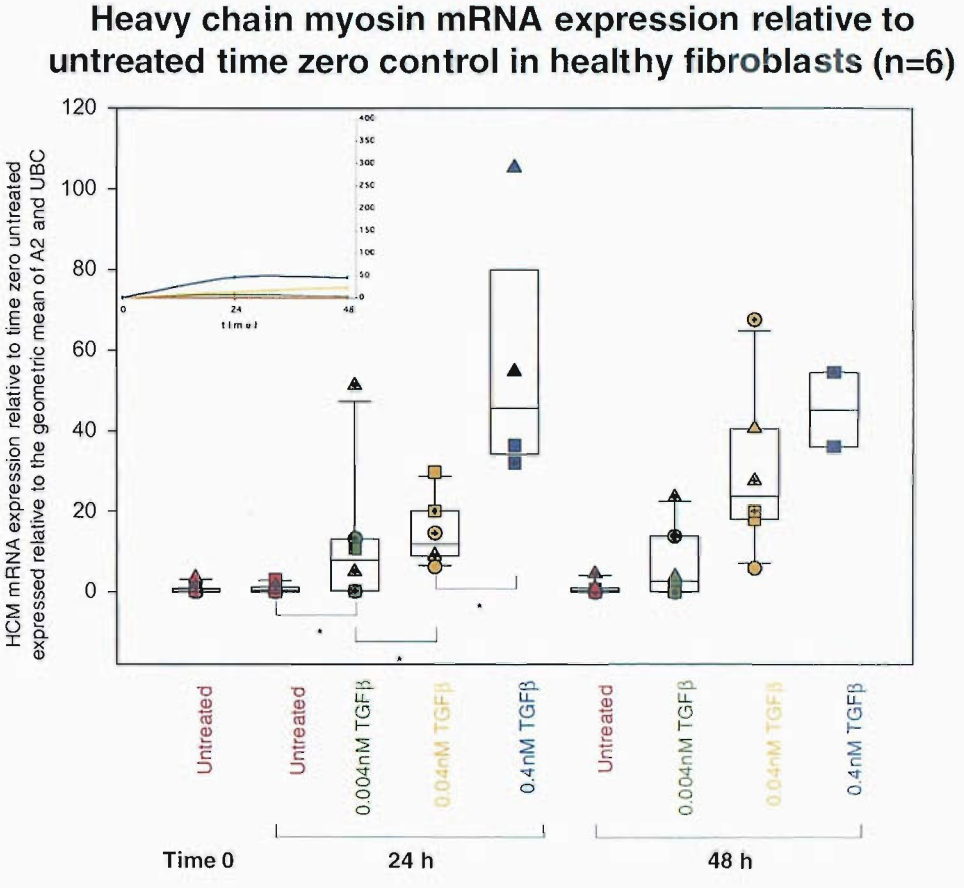


Fig 3.6a HCM mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median HCM expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

Heavy chain myosin mRNA expression relative to untreated time zero control in asthmatic fibroblasts (n=7)

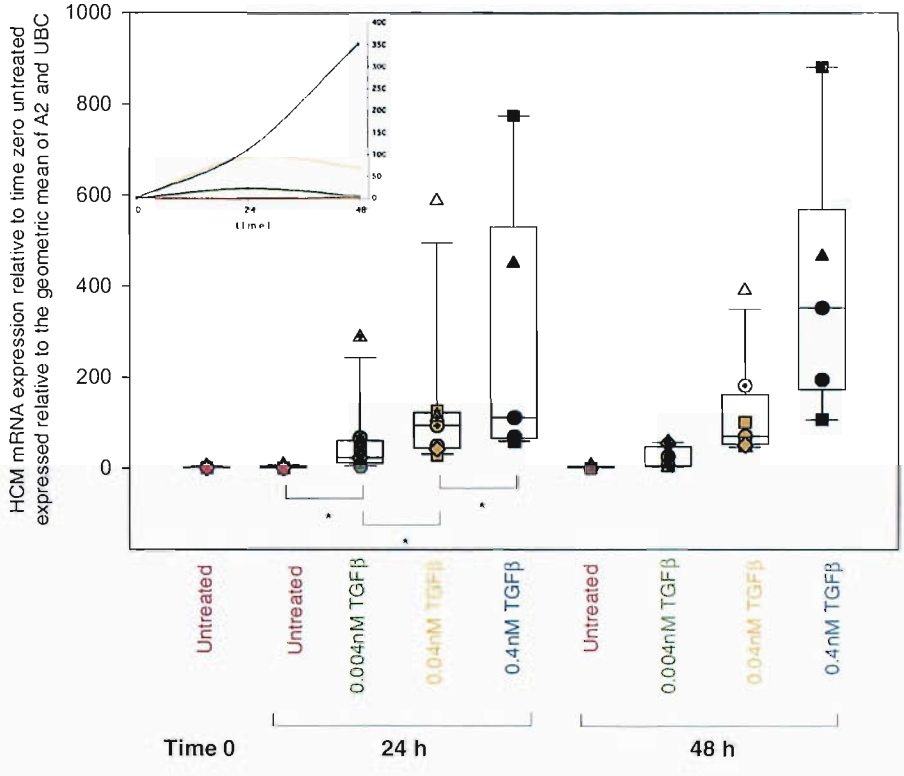


Fig 3.6b HCM mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median HCM expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

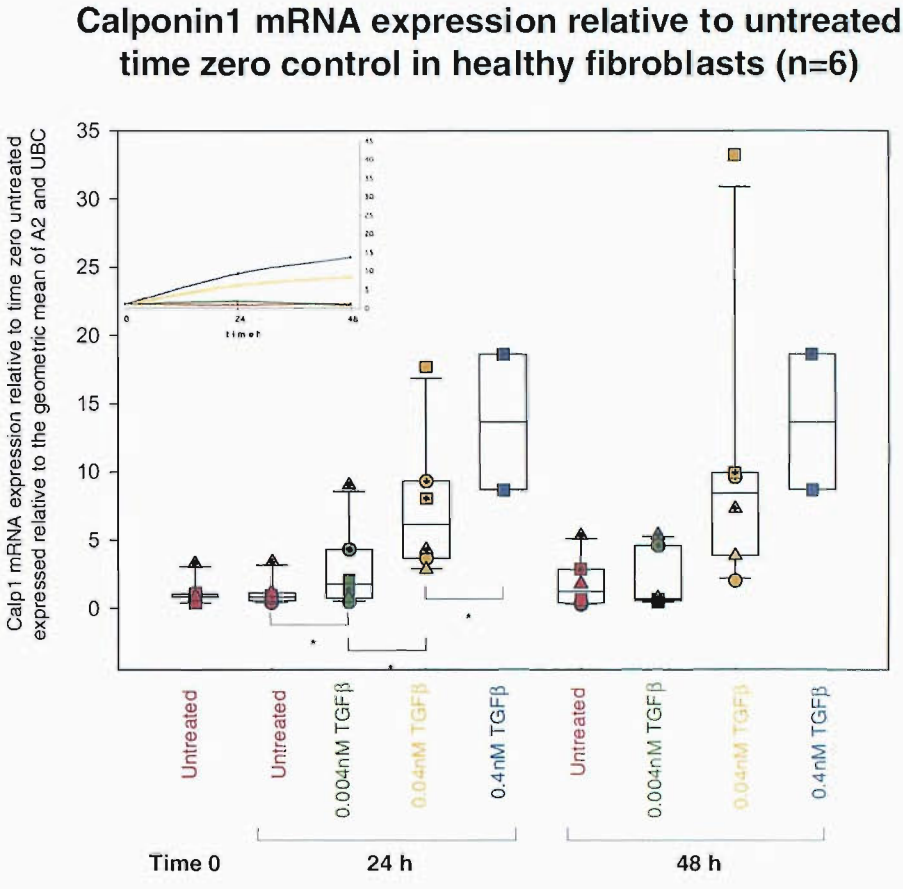


Fig 3.7a Calponin1 mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median Calponin1 expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

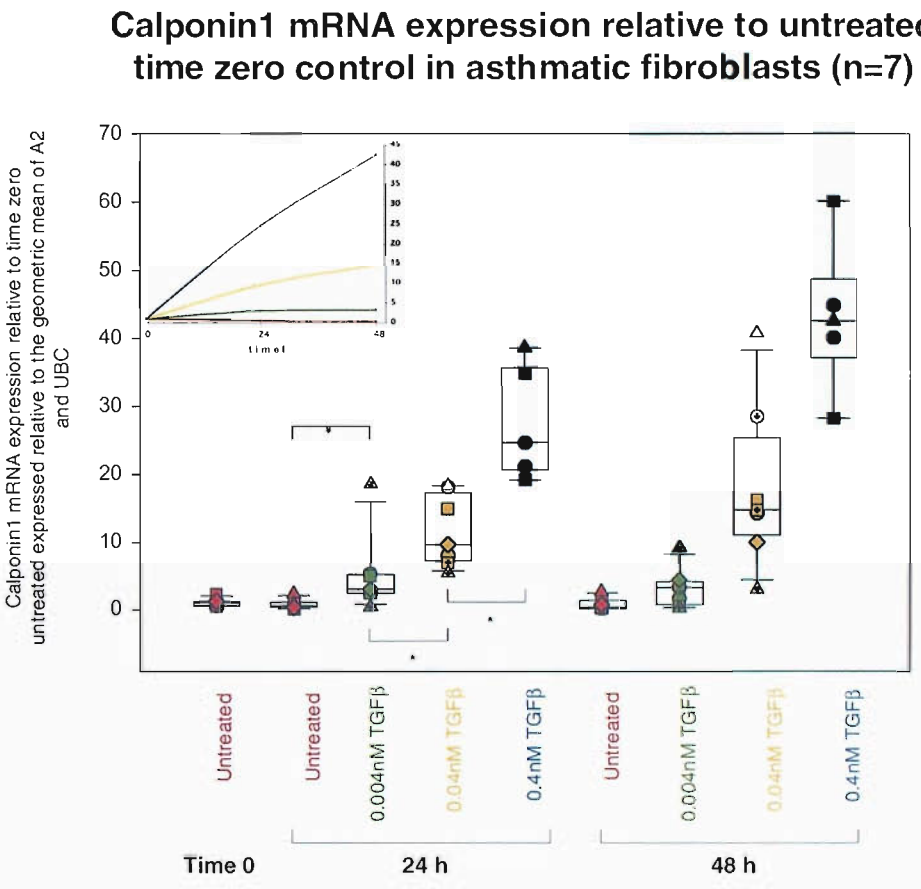


Fig 3.7b Calponin1 mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median Calponin1 expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

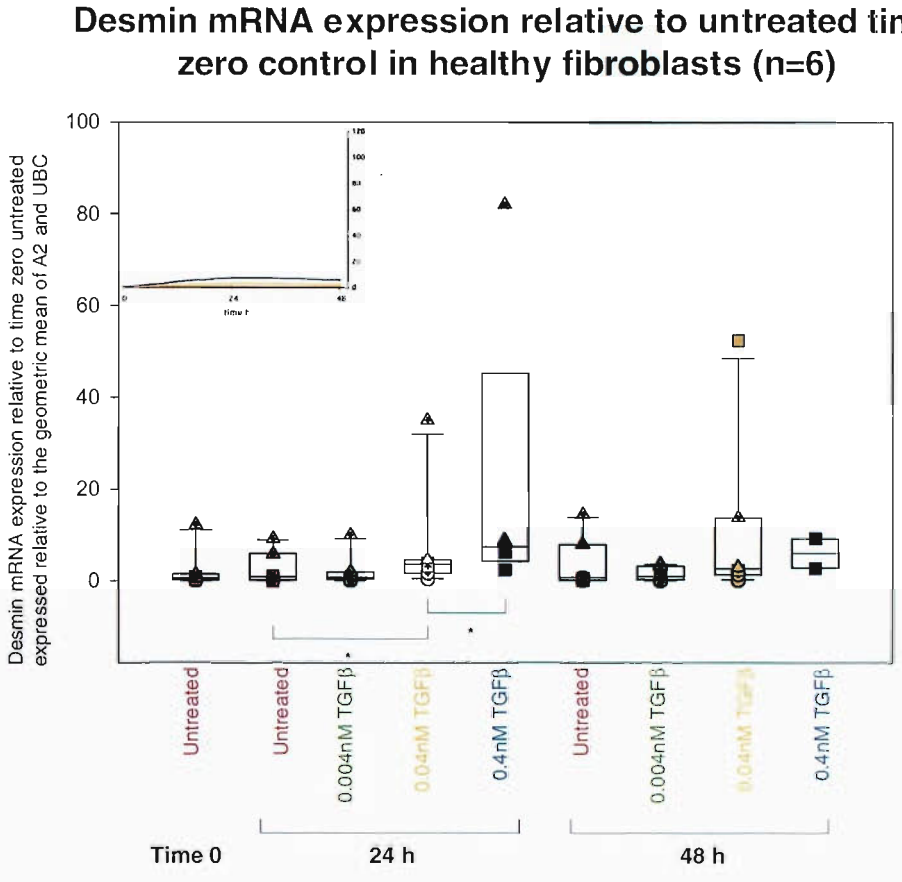


Fig 3.8a Desmin mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median desmin expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

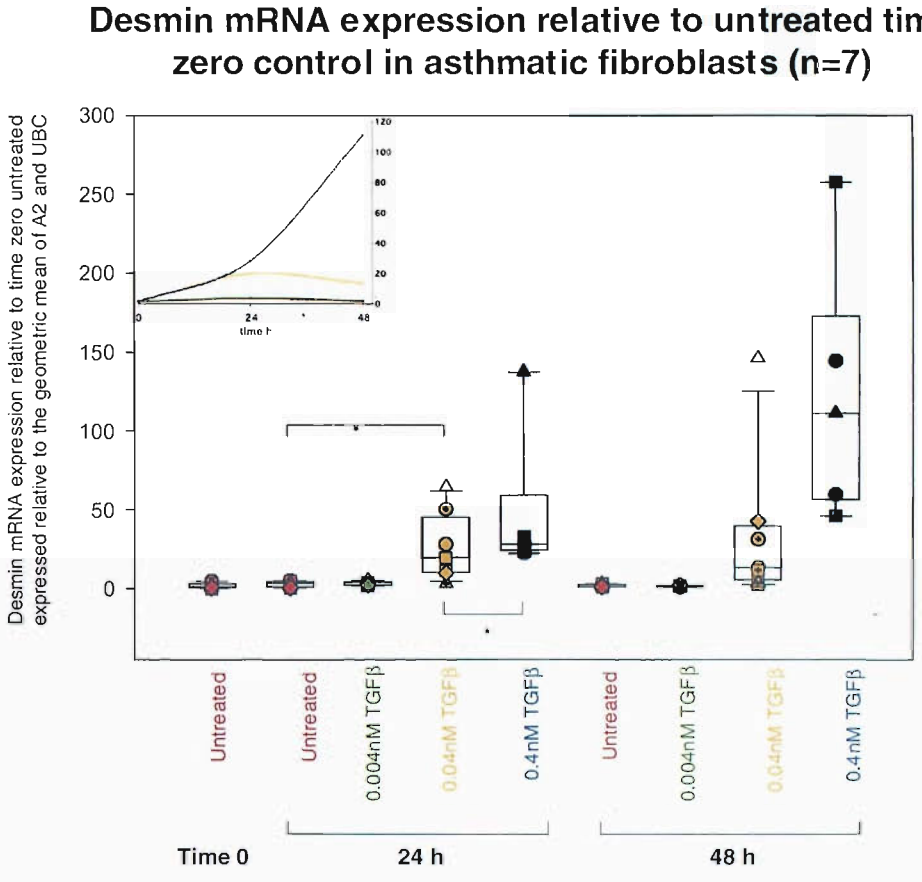


Fig 3.8b Desmin mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median desmin expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

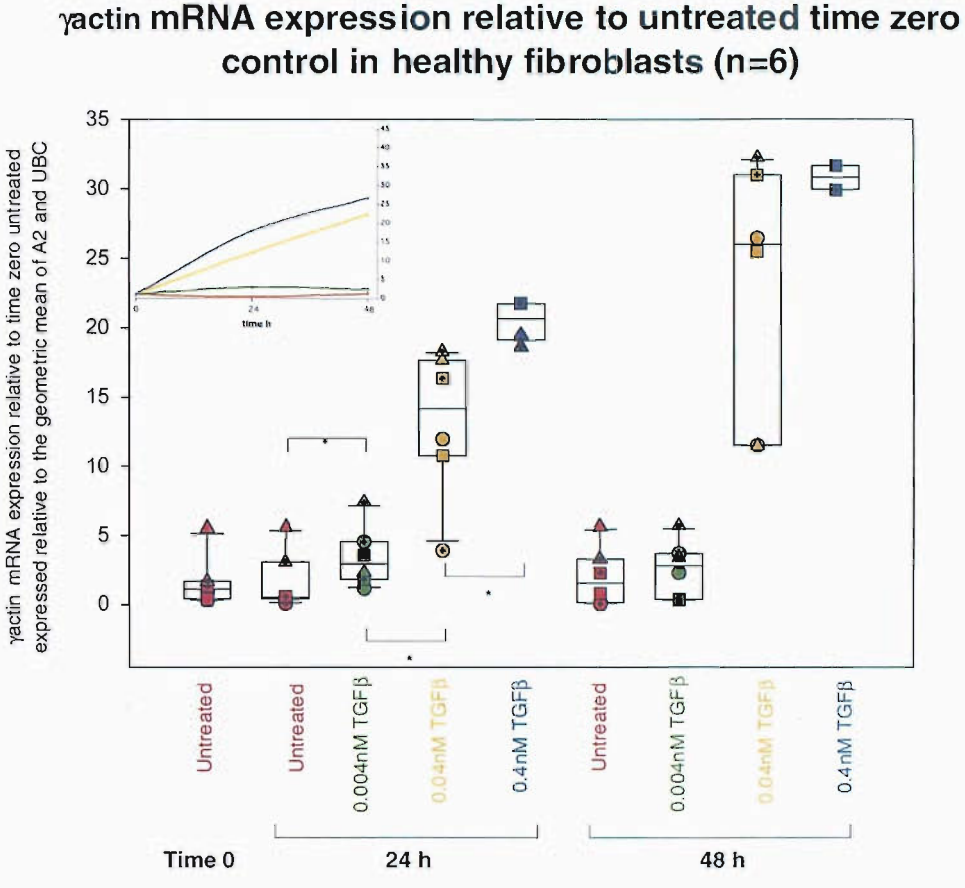


Fig 3.9a γactin mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median γactin expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

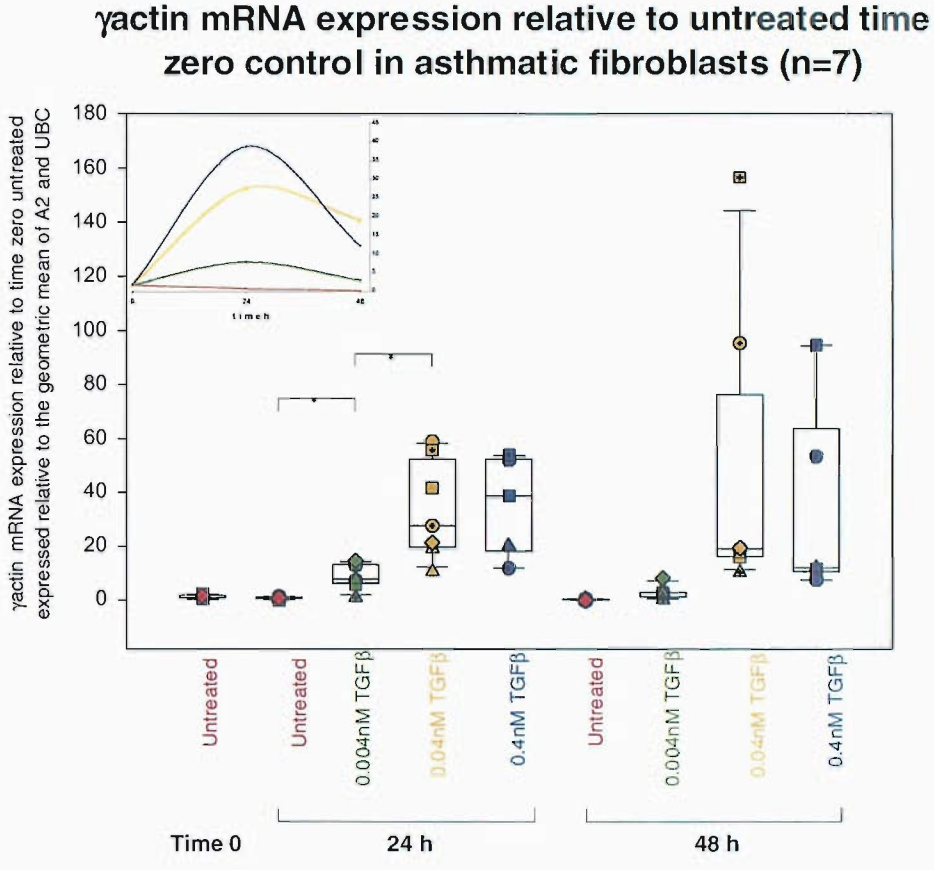


Fig 3.9b γactin mRNA expression was measured by RT-qPCR relative to untreated time zero control. Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with TGFβ 0.004nM (green), TGFβ 0.04nM (yellow) or 0.4nM TGFβ (blue) for 0, 24 or 48 hours.

Inset: Median γactin expression over time course at four different concentrations of TGFβ.

Asterisk indicates statistical significance of $p<0.05$. Friedmanns test was used.

"Smooth muscle cell marker" gene expression; Asthma vs Normals.

Graphically, comparison of asthma vs. normal gene expression is facilitated by examining the inset line graph on Figs 3.4-3.9 in which identical y-axis scaling exists between healthy and diseased-cell data.

A number of disease-related differences in "smooth muscle marker" gene expression were identified (Fig 3.4-3.9);

αSMA and γActin gene expression were maximally induced at 0.04nM TGFβ in asthmatic fibroblast cultures (Fig 3.5b and 3.9b) whilst an increase in TGFβ dose continued to induce further expression of these genes in healthy control fibroblasts (Fig 3.5a and 3.9a).

The expression of HCM, calponin 1, and desmin mRNA was induced to a significantly greater extent in asthmatic fibroblast cultures than in normal control cultures ($p<0.05$ in all cases) when treated with either 0.04nM or 0.4nM TGFβ for 48 hours (Fig 3.6-3.8). γActin mRNA expression was induced to a significantly greater extent ($p<0.05$) by 0.04nM TGFβ treatment for 24 hours in asthmatic fibroblast cultures than in healthy controls (Fig 3.9).

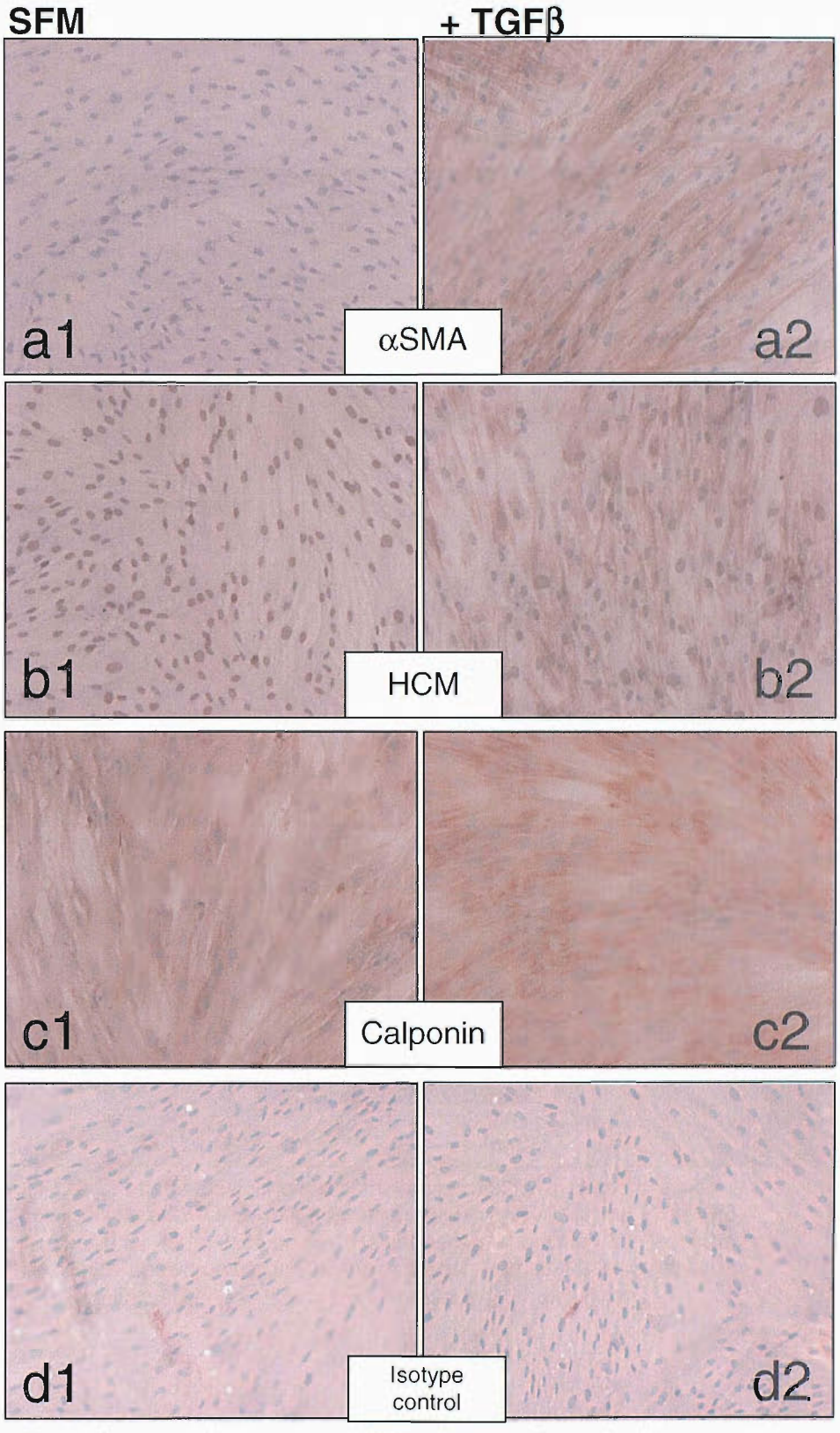


Fig 3.10 Immunohistochemical staining of primary airway fibroblasts grown in serum free medium with (**a2-d2**) or without (**a1-d1**) TGFβ (0.4nM) for 48hrs. **a**. αSmooth muscle actin **b**. heavy chain myosin **c**.calponin **d**.isotype match control. Representative of 6 repeats. No asthma vs healthy were detectable. mag x20.

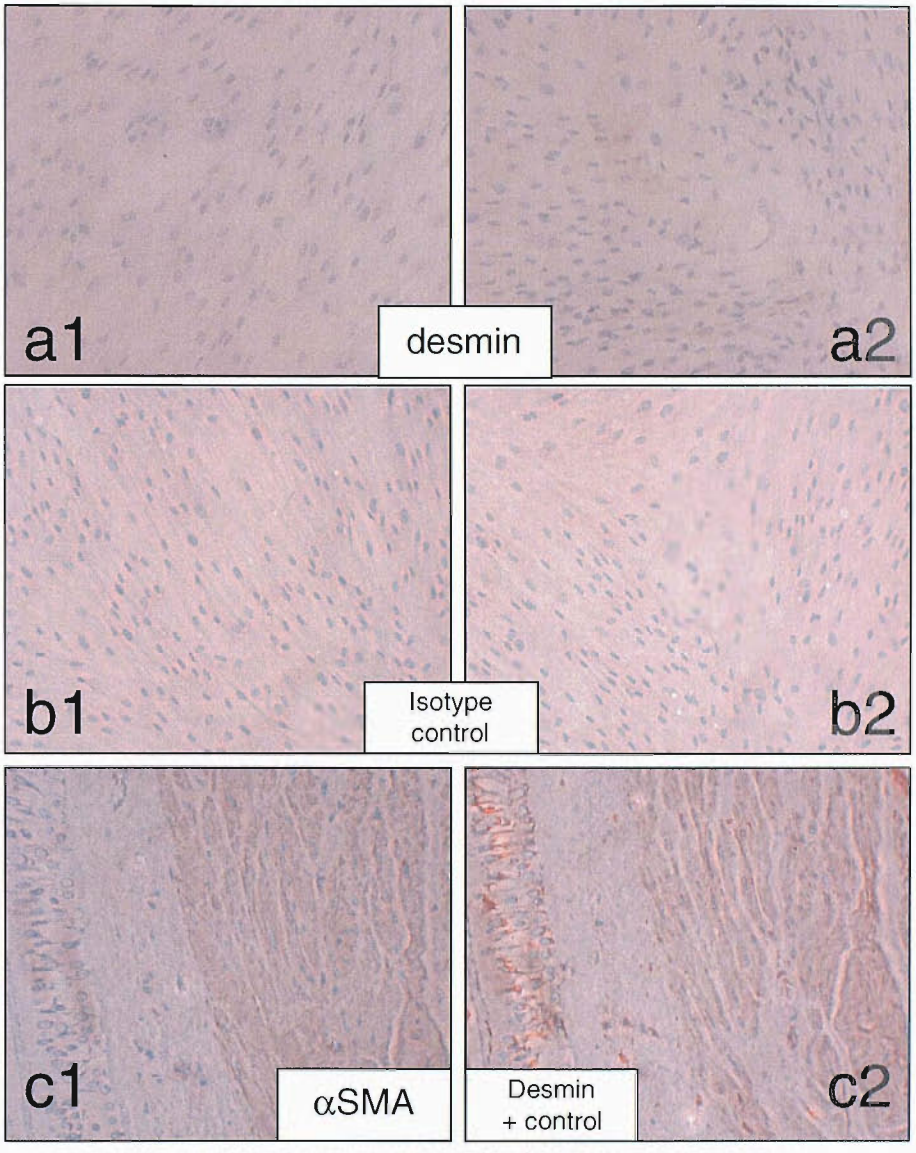


Fig 3.11 Immunohistochemical staining for desmin in primary airway fibroblasts grown in serum free medium with (**a2**) or without (**a1**)TGFβ (0.4nM) for 48hrs. Isotype match controls are shown (**b1-b2**) Effectivity of antibody shown on bronchial biopsy section (**c2**). αSMA staining in a sequential section to identify smooth muscle is shown (**c1**).

Fig 3.12 Primary human airway fibroblasts immunofluorescently stained for αSMA. Nuclei are counterstained with 7-AAD. Confluent cultures were treated for 48hours with a. no treatment b. 0.04nM TGFβ, c. 0.2nM TGFβ, d. 0.4nM TGFβ.

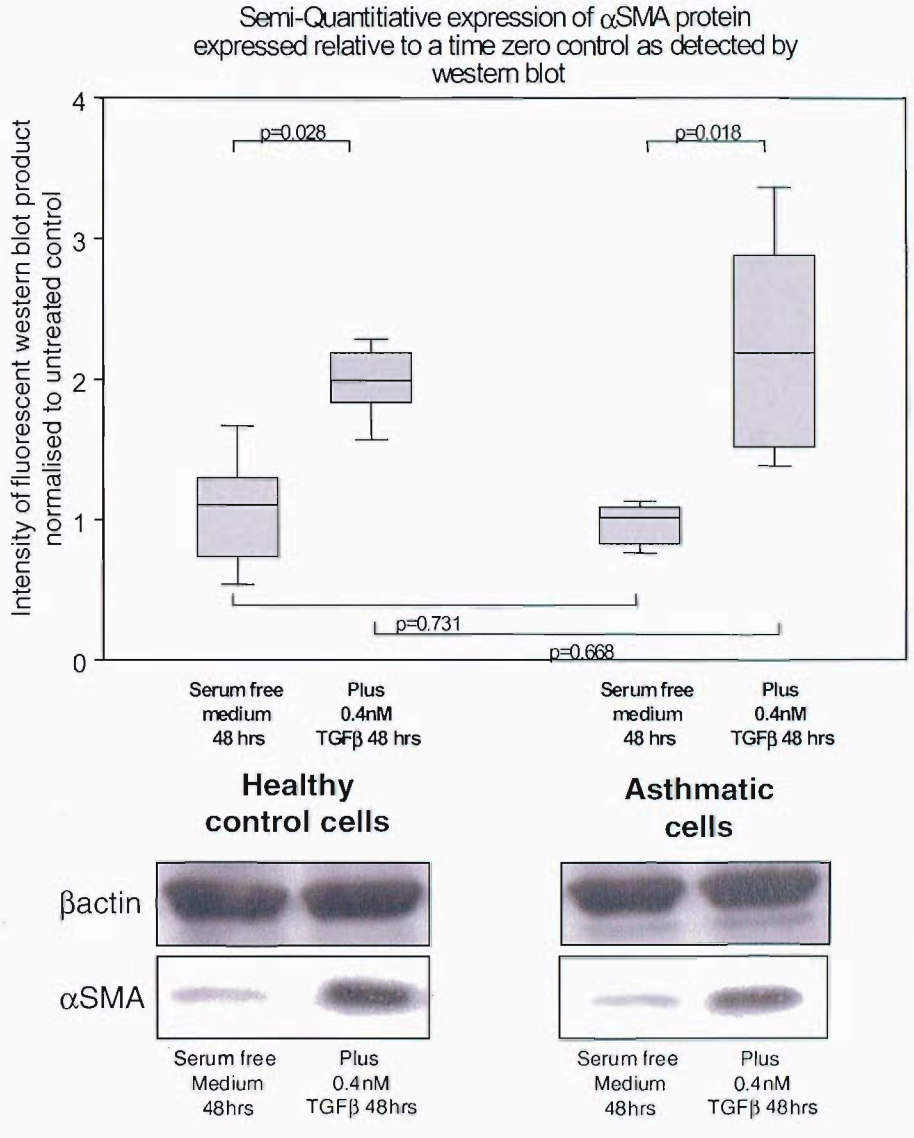


Fig 3.13 Healthy control (n=9) and asthmatic fibroblast (n=9) cultures were untreated or treated with TGFβ 0.4nM for 48 hours. Cell lysates were subject to SDS-page and probed with anti-αSMA antibodies for Western blot analysis (representative western blots shown. βActin is loading control). The intensity of the fluorescent product was then quantified using geneTools software. The investigator was blinded to the identity of the samples.

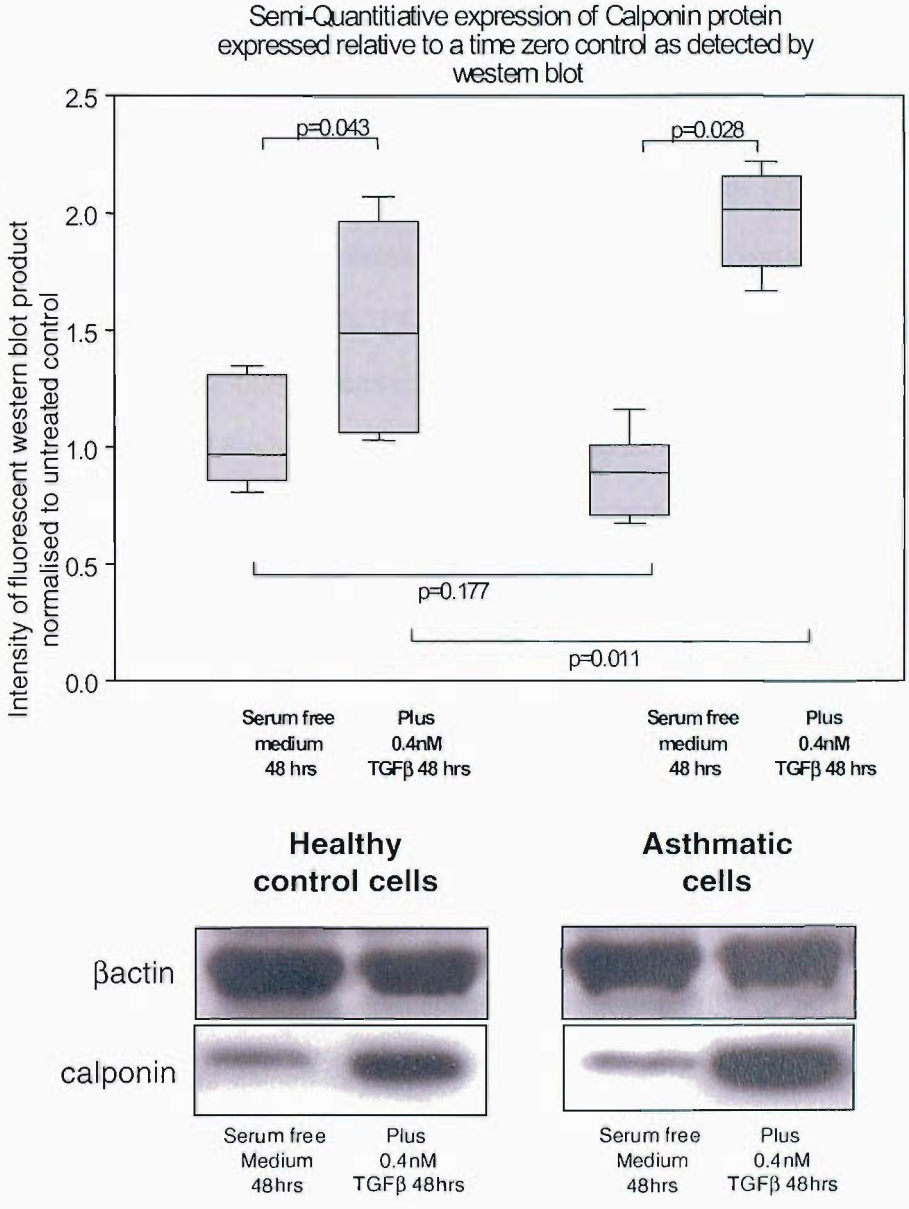


Fig 3.14 Healthy control (n=9) and asthmatic fibroblast (n=9) cultures were untreated or treated with TGFβ 0.4nM for 48 hours. Cell lysates were subject to SDS-page and probed with anti-calponin antibodies for Western blot analysis (representative western blots shown. βActin is loading control). The intensity of the fluorescent product was then quantified using geneTools software. The investigator was blinded to the identity of the samples.

"Smooth muscle cell marker" protein expression.

To determine if the upregulation of mRNA for each of the "smooth muscle marker genes" resulted in a corresponding upregulation in protein, primary airway fibroblasts treated with or without TGFβ (0.4nM) for 48 hours were immunohistochemically probed.

Protein staining for αSMA was absent in >99% of untreated cells (Fig 3.10a1). Within 0.4nM TGFβ treated fibroblasts defined filamentous staining was present within >90% of cells (Fig 3.10a2). HC myosin staining was present within the nuclei of untreated cells but not cytoplasmically detected (Fig 3.10b1). Treatment with 0.4nM TGFβ induced the formation of filaments of myosin within the cytoplasm of >80% of cells (Fig 3.10b2). Diffuse calponin staining was present within the cytoplasm of all untreated cells (3.10c1). Upon TGFβ stimulation the intensity of this staining increased and localised to resemble the filamentous staining of αSMA and HC Myosin (Fig 3.10c2). Desmin protein staining was absent from treated and untreated cells (Fig 3.10d).

In all cases isotype control antibodies produced no staining (Fig 3.10 and 3.11). Although no desmin protein was detected in either treated or untreated fibroblast cultures, the reactivity of the anti-desmin antibody was confirmed by immunohistochemical staining of bronchial biopsy section (Fig 3.11 c2). A sequential section of biopsy was stained for αSMA to identify smooth muscle within the section (Fig 3.11 c1)

"Smooth muscle cell marker" semi-quantitative protein expression.

To quantify the level of protein induction by TGFβ in primary airway fibroblast cultures the two proteins for which suitable antibodies were available (anti-αSMA and anti-calponin) were studied by western blot analysis. Semi-quantitative analysis of

the expression levels was achieved by measuring the intensity of the chemiluminescent product, which was quantified using the Genetools software in conjunction with a geneGnome scanner. β-Actin was used as a loading control for each sample. The investigator was blinded to the codes during this analysis.

The intensity of the αSMA bands as detected by Western blot analysis was significantly induced in both asthmatic and healthy control fibroblast cultures ($p=0.028$ and 0.018 respectively) upon treatment with 0.4nM TGFβ for 48 hours. Consistent with the mRNA data, there was no difference in the fold increase in band intensity upon TGFβ stimulation between asthmatic and healthy control cells (Fig 3.13).

Similarly the intensity of the calponin bands (Fig 3.14) was significantly increased in asthmatic and healthy control fibroblast cultures ($p=0.028$ and 0.043 respectively) following treatment with 0.4nM TGFβ. However the fold increase in calponin was significantly greater in asthmatic fibroblast cultures compared to healthy control cultures ($p=0.011$). This suggests that the observed difference in calponin mRNA expression in asthmatic fibroblasts, results in higher levels of calponin protein.

Discussion

Normalising gene selection using geNorm analysis

During the process of differentiation, a large number of genes are differentially regulated, as illustrated by recent literature showing the expression of 146 genes to be upregulated by TGFβ treatment(Chambers et al., 2003). Within such an assay it is likely that the expression of commonly used housekeeping genes will also be regulated. Thus, accurate gene expression may be hampered by inappropriate normalising gene selection. Therefore initial studies were undertaken using geNorm analysis to establish the most accurate and practically useful housekeeping genes to use in this model of fibroblast differentiation.

GeNorm analysis showed the average expression stability (M) of the control genes measured was lower in the fibroblast or myofibroblast alone populations than in the combined analysis. Although the increases in M values were only modest, this indicates that the control genes were differentially expressed following TGFβ treatment. This point is illustrated best by following individual genes as examples. B2M is ranked as the most stable gene in undifferentiated fibroblast, third in myofibroblast cultures, but drops to eighth in the combined analysis indicating that B2M is regulated during differentiation. By contrast SDHA is ranked only seventh, in terms of stability within the undifferentiated fibroblast analysis, third within myofibroblasts but is the best in the combined fibroblast/myofibroblast analysis. SDHA is therefore more stably expressed during differentiation than the other genes that it has "overtaken" in the rankings.

The geNorm analysis indicated that the optimum number of house keeping genes to measure within a combined fibroblast/myofibroblast culture would be eight. However the criteria suggested by the geNorm authors of a pairwise variation analysis between NF_n and NF_{n+1} of less than 0.15 was met by the inclusion of a fifth gene. In fact the inclusion of only the best three genes gave a pairwise variation analysis between NF_n and NF_{n+1} of 0.157. It seems therefore that the measurement of a large

number of housekeeping genes is not necessary within this setting. αSMA induction was measured following 48 hours of treatment with TGFβ when normalised to the geometric mean of an increasing number of the five best genes. In this case the inclusion of more housekeeping genes did not significantly alter the calculated induction of αSMA mRNA expression. Calculation of αSMA mRNA fold induction normalised to the geometric mean 18S rRNA and 28S rRNA, which are commonly selected controls did show αSMA induction but gave a result very different in appearance to the better housekeeping assays. From this result it is concluded that although measuring eight genes would give optimal normalisation, the use of the geometric mean of any two of the top five housekeeping genes will give excellent normalised results within this assay. Selection of housekeeping genes often reflects a balance between what is scientifically desirable with what is practically achievable. For a number of reasons the geometric mean of A2 and UBC was chosen for the remaining analysis. Firstly A2 and UBC are two of the best three genes in the combined fibroblast/myofibroblasts analysis. Furthermore, A2 and UBC have similar levels of expression making them ideal for a multiplex assay allowing simultaneous detection of both genes. This adds a further degree of accuracy by minimising any sampling differences due to pipetting error and more importantly minimises the amount of cDNA required to measure the two genes. When using precious cDNA from primary cell cultures this is of particular importance.

"Smooth muscle marker" expression in differentiating fibroblasts

Analysis of "smooth muscle marker" gene expression at mRNA and protein level gives us a revealing insight to the nature and extent of the differentiating fibroblast's switch towards a smooth muscle phenotype.

Stimulation with TGFβ significantly induced mRNA expression for all "smooth muscle markers" that were measured. However not all "smooth muscle markers" were induced at the protein level.

αSMA expression is the key marker for the differentiated (as opposed to the proto-) myofibroblast phenotype. Undifferentiated fibroblast cultures expressed a very high level of αSMA mRNA, almost identical to the highly abundant housekeeping genes used for normalisation. However the αSMA protein is present within only a minute proportion of these untreated cells. Upon stimulation with TGFβ αSMA mRNA expression increases further. Subsequently the protein expression is vastly induced. These data suggest that undifferentiated fibroblasts are primed for myofibroblast differentiation due to the presence of a large pool of αSMA mRNA. Upon appropriate stimulation with TGFβ, fibroblasts quickly differentiate to a proto-myofibroblast phenotype, by the translation of existing transcript and the simultaneous induction of nascent mRNA.

A morphological change in the fibroblasts occurs alongside this upregulation of αSMA expression. The cells begin to "line up" with one another and form swathes. These cells may be forming a contractile unit, similar to smooth muscle bundles seen in the airways.

The responses of HC myosin and calponin to TGFβ stimulation are more conventional. HC myosin mRNA and protein expression were both very low in fibroblasts and were both significantly induced during differentiation. Calponin expression at a mRNA and protein level was relatively high in untreated fibroblasts and during differentiation mRNA and protein expression increased further. However, the distribution of protein changed from diffuse cytoplasmic staining to a filamentous staining pattern, which is likely due to the recruitment of calponin in to the rapidly assembling contractile apparatus within the cell. HC myosin mRNA and protein are both good indicators of the myofibroblast phenotype, whilst calponin is widely expressed prior to TGFβ treatment and is therefore a poor marker of differentiation. Desmin is a key intermediate filament found in contractile smooth muscle cells. Desmin mRNA expression is very low in untreated fibroblasts but increased

significantly upon TGFβ stimulation. However there was no induction of desmin protein within the cells. γActin mRNA was relatively abundant within untreated cultures and was further induced upon stimulation with TGFβ. No antibody to γActin is currently commercially available so no protein data for this molecule is available. However γActin is generally considered an excellent marker for fully differentiated smooth muscle cells(Sawtell and Lessard, 1989, Qian et al., 1996) and is unlikely to be present in these myofibroblast cultures.

Overall, TGFβ treatment of primary airway fibroblasts did not achieve a complete phenotypic switch to a differentiated smooth muscle cell proven by the absence of essential protein markers. However mRNA pools exist for such markers but appear not to be translated. The absence of desmin protein, for example, but an abundance of mRNA in activated fibroblasts is analogous to the large pool of untranslated αSMA mRNA that is present in untreated fibroblasts. It is possible therefore that fibroblasts are primed for phenotypic differentiation into myofibroblasts and this may be achieved by TGFβ treatment. Likewise myofibroblasts are primed for further differentiation but lack the appropriate stimulation for the translation to a *bone fide* differentiated smooth muscle phenotype.

This work highlights the fact that an altered expression of mRNA does not always correlate with an altered expression of protein. This was the case, for example, with desmin. Therefore in validating the significance of these disease-related differences in mRNA expression for some markers of smooth muscle differentiation it is important that these differences translate to a difference in protein expression also. This was demonstrated by the observation that calponin expression as analysed by western blot was induced to a significantly greater extent in asthmatic fibroblast cultures than in healthy control cells.

Serum deprivation and mechanical stretch have both been shown to be an important differentiating stimuli in smooth muscle cells(Camoretti-Mercado et al., 2000, Yang et

al., 2000) by altering cellular distribution of SRF. It would therefore be useful to investigate the effect of serum deprivation and/or stretch on the differentiation of fibroblasts and potential further differentiation of myofibroblasts. For example it is possible that either of these stimuli may cause the translation of desmin mRNA which accumulates upon stimulation of primary airway fibroblasts with TGFβ.

What relevance for asthma?

The TGFβ dose dependency of the induction of the differentiated myofibroblast phenotype emphasises the importance of the role of TGFβ in the asthmatic airways. It is known that TGFβ levels are increased in the asthmatic airway(Redington et al., 1997). These data show that more TGFβ within the airways is likely to lead to a greater number of myofibroblasts, each expressing more αSMA, HC myosin, and calponin1 protein, each therefore with a greater contractile potential.

Assuming the discussed model whereby fibroblasts are primed for phenotypic transition to smooth muscle by the accumulation of mRNA pools for markers of a highly differentiated smooth muscle cell phenotype, the disease related differences in the mRNA expression of these markers may provide some insight into the pathogenesis of asthma.

HC Myosin, calponin 1, and desmin gene expression were all induced by TGFβ to a significantly greater extent in asthmatic fibroblast cultures than in healthy control cultures. In some cases this induction of transcript translates functionally to a significantly greater induction of "smooth muscle marker" *protein,* as demonstrated by the greater induction of calponin expression following TGFβ stimulation in asthmatic fibroblast cultures versus healthy control cells. It seems then that asthmatic fibroblasts are "primed" by TGFβ to a greater extent than healthy fibroblasts by the transcription of larger mRNA-pools for markers of a differentiated smooth muscle phenotype. If appropriate stimulation for further smooth muscle phenotype transition exists *in vivo*, it would be fair to hypothesise that fibroblasts within the asthmatic

airway would be better primed for this transition than in a healthy subject. The sensitivity of asthmatic fibroblasts to TGFβ "priming" is emphasised further by the fact that αSMA and γActin mRNA were maximally induced by only 0.04nM TGFβ, where as healthy control fibroblasts required a greater dose to achieve maximal transcription of mRNA for these genes. Within the asthmatic airway therefore two processes may be acting in concert to promote myofibroblast differentiation. Firstly asthmatic fibroblasts will respond more sensitively by inducing maximal expression of actins α and γ mRNA with a lesser exposure to TGFβ than healthy fibroblasts. Secondly induction of mRNA for other markers of a highly differentiated fibroblast phenotype will be greater in asthmatic cells than those of a healthy individual.

A recently published study identified ADAM33 (a Disintegrin and Metalloprotease) as a gene, polymorphisms within which, are significant in an individuals susceptibility to asthma. At the time of publication this was the only such asthma susceptibility gene identified to be expressed in mesenchymal cells such as fibroblasts, myofibroblasts and smooth muscle cells. The linkage analysis associating ADAM33 with asthma was particularly strong when a phenotype of bronchial hyperresponsiveness rather than asthma alone was used. Functional studies of a similar ADAM, ADAM12, have shown the protein to be involved in the differentiation and fusion of myocytes in to contractile skeletal muscle(Lafuste et al., 2004). No functional data as to the role of ADAM33 is available, and data from this chapter indicates that the process of TGFβ induced myofibroblast differentiation towards a smooth muscle-like phenotype *in vitro* is abnormal in asthmatic cells compared to healthy controls. It is logical therefore to investigate the expression of ADAM33 in healthy and asthmatic fibroblast cultures before and during TGFβ induced differentiation to assess any potential role for ADAM33 in this process.

Summary of results and novel findings

- Of the 12 commonly used normalising control genes that were measured SDHA, UBC, A2, βActin and GAPDH are the top five most stably expressed in a differentiating fibroblast culture.

- The geometric mean of any pair of these top five genes will act as an excellent normalising control when measuring gene expression in differentiating fibroblasts.

- There is no difference at baseline in the expression of "smooth muscle marker" genes between asthmatic and healthy control fibroblast cultures.

- αSMA, HC myosin, calponin 1, desmin and γActin are all significantly upregulated at a mRNA level in primary airway fibroblast cultures by stimulation with TGFβ in a dose dependent manner.

- αSMA and γActin mRNA expression is maximally induced by a lower dose of TGFβ in asthmatic fibroblast cultures than in healthy control cultures.

- HC myosin, calponin 1, desmin and γActin mRNA expression are all increased to a greater extent in asthmatic fibroblast cultures than in healthy control cultures.

- The greater induction of smooth muscle marker gene transcripts in asthmatic fibroblast cultures versus healthy control cells subsequently translates to a greater induction of protein as illustrated by the example of calponin 1.

- Protein expression of αSMA , HC myosin and calponin1 in primary airway fibroblast cultures is induced/increased by stimulation with TGFβ.

- Desmin protein expression is not induced by treatment with TGFβ thus highlighting the fact that an increase in mRNA does not always correlate with a subsequent increase in protein.

Results Chapter 2

Results Chapter 2

Background

ADAM33 is a recently identified gene whose polymorphic variation has been shown to be highly significant in influencing an individuals susceptibility to developing asthma (Van Eerdewegh et al., 2002). However, no information about the normal functional role of ADAM33, or how this is modified in asthma, is currently available. ADAM33 expression is found only in fibroblasts, myofibroblasts, smooth muscle cells and neuronal cells within the airways, suggestive of a role in remodelling. It has therefore been hypothesised that the functional effect of polymorphism's within the ADAM33 gene will be seen within the smooth muscle cells of the airway as a cell type with potential links to bronchial hyperresponsiveness.

Studies of ADAM12, which shares homology with ADAM33, have found ADAM12 to be central to the differentiation of the myogenic precursor cell, the myocyte, in to functional skeletal muscle(Lafuste et al., 2004, Yagami-Hiromasa et al., 1995). The mechanism by which ADAM12 initiates adhesion of myocytes is $\alpha 9\beta 1$ integrin dependent. A recent study has shown ADAM33 to be capable of binding $\alpha 9\beta 1$ integrin by a similar chemistry(Bridges et al., 2004). ADAM12 expression increases upon differentiation of myocytes, so consequently I have hypothesised that as the expression of smooth muscle markers increases within TGFβ treated populations of fibroblasts as illustrated in results chapter 1, so will the expression of ADAM33.

ADAM33 is expressed as a number of splice variants(Van Eerdewegh et al., 2002, Powell et al., 2004). It is essential therefore to examine the expression of these transcripts in addition to the global expression levels of the molecule.

An increasing volume of literature illustrates the importance of the interaction of ADAM proteins and the extra cellular matrix (ECM) (Wolfsberg and White, 1996, Nath et al., 2000). It is possible that native ADAM33 expressed on the cell surface may interact with the surrounding ECM, either cleaving it or disrupting cell-matrix

interactions. The expression of such a molecule may be regulated by signals derived from extracellular sources. In addition the make-up of the surrounding extracellular matrix has been shown to be important in affecting mesenchymal cell differentiation (Johnson et al., 2001, Hayward et al., 1995, Yang et al., 2000, Scaffidi et al., 2001). If a role for ADAM33 in myofibroblast differentiation exists it is possible that ECM composition may effect ADAM33 expression.

Therefore I will examine any effects of extra cellular matrix composition upon ADAM33 expression by culturing cells upon differing extracellular matrix components.

The aims of this chapter will be to examine the overall expression of ADAM33 as well as of particular splice variants in primary airway fibroblasts, comparing asthmatic cells with healthy control cells. To examine the expression of ADAM33 globally as well as of particular splice variants over the course of myofibroblast differentiation induced by TGFβ_2 and to examine the effects of culturing fibroblasts upon differing extra cellular matrix components evaluating any subsequent effect upon ADAM33 expression.

Results

ADAM33 mRNA expression at baseline

mRNA samples from six untreated healthy control and seven untreated asthmatic subjects were analysed to determine any disease specific differences in the overall levels of ADAM33 expression. This was quantified using a TAQman® primer and probe set targeting the 3'UTR of the molecule (Fig 2.4), this region being predicted to be present in all transcripts(Powell et al., 2004).

There was no difference in the global expression of ADAM33 mRNA in untreated asthmatic fibroblasts and healthy control fibroblasts (Fig 4.1). ADAM33 mRNA expression had a ΔCt (gene of interest Ct value- normalising Ct value) of ~5. This equates to a gene expressed at around 3% of the level of the abundant housekeeping genes used to normalise gene expression. i.e. ADAM33 transcripts are not abundantly expressed but at the same time are not particularly rare .

ADAM33 mRNA expression over TGFβ time course

The expression of ADAM33 mRNA in healthy and asthmatic fibroblast cultures was measured over a 48 hour period of TGFβ treatment to assess the expression levels of ADAM33 during the process of TGFβ mediated myofibroblast differentiation.

Treatment with TGFβ caused a dose-dependent down regulation of ADAM33 mRNA expression in both asthmatic and healthy control fibroblasts compared to untreated (serum starved) conditions (Fig 4.2). This down-regulation was evident by 4 hours in healthy control fibroblast populations and reached statistical significance by 8 hours ($p=0.014$)(Fig 4.2b). The same trend was seen in asthmatic cultures although statistical significance was achieved by 24 hours ($p=0.046$)(Fig 4.2b).

Within the untreated cultures, which were in serum free medium, there was an increase in ADAM33 mRNA expression by approximately 2-fold relative to time zero. This increase reached statistical significance in asthmatic cultures by 8 hours

(p=0.014) (Fig 4.2b). Although not statistically significant the same trend was seen in the healthy control cultures (Fig 4.2a).

ADAM19 mRNA expression

The dose dependent down-regulation of ADAM33 in response to TGFβ is not typical of other ADAMs(Le Pabic et al., 2003, Fritsche et al., 2003). To illustrate this point, the expression of another ADAM, ADAM19, was measured over the same period of TGFβ treatment in healthy control cells.

ADAM19 mRNA expression was induced by treatment with TGFβ in a dose dependent manner. This induction reached significance by 8 hours of treatment with a dose of 0.04nM or 0.4nM TGFβ (p=0.011). This effect persisted, although it became less significant, over the remainder of the 48 hour time course (Fig 4.3).

ADAM33 splice variant mRNA expression at baseline

ADAM33 is known to exist at the mRNA level as a number of alternative splice forms(Powell et al., 2004, Van Eerdewegh et al., 2002). It is not known if the expression of these is differentially regulated. Therefore three assays were used to detect splice variants of ADAM33 (The βform, soluble form and all forms containing the metalloprotease domain) (Fig 4.4). These assays were validated and shown to be of equal efficiency in a recent study by Powell *et al*(Powell et al., 2004) from our own laboratory. The expression of each variant was calculated relative to the global expression levels of ADAM33 as detected by the assay targeting the 3' UTR of ADAM33. In healthy control cells the β-form was detected at around 20% the detection level of total ADAM33 transcript. The putative soluble form was detected at less than 1% whilst the metalloprotease containing transcripts were detected at less than 5% of total ADAM33 transcript level. No differences in expression levels of splice variants were detected between healthy control and asthmatic cell populations.

ADAM33 splice variant mRNA expression upon TGFβ treatment.

The expression of the three splice variants was followed over a 48 hour exposure to TGFβ at three different concentrations in order to detect any differential regulation of ADAM33 splice variant expression during the process of myofibroblast differentiation. However the expression of all three splice variants was regulated in the same manner as global ADAM33 mRNA levels. i.e. their expression was down regulated in dose dependent manner by treatment with TGFβ, whilst there was a tendency for their expression to be increased by culturing them in serum deprived conditions. There was no difference in expression of any splice variant at any dose or time point between asthmatic cells and cultures of healthy control fibroblasts.

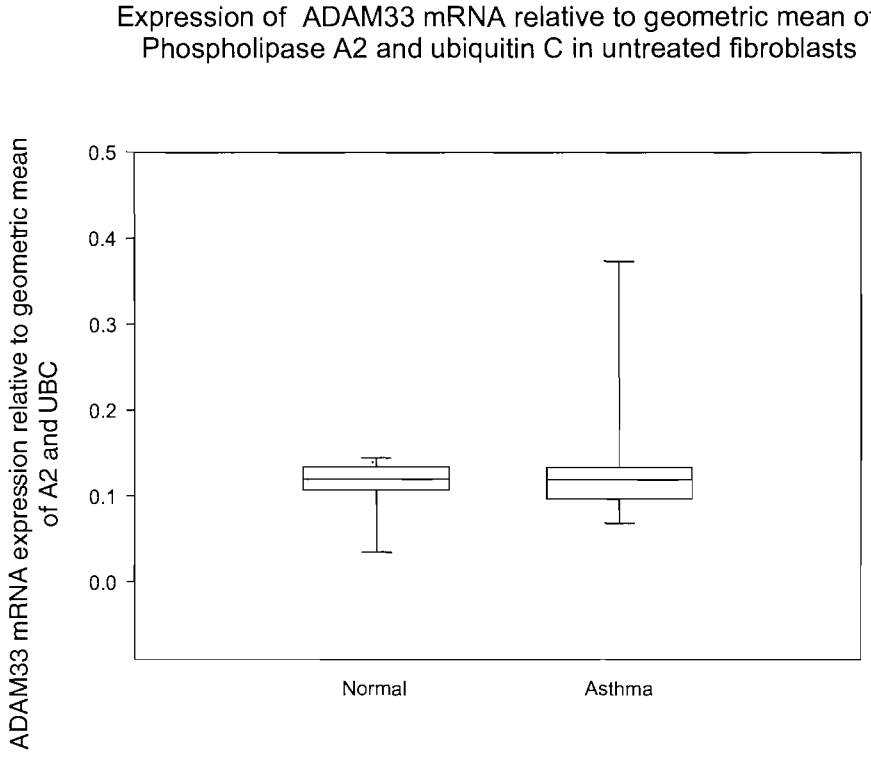


Fig 4.1 mRNA expression levels of ADAM33 was measured in untreated conditions at time-zero in asthmatic (n=7) and healthy control (n=6) primary airway fibroblast lines. mRNA expression was calculated relative to the expression of phospholipase A2 and ubiquitin C.

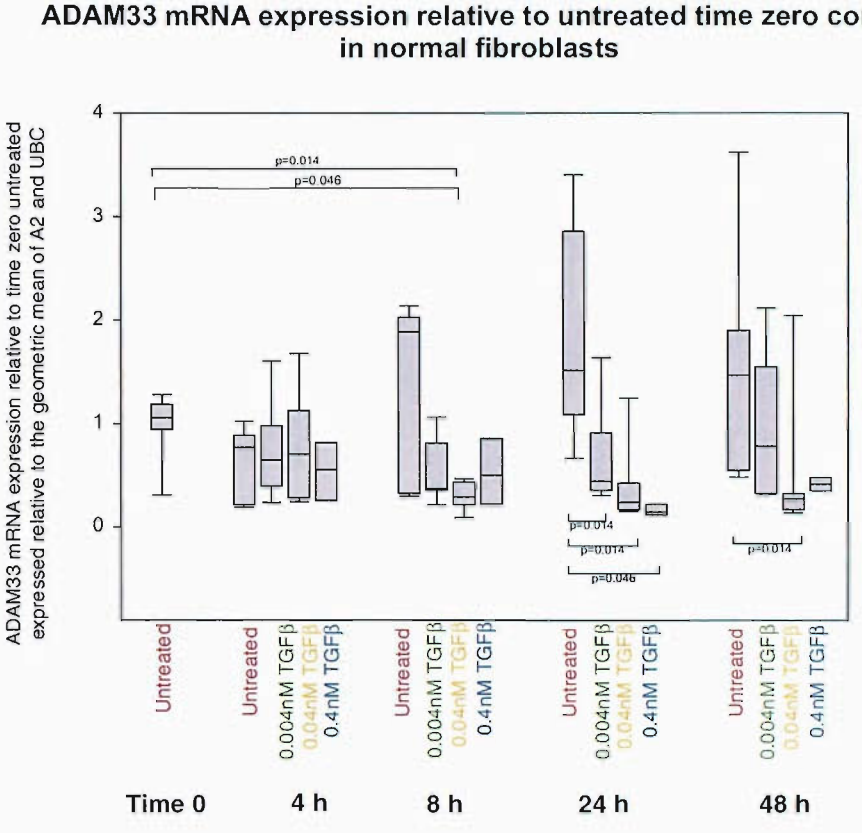


Fig 4.2a ADAM33 mRNA expression was measured by RT-qPCR relative to untreated time zero control in healthy control fibroblasts (n=6). Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with 0.004nM TGFβ (green), 0.04nM TGFβ (yellow) or 0.4nM TGFβ (blue) for 0, 4, 8, 24 or 48 hours.
p values calculated using Friedmanns test.

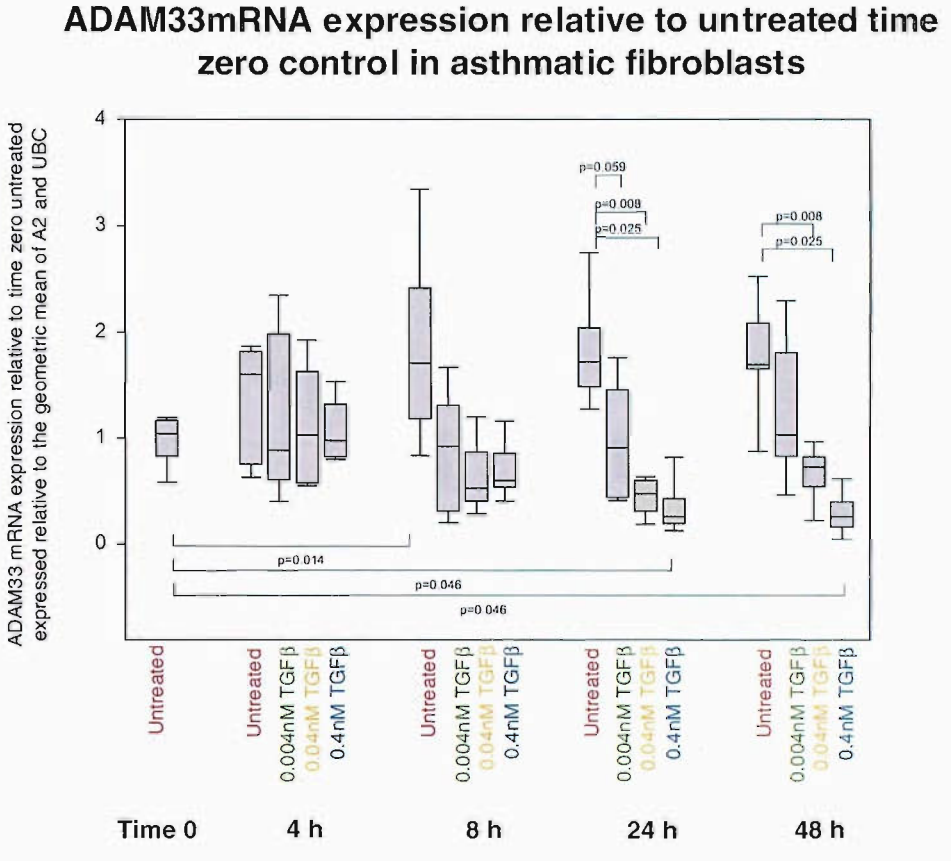


Fig 4.2b ADAM33 mRNA expression was measured by RT-qPCR relative to untreated time zero control in asthmatic fibroblasts (n=7). Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with 0.004nM TGFβ (green), 0.04nM TGFβ (yellow) or 0.4nM TGFβ (blue) for 0, 8, 24 or 48 hours.
p values calculated using Friedmanns test

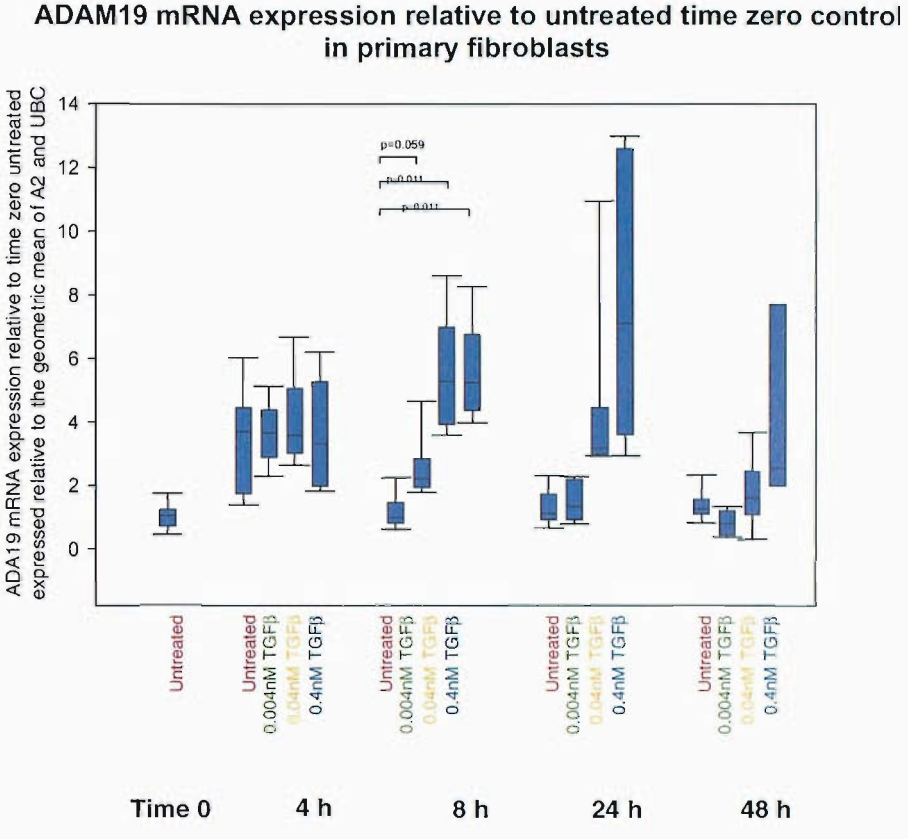


Fig 4.3 ADAM19 mRNA expression was measured by RT-qPCR relative to untreated time zero control in healthy control fibroblasts (n=6). Expression was normalised to the expression of A2 and UBC. Fibroblasts were untreated (red) or treated with 0.004nM TGFβ (green), 0.04nM TGFβ (yellow) or 0.4nM TGFβ (blue) for 0, 8, 24 or 48 hours.
p values calculated using Friedmanns test.

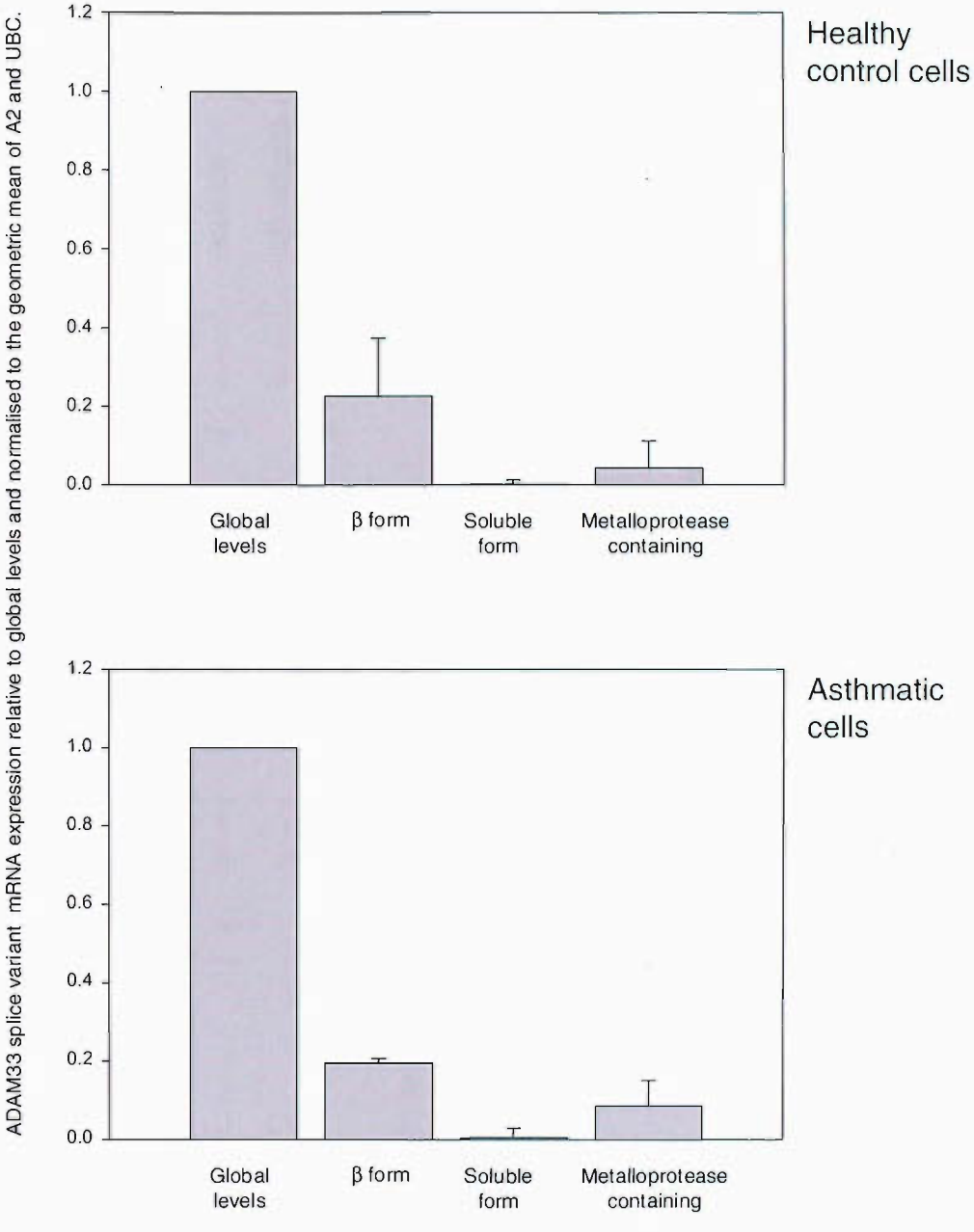


Fig 4.4 ADAM33 mRNA splice variant expression in healthy control (n=6) or asthmatic (n=7) primary airway fibroblasts was measured by RT-qPCR relative to global levels of ADAM33 mRNA as detected by assay targeting the 3'UTR of the molecule. Expression was normalized to the expression of A2 and UBC.

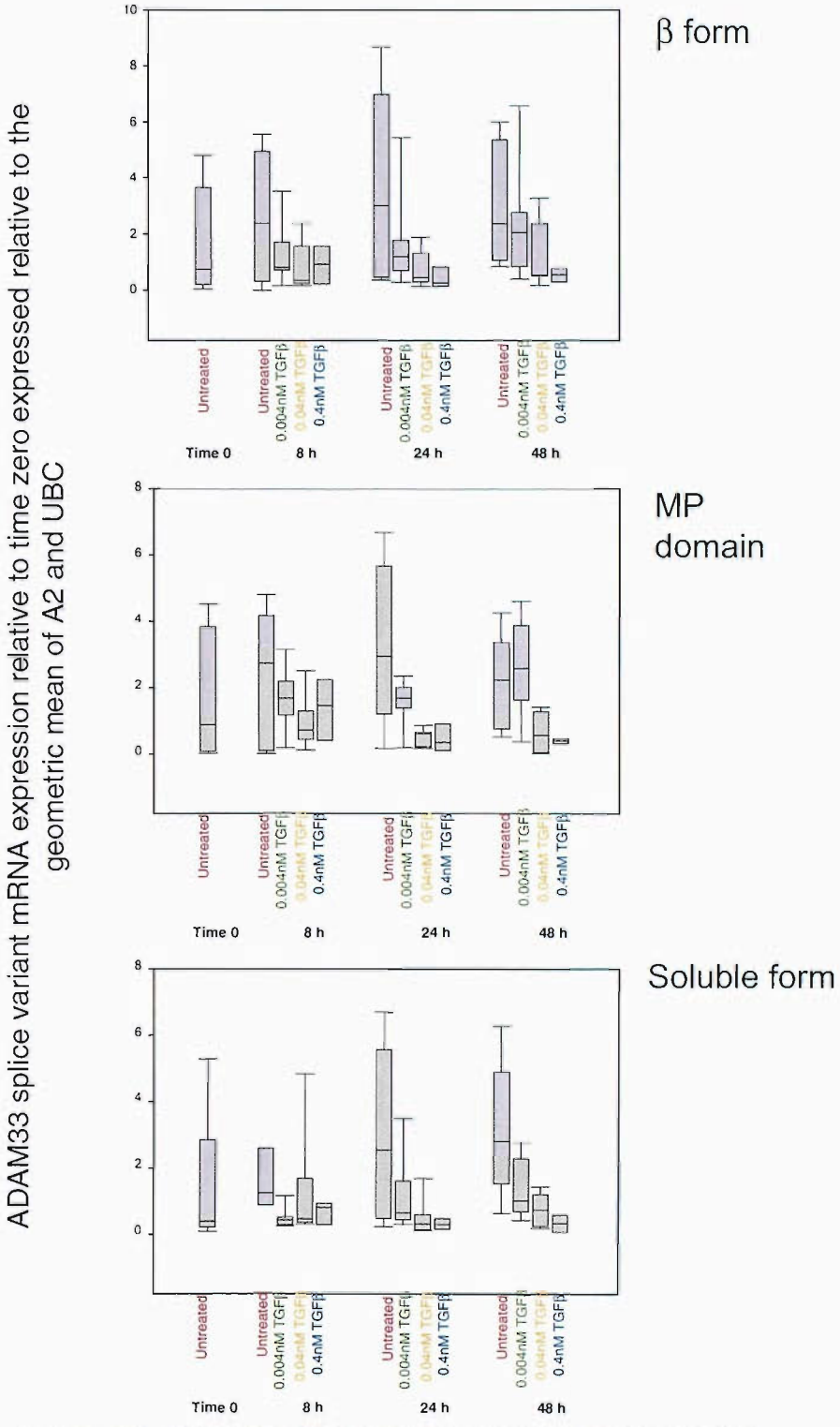


Fig 4.5a ADAM33 mRNA splice variant expression in healthy control (n=6) primary airway fibroblasts was measured by RT-qPCR relative to time 0 in untreated cells. Expression was normalised to the expression of A2 and UBC. Cells were untreated (red) or treated with 0.004nM TGFβ (green), 0.04nM TGFβ (yellow) or 0.4nM TGFβ for 0, 8, 24 or 48 hours.

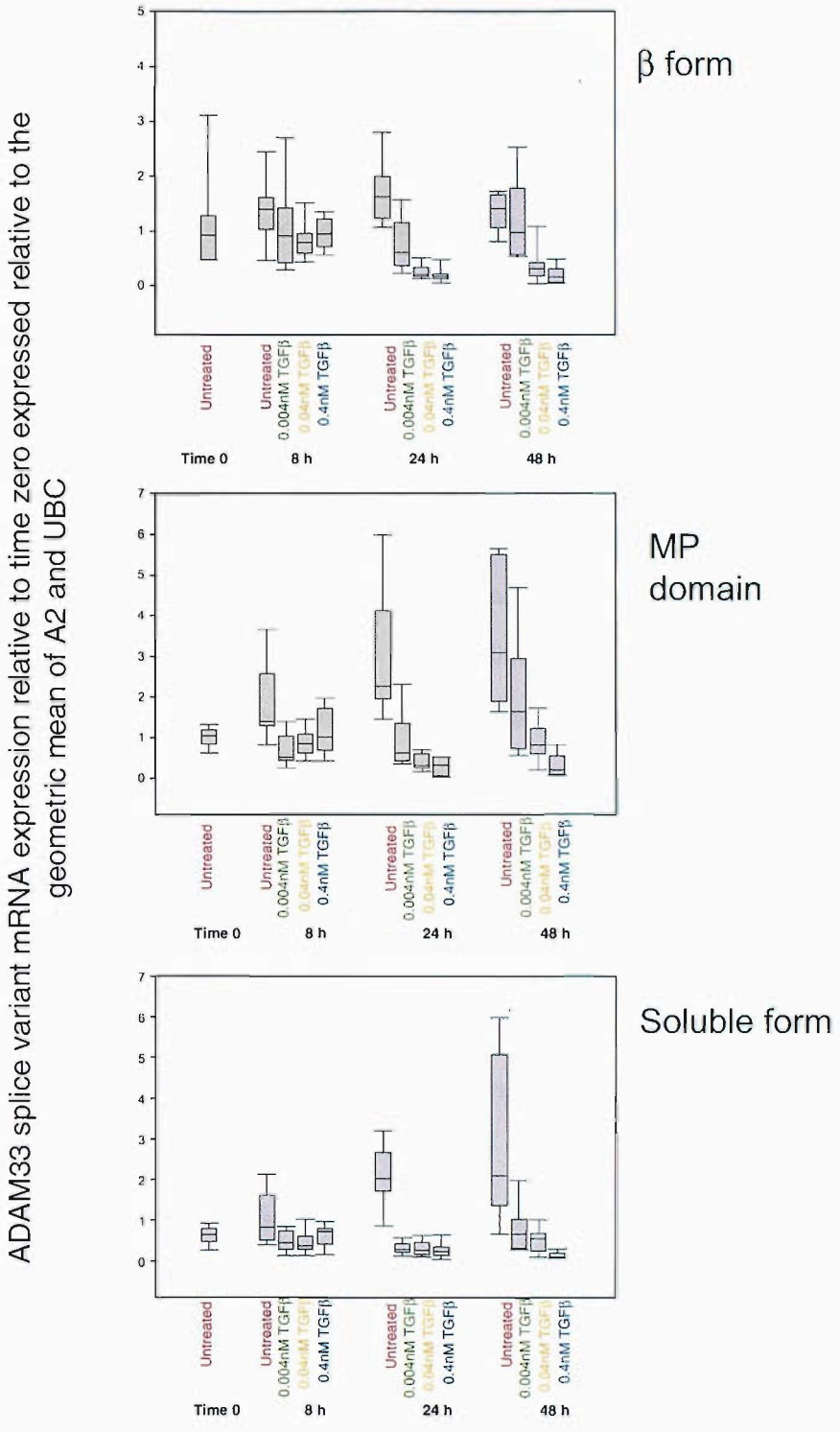


Fig 4.5b ADAM33 mRNA splice variant expression in asthmatic (n=7) primary airway fibroblasts was measured by RT-qPCR relative to time 0 in untreated cells. Expression was normalised to the expression of A2 and UBC. Cells were untreated (red) or treated with 0.004nM TGFβ (green), 0.04nM TGFβ (yellow) or 0.4nM TGFβ for 0, 8, 24 or 48 hours.

αSMA mRNA expression upon differing extracellular matrix components.

It is documented that the ECM in which cultured mesenchymal cells are grown can regulate the process of differentiation(Hayward et al., 1995, Zhang et al., 1999). To further our understanding of the biology of ADAM33, the expression of ADAM33 in cells grown on different matrices was measured to assess any effect this may have on the regulation of ADAM33 expression.

As a positive control for the process of myofibroblast differentiation αSMA mRNA expression was measured in fibroblasts treated with or without TGFβ on three ECM components (collagen, fibronectin and laminin) as well as uncoated plastic tissue-culture dishes (Fig 4.6 and 4.7). There was no difference in αSMA expression at baseline between the four conditions or between asthmatic or healthy control cells. αSMA actin mRNA expression was induced 5-7 fold by treatment with TGFβ on all matrices. There was also no difference between asthmatic myofibroblast populations and healthy control cells. Cells grown in the serum free medium alone showed no change in the expression of αSMA mRNA.

ADAM33 mRNA expression upon differing extracellular matrix components.

Total ADAM33 mRNA expression was also measured following growth upon the three ECM components, as well as tissue culture plastic. At time zero there was no difference in ADAM33 mRNA expression upon the differing matrices in healthy control cell cultures. In asthmatic cultures ADAM33 expression was significantly lower in fibroblasts grown on fibronectin and laminin than those on collagen or plastic ($p=<0.05$).

Upon treatment with TGFβ ADAM33 expression was similar on all matrices. This expression was suppressed relative to time zero on all growth matrices although in

this case significant (p=<0.05) suppression was achieved only on plastic and collagen in both healthy and asthmatic cells. Significant suppression of ADAM33 mRNA expression did not occur on fibronectin or laminin in either asthmatic or healthy control cells.

Cells grown in serum free medium showed a tendency to increase ADAM33 mRNA expression over the 48-hour period. In healthy control cells this increase in expression reached significance (p=<0.05) in cells grown on fibronectin. In asthmatic cells this increase in expression reached significance in cells grown on fibronectin and laminin.

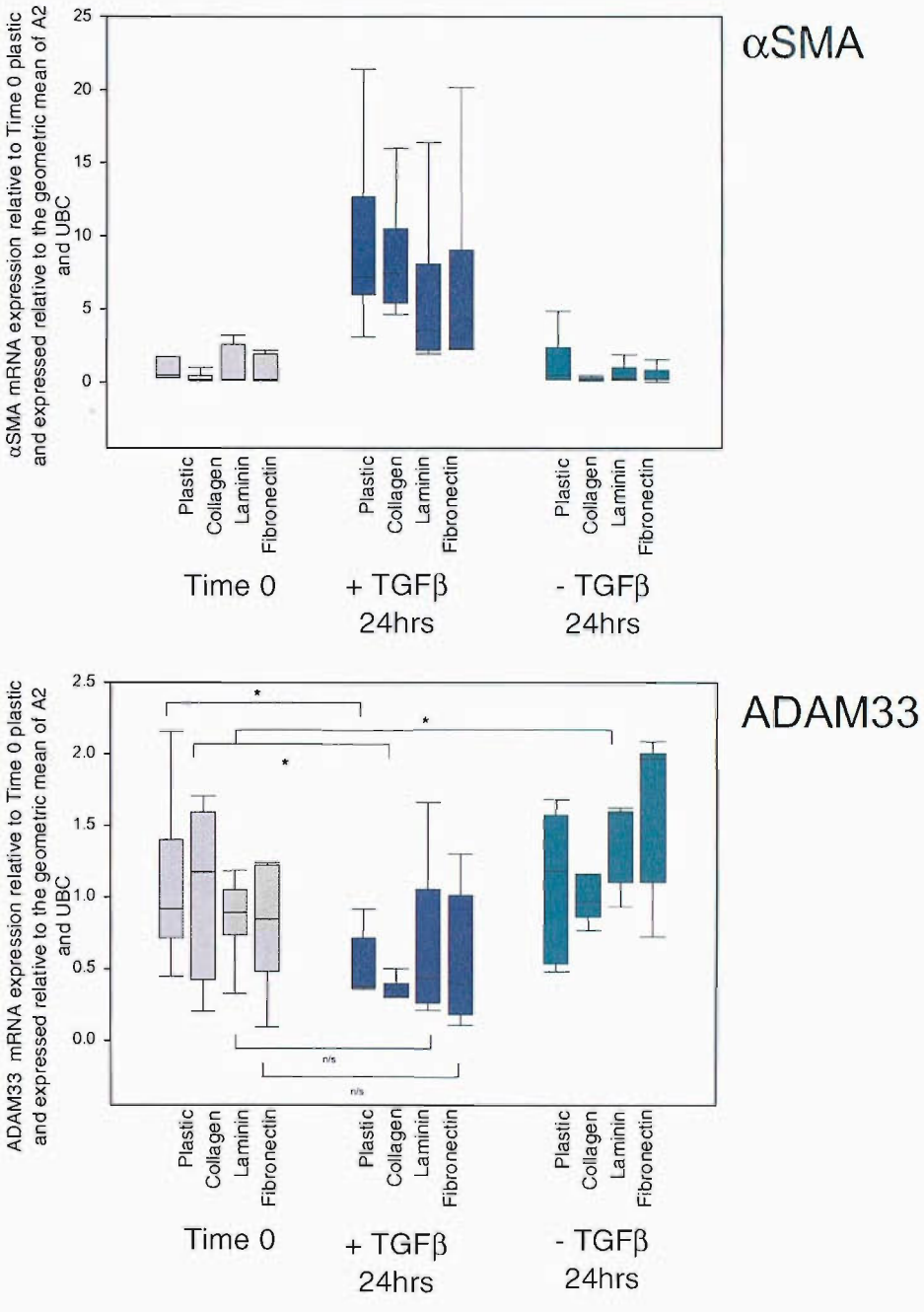


Fig 4.6 αSMA or ADAM33 mRNA expression in healthy control primary airway fibroblasts (n=5) was measured by RT-qPCR relative to untreated time zero cultures grown on tissue culture plastic. Expression was normalised to the expression of A2 and UBC. Cells were grown on tissue culture plastic, either uncoated or coated with collagen, laminin or fibronectin and either untreated or treated with 0.4nM TGFβ for 24 hours.

* indicates statistical significance $p<0.05$.

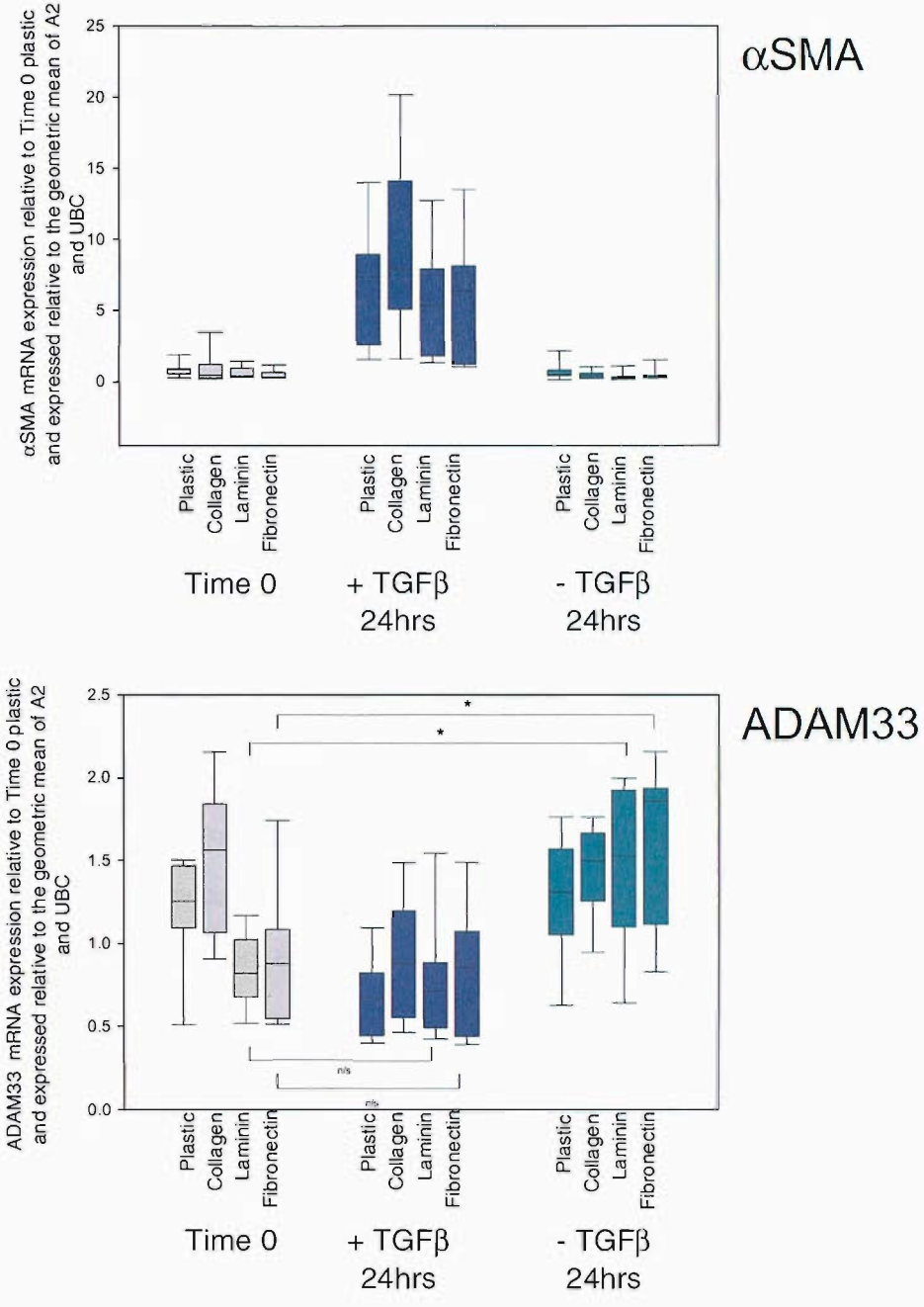


Fig 4.7 αSMA or ADAM33 mRNA expression in asthmatic primary airway fibroblasts (n=6) was measured by RT-qPCR relative to untreated time zero cultures grown on tissue culture plastic. Expression was normalised to the expression of A2 and UBC. Cells were grown on tissue culture plastic, either uncoated or coated with collagen, laminin or fibronectin and either untreated or treated with 0.4nM TGFβ for 24 hours.

* indicates statistical significance $p<0.05$.

Discussion

ADAM33 gene expression in differentiating fibroblasts.

The fact that ADAM33 mRNA expression is comparable between asthmatic and normal fibroblasts either at baseline or during differentiation suggests that the overall level of expression is unlikely to be the cause of the abnormalities in asthma in fibroblasts at least. This is unsurprising as the SNP's (single nucleotide polymorphisms) within the ADAM33 molecule most highly associated with asthma are found towards the 3' end of the molecule and not in the promoter. However, the fact that TGFβ treatment leads to a dose-dependent down-regulation of ADAM33 mRNA expression is an interesting finding. As ADAM33 is known to be expressed as a number of alternatively spliced transcripts it was important to evaluate this finding in the context differentially spliced variants of ADAM33. Having done this analysis these data show the response of the cell to TGFβ treatment in terms of ADAM33 splice variant mRNA expression was consistent amongst all the key splice variants tested. The data provided no evidence of differential regulation of splicing during TGFβ driven differentiation. Hence it seems evident that the cell systematically down-regulates the expression of all ADAM33 mRNA transcripts upon treatment with TGFβ.

What relevance for asthma?

It is possible that within an environment in which TGFβ is abundant, such as the asthmatic airways, that ADAM33 expression may be significantly suppressed. This may be reason to hypothesise that a loss of ADAM33 function contributes to abnormal lung function in asthma. Given that an increase in TGFβ may arise from tissue injury, the effect of TGFβ on ADAM33 may highlight an important gene-environment interaction.

The expression of ADAM19 mRNA is altered in an entirely opposite fashion from

ADAM33. ADAM19 mRNA expression is induced upon treatment with TGFβ in a dose dependent manner. This finding is consistent with findings relating to other ADAMs (Fritsche et al., 2003, Le Pabic et al., 2003) and illustrates the unusual nature of the response of the cell in downregulating ADAM33 expression upon TGFβ treatment.

Although SNPs have been identified in the ADAM33 gene that may alter splicing in these cases no asthma related anomalies in splicing were identified. However SNP-typing analysis data for the individual subjects in this study were not available and hence it is not possible to conclude precisely that disease related differences in splice variant expression will not exist. In addition the number of subjects available for this experiment was small (n=7 asthmatic, 6 healthy) and may not have shown very subtle differences in mRNA expression. Expression of alternative ADAM33 splice variants was regulated in a manner that mirrors the overall expression of ADAM33 in response to TGFβ treatment. This suggests that the mechanism responsible for the reduction in ADAM33 mRNA transcription during TGFβ induced myofibroblast differentiation targets the ADAM33 promoter reducing ADAM33 mRNA levels as a whole, rather than altering the level of particular variants.

TGFβ signalling occurs through the recruitment of TGFβ receptors into a heteromeric complex that then signal internally via a cascade of Smad proteins(Wurthner et al., 2001). Little is currently understood about the regulation of ADAM33 at the promoter level although no Smad binding sites are present within the promoter region. Hence it is difficult to hypothesise further as to the mechanism by which TGFβ stimulation results in a decrease in ADAM33 mRNA expression. As the TGFβ mediated down-regulation of ADAM33 mRNA expression is a relative slow effect (~24-48hours) and no Smad binding sites are present in the ADAM33 promoter region, it is likely to be a secondary effect caused by a Smad mediated pathway upregulating the expression of other molecules thus preventing transcription ADAM33 mRNA.

Having observed that the cell's response to TGFβ treatment is that ADAM33 expression is down-regulated within the cell, one hypothesis is that ADAM33 somehow inhibits the process of differentiation and hence it is necessary for the cell to inhibit the expression of the molecule, in order to proceed with the differentiation process. However, as illustrated in results chapter 1, changes in mRNA expression do not always equate to a subsequent change in protein expression. Therefore, as will be discussed in the following chapter, it is essential to examine the expression of ADAM33 protein in the differentiating fibroblast as a next step towards testing this hypothesis.

The effects of the extracellular matrix upon myofibroblast differentiation and ADAM33 expression.

The make-up of the extracellular matrix on which mesenchymal cells in culture are grown has been shown to be a regulatory factor in the process of differentiation. For example fibronectin and in particular laminin have been shown to maintain a contractile phenotype in cultured smooth muscle cells(Hayward et al., 1995, Zhang et al., 1999). Hence as these data have shown ADAM33 expression to be altered during the differentiation process, it is logical to examine the effect that altering the ECM may have on ADAM33 expression. Such data may provide evidence about the fundamental biology of ADAM33. Although the sample size in this experiment was small these data provided some interesting findings.

As ADAM33 expresses a potentially functional disintegrin-like domain, barring any selective splicing-out of the domain, it is possible that integrin interactions with the molecule may regulate ADAM33 expression in a homeostatic manner. The presence of particular ECM components has been shown to effect gene expression in *in vitro* studies(Xiang et al., 2004, Ortega-Velazquez et al., 2004), so it is possible that extracellularly derived signals transmitted via the disintegrin-like domain, or disruption of other integrins via the disintegrin-like activity of ADAM33, may alter

ADAM33 transcription within the cell. Similarly if the function of ADAM33 is in the cleavage of ECM components the presence of a particular component may drive the upregulation of ADAM33 expression by the cell.

Therefore it was important to assay for the expression of ADAM33 mRNA in cells cultured on alternative ECM components. The data acquired indicated that the matrix upon which primary fibroblasts are cultured had no significant effect on the potency of TGFβ to induce the myofibroblast phenotype as indicated by the induction of αSMA mRNA. And consistent with data under standard culturing conditions (see results chapter 1) αSMA mRNA expression was induced to the same extent in asthmatic cells compared to healthy control fibroblasts.

The effect of treating primary airway fibroblasts grown on plastic or collagen with TGFβ was to reduce ADAM33 mRNA expression. This is consistent with the detailed data illustrated above (Figs 4.2a and 4.2b). However growth of the cells upon fibronectin or laminin led to an inhibition of the significant TGFβ initiated down-regulation of ADAM33 mRNA expression. In cultures of serum starved cells, growth upon fibronectin or laminin however led to a significant upregulation of ADAM33 relative to time zero. This was not evident in cells grown on plastic or collagen. These data suggest a possible role for fibronectin and laminin in promoting ADAM33 stability in the pro-myofibroblastic environment of TGFβ treatment, whilst increasing ADAM33 expression in the pro-smooth-muscle-differentiation environment of serum deprivation(Camoretti-Mercado et al., 2000). As fibronectin and laminin have been shown to promote smooth muscle cell differentiation *in vitro*(Hayward et al., 1995, Zhang et al., 1999) these data may suggest ADAM33 expression would be greater in differentiated smooth muscle cells. This would be consistent with the linkage analysis between ADAM33 polymorphism expression and bronchial hyperresponsiveness (Van Eerdewegh et al., 2002).

This work identified two situations in which ADAM33 mRNA expression was altered. That of TGFβ treatment and continued culture in a serum deprived environment. It is important to relate these findings to any subsequent change in protein expression since data from results chapter 1 highlights a scenario where, although mRNA expression is increased, no increase in functional protein occurs. Hence analysis of ADAM33 protein expression under both of these conditions may add weight to the findings of this chapter as well as yield novel data about the expression of ADAM33 protein. Therefore the next aim of this study to generate/characterise antibodies against ADAM33 so that accurate analysis of protein expression may be achieved.

Summary of results and novel findings

- ADAM33 mRNA expression was comparable in asthmatic primary airway fibroblast cultures compared to healthy controls.

- Alternative splicing of the ADAM33 gene was not found to be different in asthmatic primary airway fibroblast cultures compared to healthy controls and was not differentially regulated by treatment with TGFβ.

- ADAM33 mRNA expression in primary airway fibroblast cultures was significantly down-regulated in a dose responsive manner by treatment with TGFβ. This was opposite to the expression of another ADAM, ADAM19, which was upregulated.

- Growth on fibronectin or laminin prevented the significant TGFβ induced down-regulation of ADAM33 mRNA expression in primary airway fibroblasts.

- ADAM33 mRNA expression is significantly increased by serum deprivation in asthmatic primary airway fibroblast cultures grown on fibronectin and laminin.

- Altering the ECM matrix did not affect the process of TGFβ induced differentiation of primary airway fibroblasts.

Results Chapter 3

Results Chapter 3

Background

Data from the previous chapter indicated that ADAM33 mRNA expression is down regulated in dose dependent manner by treatment with TGFβ. This may prove to be an important observation in a disease state such as asthma where TGFβ levels are known to be elevated. However, as highlighted in results chapter 1, it is important not to assume that this regulation of mRNA expression will correspond with a subsequent change in protein expression. So to test the hypothesis that TGFβ treatment will leads to a functionally relevant down-regulation of ADAM33 protein expression, it is crucial to establish reliable antibody preparations to detect and quantify ADAM33 protein levels. This is the major aim of this chapter.

The ADAMs are a structurally complex family of proteins, each consisting of a number of potential functional domains. The full length ADAM33 molecule is predicted to contain a pro-domain, a metalloprotease domain, a disintegrin domain, an EGF-like domain, a cysteine-rich domain, a transmembranous domain and a cytoplasmic domain (Fig 1.6).

Very little is known about the cellular localisation of ADAM33 although it is likely that at least some ADAM33 transcripts are expressed on the cell surface(Garlisi et al., 2003). The *in vivo* function of ADAM33 is unknown although the metalloprotease domain has recently been shown to be active *in vitro* (Zou et al., 2003). Domain specific antibodies to ADAM33 would be enormously useful in determining the cellular localisation of ADAM33 in primary airway cells, whilst antibodies that bound to individual domains within the extra cellular domain of ADAM33 and neutralised their respective activity would also be potentially useful in determining any function for ADAM33.

A number of alternatively spliced ADAM33 transcripts have already been identified (Van Eerdewegh et al., 2002) (Zou et al., 2003). Within some of these alternative splice variants one or more of the functional domains is absent. Little is known about which of these splice variants will be translated in to protein and therefore three of the functional domains, the metalloprotease domain, the disintegrin domain and the cytoplasmic tail, have been targeted for antibody production.

At the time of this study, no work had been carried out on the folding of the ADAM33 molecule (although subsequently the crystal structure of the metalloprotease domain has been established (Orth et al., 2004)). It is not known therefore which domains are likely to be physically accessible in any native ADAM33 protein. This is a further reason for targeting a number of different areas of the molecule for antibody production.

To achieve these objectives, four rabbits and two chickens were immunised with peptides corresponding to the metalloprotease, disintegrin or cytoplasmic tail domain or a mixture of peptides corresponding to the metalloprotease and disintegrin domain. For the immunisations of the four rabbits, a short peptide sequence from each domain was chosen based upon corresponding regions from ADAM 19 that were successfully used to produce antibodies in another study(Zhao et al., 2001). BLAST searching revealed that the selected sequences were unrelated to any protein other than ADAM33. A similar strategy was employed to design peptides for the immunisation of the two chickens, although in addition these peptides were chosen for their homology to the corresponding sequence in mouse ADAM33. Thus potentially generating a reagent suitable for studying ADAM33 expression in an animal model.

By using both rabbit and chickens to generate anti-ADAM33 immunoglobulins it was hoped that it would be possible to double stain cells for different ADAM33 domains using different secondary antibodies for detection.

The aim of this chapter is therefore to develop and characterise useful preperations of antibodies to ADAM33.

Results

Antibody production

Four rabbits and two chickens were immunised with peptide corresponding to a number of regions of ADAM33 in an attempt to produce antibodies against the molecule.

Animal	Peptides used for immunisation
Rabbit A	M
Rabbit B	D
Rabbit C	C
Rabbit D	M & D
Chicken A	M
Chicken B	C

Table 5.1. Animals were immunised with one or a combination of ADAM33 peptides corresponding to the metalloprotease domain (M), the disintegrin-like domain (D) or the cytoplasmic tail (C). See Table 2.8 (Materials and Methods).

Following immunisation with carefully designed synthetic ADAM33 KLH-linked peptides and a series of "booster" immunisations, sera from each rabbit was partially purified by means of ammonium sulphate precipitation.

Then sera from all four rabbits, along with the partially purified immunoglobulin and pre-immune sera were tested against immunising peptides and a negative peptide of similar length by means of ELISA. IgY from both chickens were also tested against their respective immunising peptides and a negative peptide of similar length.

Rabbit immunoglobulin characterisation by ELISA

Analysis of each sample by ELISA identified three rabbit sera that showed specific antibody interaction with the relevant ADAM33 immunising peptide (Fig 5.1). Rabbit B serum was shown to contain a relatively high titre of antibody specific to the disintegrin domain peptide (peptide D). Serum from rabbit C showed specific binding to the cytoplasmic tail peptide (peptide C) although the titre was low. Rabbit D serum also bound specifically to the disintegrin domain peptide. Neither serum from rabbit A or D showed any antibody specificity with the metalloprotease domain peptide (peptide M).

Chicken immunoglobulin characterisation by ELISA.

Following this analysis two chickens were immunised by AvesLabs in an attempt to produce an antibody with specificity to the metalloprotease domain and to improve on the relatively low titre antibody obtained against the cytoplasmic tail antibody. ELISA analysis of these chicken antibodies isolated from egg yolk (Fig 5.2) showed Chicken A to contain high titre of antibody against the metalloprotease domain, whilst chicken B showed no specific binding to the cytoplasmic tail peptide and hence failed to improve on the sera from rabbit C.

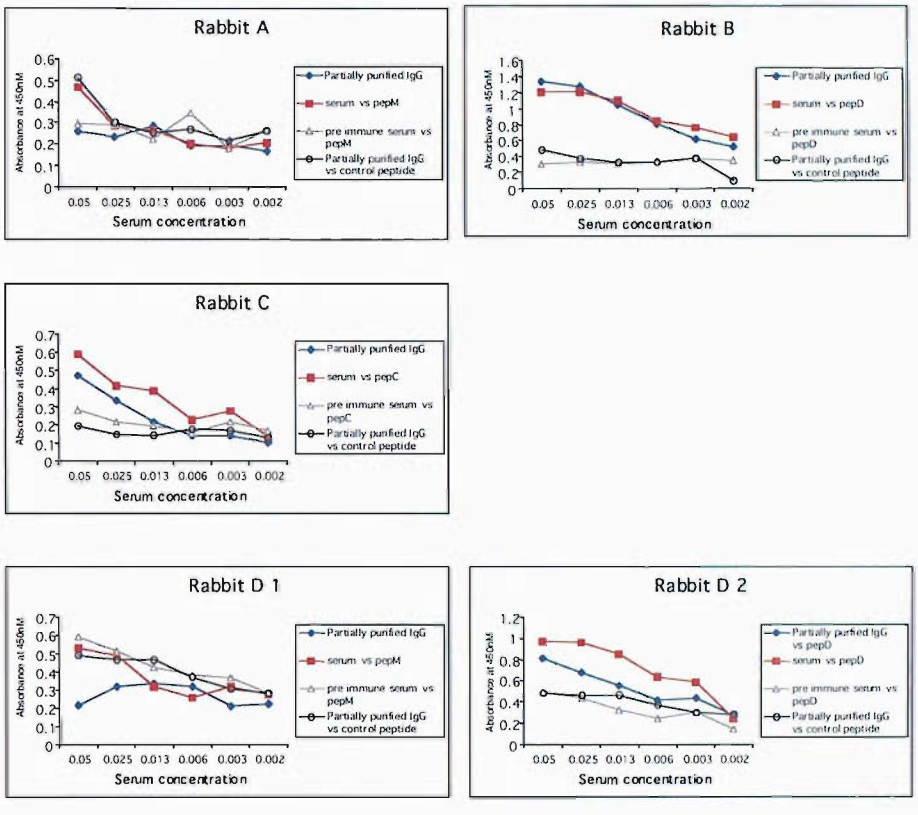
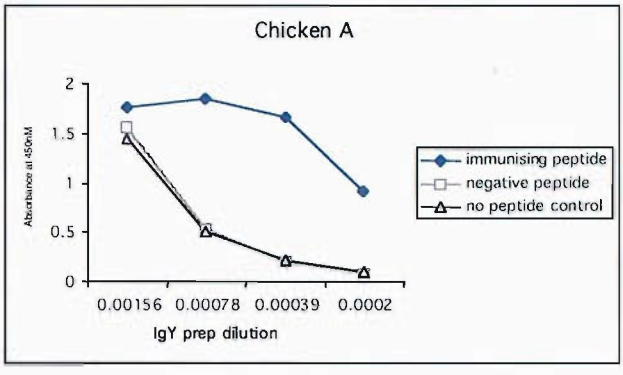


Fig 5.1 ELISA data showing binding of raw sera and partially purified immunoglobulin from Rabbits A-D to their respective immunising peptides or a negative peptide control. A pre immune serum control from each Rabbit is also present. Rabbit A was immunised with peptide M, Rabbit B with peptide D, Rabbit C with peptide C and Rabbit D with both peptides M and D.



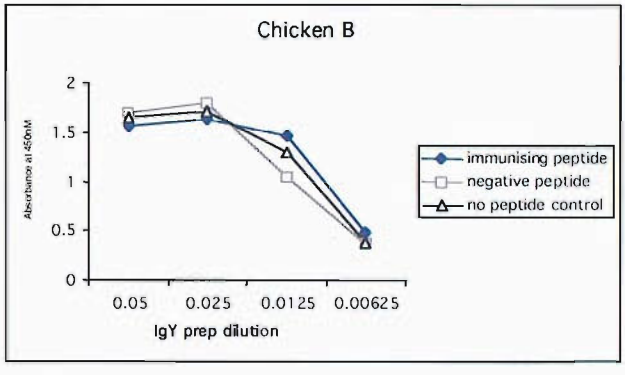


Fig 5.2 ELISA data showing the binding of IgY preparations from both chickens to their respective immunising peptides or a negative peptide control. A no peptide control is present in each case also.

Chicken A was immunised with a peptide from the mettaloprotesase domain. Chicken B was immunised with a peptide from the cytoplasmic tail.

Characterisation of immunoglobulins by Western Blot analysis.

Having established by ELISA that at least some of the immunoglobulin preparations generated contained a good titre of antibody against their respective immunising peptide, it was important to show that these preparations were capable of detecting full-length ADAM33 protein.

To further characterise these antibodies and to verify their reactivity against ADAM33 protein, partially purified immunoglobulin from all four rabbits and antibodies from both chickens, were tested by means of western-blot against cell lysate from COS-7 or HEK293 cells transfected with full length ADAM33. Mock transfected cell lysate was used as a negative control. A positive control was provided by probing the nitro-cellulose membrane with a sample of anti-ADAM33 antibody provided by the Schering-Plough research institute(Garlisi et al., 2003).

Partially purified immunoglobulin from all four rabbits detected a number of bands in both the positive and negative control lysates. However, the positive control full-length wild-type ADAM33 was not detected by any of the rabbit sera (Fig 5.3). Likewise neither chicken sera was able to detect full length ADAM33 by Western blot analysis (Fig 5.4).

In all cases immunoglobulin preparations were titrated in an attempt to establish a suitable working concentration.

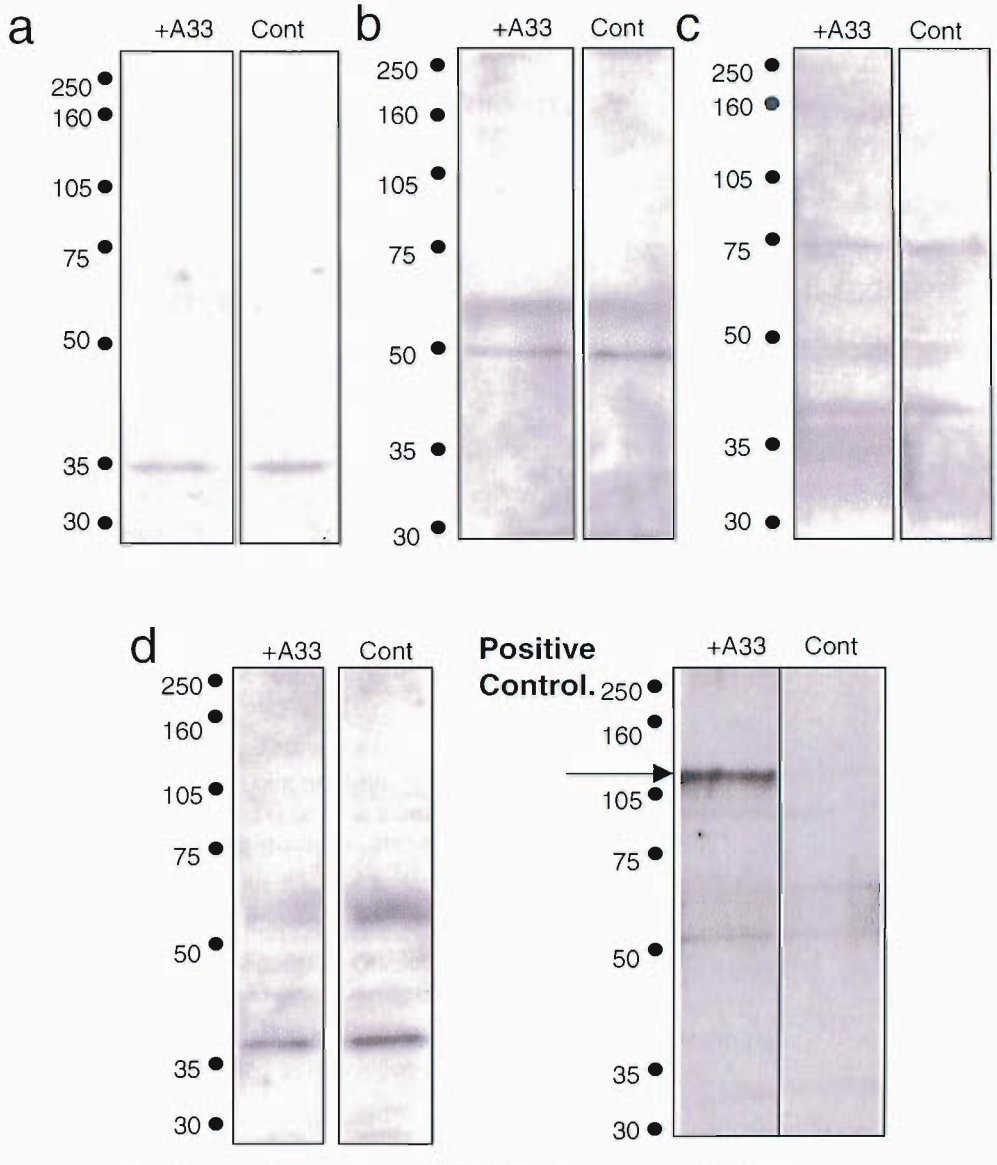


Fig 5.3 Western blot analysis of COS-7 cells transfected with full-length ADAM33 (+) or a no insert control (-). Primary antibodies were partially purified IgG from rabbits **a-d** respectively.

Rabbit **a** was immunised with a peptide from the ADAM33 metalloprotease domain, Rabbit **b** from the disintegrin domain, Rabbit **c** from the cytoplasmic tail and Rabbit **d** from both the metalloprotease domain and disintegrin domain.

An anti-ADAM33 cytoplasmic tail antibody (a gift from Sherrring Plough) was used as a positive control.

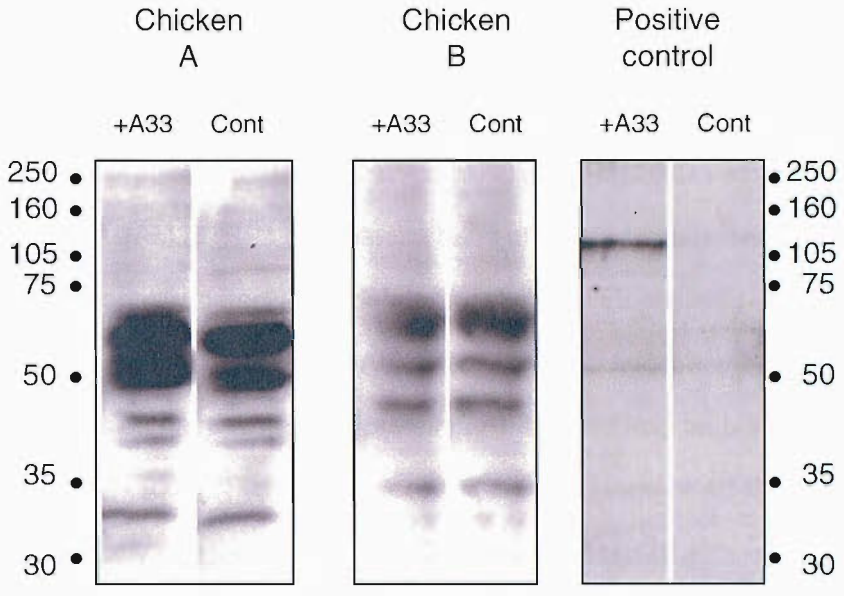


Fig 5.4 Western blot analysis of COS-7 cells transfected with full-length ADAM33 (+) or a no insert control (-). Primary antibodies were partially purified IgG from Chickens **A** and **B** respectively.

Chicken **A** was immunised with a peptide from the ADAM33 metalloprotease domain. Chicken **B** was immunised with a peptide from the ADAM33 cytoplasmic tail.

An anti-ADAM33 cytoplasmic tail antibody (a gift from Sherrring Plough) was used as a positive control.

Characterisation of commercial antibodies by Western Blot analysis.

During the characterisation of these "in-house" antibodies, three anti-ADAM33 antibodies became available commercially (Triple bilogica). Three antibodies labelled RP1, RP2 and RP3 are designed to detect the pro-domain, metalloprotease domain and cytoplasmic tail of ADAM33 respectively. Each anti-ADAM33 antibody was tested against full length ADAM33 transfected HEK293 cell lysate (Fig 5.5). RP1 would be predicted to detect a single band around 120kDa which represent unprocessed ADAM33. RP1 did detect a band of approximately this size (Fig 5.5). RP2 could potentially detect 2 bands representing the processed (~100 kD) and unprocessed (~120 kD) form of ADAM33. RP2 did not detect any bands of the predicted sizes of these respective proteins. RP3 would also be predicted to detect the processed (~100 kD) and unprocessed (~120 kD) form of ADAM33. However in these lysates only one band of around 120kDa was detected although this band was intense and may have shielded the presence of a second band. The anti-ADAM33 positive control antibody (donated by Schering Plough research institute) detected a single band of ~120kDa.

As well as RP3 appearing to have the greatest affinity for full length ADAM33 it was also the most useful antibody preparation for this project, as the cytoplasmic tail of ADAM33 is predicted to be present in the majority of ADAM33 transcripts(Powell et al., 2004). The pro-domain will presumably be processed and therefore absent in functional ADAM33, hence making RP1 a less useful tool. Similarly RP2 may not be as useful a reagent as the metalloprotease domain is predicted to be absent from the majority of ADAM33 isoforms(Powell et al., 2004).

RP3 detected several other bands were detected at lower molecular weights although the intensity of these bands was minimal relative to the two major bands. These bands may represent degradation products of the ADAM33 protein.

ADAM33 detection in primary airway fibroblasts by RP3.

As RP3 was shown to have the highest affinity for full length ADAM33 by western blot and was considered to be the most useful of the antibodies, a preliminary examination of ADAM33 protein expression in primary airway fibroblast cell lysate was carried out. RP3 detected a number of bands (Fig 5.6). However none of these bands correspond to the predicted full length or processed form of ADAM33 observed in transfected cells. Detection of these bands was apparently specific as detection was not evident with a pre-absorbed antibody control.

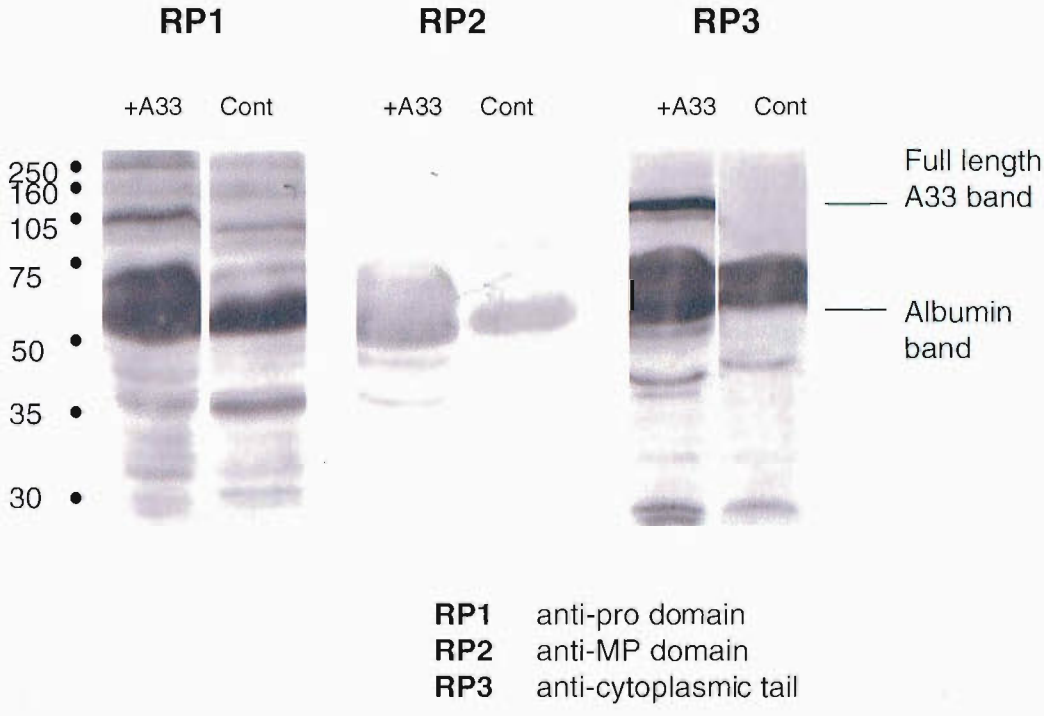


Fig 5.5 Characterisation of three commercially available anti-ADAM33 antibodies by western blot. Antibodies were titrated (data not shown) and tested against HEK293 cells transfected with full length ADAM33 or control untransfected HEK293 cells.

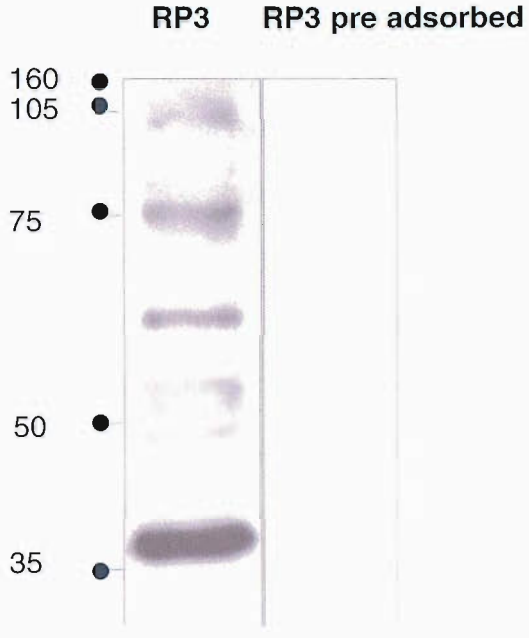


Fig 5.6 Further western blot analysis of commercially available anti-ADAM33 cytoplasmic tail antibody RP3.

This antibody detected a number of bands in primary airway fibroblast cell lysate. Pre-adsorption of the antibody with the immunising peptide resulted in the blocking of these bands.

Characterisation of commercial antibodies by Immunofluorescent staining.

In order to examine the ability of RP3 to detect ADAM33 in whole cells by immunofluorescent staining, cells were transfected with a GFP-tagged ADAM33 clone. Subsequently cells were stained immunofluorescently with RP3 tagged with a of Zenon alexa-fluor (Molecular probes, Oregan) fluorescent label (Fig 5.7). If RP3 was detecting ADAM33 specifically the RP3 staining would co-localise with the GFP perfectly, whilst untransfected cells provide a negative control.

Immunofluorescent staining (with RP3) of H292 cells transfected with full length ADAM33 gave rise to specific staining in approximately 10% of the cells. The percentage of stained cells was identical to the number of fluorescent cells seen in a population of cells transfected with full length ADAM33 labelled with GFP.

The pattern of staining co-localised with that of the GFP-ADAM33 transfected cells and appeared to be localised to an intracellular compartment surrounding the nucleus (Fig 5.7).

Pre-adsorption of RP3 with the corresponding immunising peptide abolished any staining with the RP3 antibody thus providing further evidence that the detection with RP3 is specific for ADAM33.

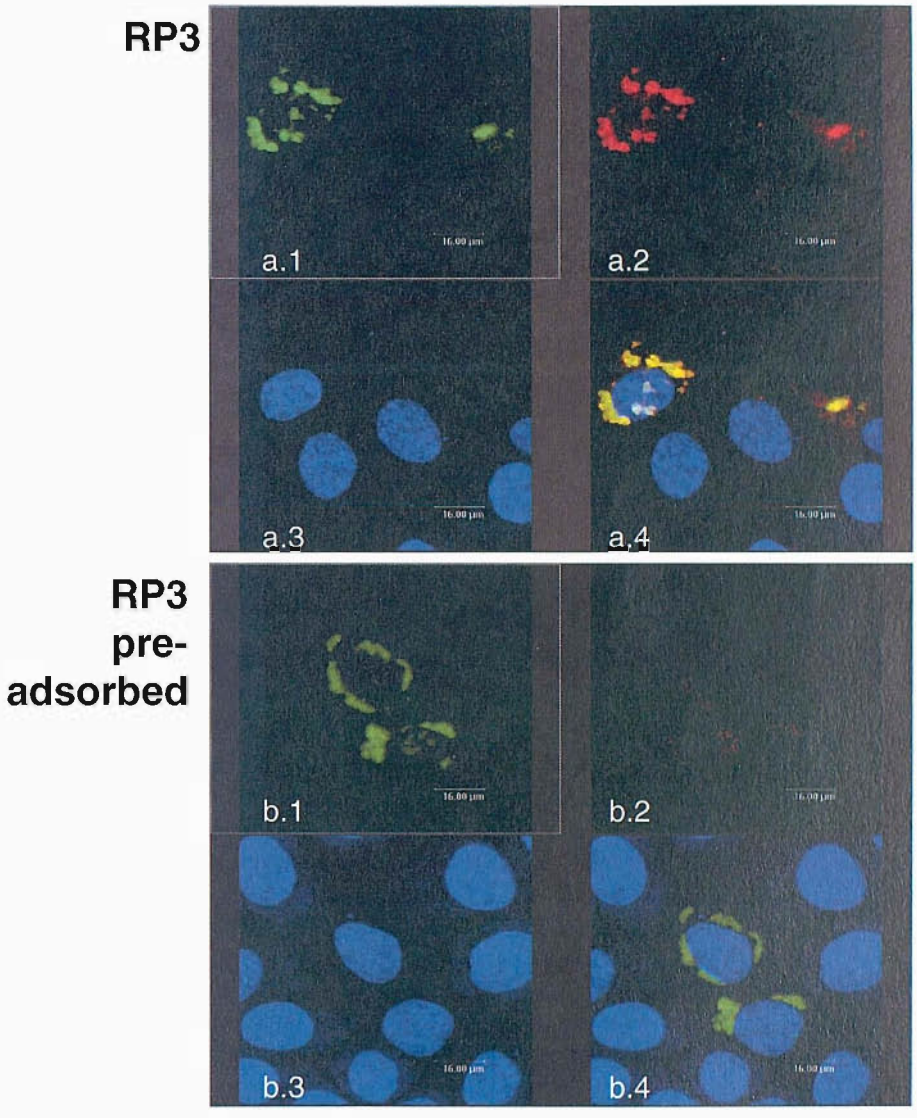


Fig 5.7 H292 cells transfected with full-length ADAM33-GFP and immunofluroescently stained with RP3 (anti-ADAM33 cytoplasmic tail antibody) or RP3 pre adsorbed with the immunising peptide. **a.1& b.1** show ADAM33-GFP. **a.2 & b.2** show RP3 staining. **a.3 & b.3** show nuclear counter-stain. **a.4 & b.4** show composite of all three colours. Red-Green co-localisation shown as yellow.

Discussion

Immunoglobulin preparation and characterisation.

As illustrated by data from Results chapter 1, it is important not to assume that mRNA expression correlates with levels of protein expression. Therefore analysis of protein expression is a vital consideration in furthering our understanding of the biology of ADAM33. In order to achieve this, a number of animals were immunised with ADAM33 peptides corresponding to various ADAM33 functional domains;

Anti-metalloprotease domain.

Rabbit A as well as Rabbit D and Chicken A were immunised with peptide corresponding to a region of the metalloprotease domain of ADAM33. Sera from Rabbits A and D however failed to detect their immunising peptide even at high concentrations in an ELISA format (Fig 5.1). IgY preparations from Chicken A however were able to detect their respective immunising peptide by ELISA (Fig 5.2). The peptide used to immunise Chicken A was 5 amino acids longer than that used to immunise Rabbits A and D. This may have made this peptide more immunogenic. Despite this neither sera from rabbits A or D or the apparently high titre IgY preparation from Chicken A were able to detect full-length recombinant ADAM33 by western blot (Fig 5.3 & 5.4). Both peptides equate to a region of the metalloprotease domain of limited tertiary structure so are likely to be accessible

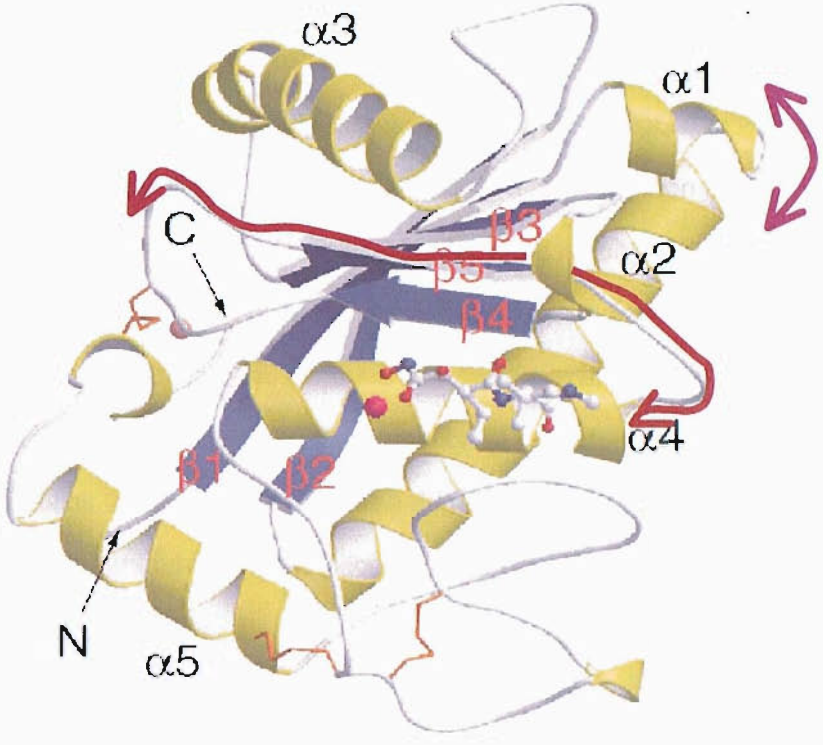


Fig 5.8 Overall structure of the metalloprotease domain of ADAM33 adapted from
Orth *et al* J. Mol. Biol (2004) **335**, 129-137.
αHelices and β-pleated sheets are shown in yellow and blue respectively.
The polypeptide termini are in indicated with N and C. Disulphide bridges are in Orange, the Catalytic zinc ion is in pink, the calcium ion in rose.
The ball and stick structure represents a substrate occupying the active site.
Location of the peptides used to immunise Rabbits A and D and Chicken A
are shown as **purple** and **maroon** respectively.

even in non-denatured protein (Fig 5.8). It is possible that the immunising peptide formed secondary structure of it's own and hence immunoglobulins generated in the animals were effective against the peptide but not the denatured full length protein following SDS-page.

Anti-disintegrin domain.

A peptide corresponding to the disintegrin domain of ADAM33 was used to immunise Rabbit B and Rabbit D. Both rabbits produced a high titre of IgG that was able to detect the immunising peptide by ELISA (Fig 5.1). It appears then that this particular peptide is highly immunogenic. Despite this promising result neither IgG preparation was able to detect full-length recombinant ADAM33 by western blot (Fig 5.3). The disintegrin domain peptide used contains four cysteines and may therefore form secondary structure of it's own via the formation of disulphide bonds. Immunoglobulins that recognise this three-dimensional structure are unlikely to detect the linear denatured protein following SDS-page.

Anti-cytoplasmic tail.

An antibody against the cytoplasmic tail of ADAM33 would be a particularly useful reagent as the cytoplasmic tail of ADAM33 is predicted to be present in the majority of ADAM33 transcripts. Hence Rabbit C and Chicken B were immunised with synthetic peptides based upon a region of the cytoplasmic tail of ADAM33. IgY from Chicken B failed entirely to discriminate between the immunising peptide and a control peptide or no-peptide control in an ELISA (Fig 5.2). However sera from Rabbit C contained a titre of IgG capable of detecting the immunising peptide over control conditions (Fig 5.1). Despite this promising finding this antibody preparation in a western blot was unable to detect full-length recombinant ADAM33 (Fig 5.3).

On the whole, these results were disappointing, as ELISA analysis of all six antibody preparations in general was promising. So why all four rabbits and both chicken sera's failed to detect full length wild-type ADAM33 is perplexing.

One explanation could be that the tertiary structure of the immunising peptides was sufficiently different from the corresponding sequence on the linear full-length wild-type ADAM33. This might affect the ability of antibodies that recognise the smaller peptides to recognise the full-length linear protein. This would explain the observation that antibodies from Rabbit B, Rabbit C and Chicken A were able to detect their respective immunising peptides by ELISA but could not detect recombinant ADAM33 on a Western Blot.

Another explanation for the lack of success in producing peptides antibodies could be that the immune system of the donor animals did not consider the immunising peptides sufficiently foreign, and thus antibody titre against the peptide was low. This might explain the fact that in neither serum from Rabbit A or Rabbit D was any antibody activity specific to the metalloprotease domain peptide detected by ELISA.

Characterisation of commercially available anti-ADAM33 antibodies.

As the cytoplasmic tail of ADAM33 is present in the majority of active ADAM33 transcripts (Powell et al., 2004) and therefore predicted to be present in processed ADAM33 protein, whilst the pro-domain and metalloprotease-domain are not. Hence, of the three commercially available antibodies, only the anti-cytoplasmic tail antibody, RP3 was further characterised.

Preliminary experiments with this anti-ADAM33 cytoplasmic tail antibody (RP3) were encouraging. This antibody detected several bands in the recombinant ADAM33 positive control lysate. The largest of these bands correspond to full length ADAM33. The smaller bands detected were minimally expressed relative to the major band and are likely to be degraded products from the full length ADAM33 molecule. This antibody was also capable of detecting ADAM33 by immunofluorescent staining in

H292 cells transfected with recombinant full length ADAM33. The pattern of staining and percentage of stained cells seen was identical to the pattern of fluorescence seen in cells transfected with GFP tagged ADAM33, thus indicating that the staining seen was indeed ADAM33 specific. Punctate staining localised to an intracellular compartment surrounding the nucleus, as was the case here, is typical of protein localised to the endoplasmic reticulum. This may indicate that ADAM33 is retained within the endoplasmic reticulum until appropriate signals trigger its transportation to its functional location. This observation is speculative however and further controls would be required to establish the significance of this observation.

Multiple bands: splicing or degrading?

When applied to primary airway fibroblast lysates this antibody was also able to detect a number of bands by western blot analysis. Pre-adsorption with the immunising peptide resulted in a reduced pattern of staining, suggesting that the bands detected were ADAM33 related.

It is possible that the multiple banding patterns seen are due to the presence of a number of alternatively spliced ADAM33 transcripts. Alternatively these bands could be degraded ADAM33 products. To establish if alternative splicing or degradation is the reason for this banding pattern it would be useful to analyse these bands by mass spectrometry. Protein sequence data would indicate any splicing events or establish points of cleavage in degraded protein. A simpler approach to answering this question however would be compare fibroblast lysates with a recombinant cell line transfected with full-length ADAM33. In such a cell line no splicing can occur as the ADAM33 cDNA clone will be intron free. If an identical banding pattern is observed by western blot analysis is can be assumed that the multiple bands seen in fibroblast lysate are indeed products of ADAM33 degradation and not selective splicing.

If degradation is shown to be the cause of the multiple banding pattern it is possible that TGFβ may upregulate this degradation alongside the down-regulation of ADAM33 mRNA expression illustrated in results chapter 2. An upregulation of ADAM33 degradation might manifest in an overall reduction in ADAM33 protein detection or an accumulation of smaller degradation products.

Therefore antibody preparations and protocols developed in this chapter will be used to assay the expression of ADAM33 protein in fibroblasts both untreated and stimulated with TGFβ to cause myofibroblast differentiation in the following chapter.

Summary of results and novel findings

- Immunisation of rabbits and chickens with small ADAM33 peptides produced antibodies with affinity for the immunising peptide but failed to generate antibodies capable of detecting full-length ADAM33.

- Commercially available anti ADAM33 antibodies were shown to be effective against recombinant ADAM33. Anti ADAM33 cytoplasmic tail antibody RP3, was the most useful preparation.

- Anti-ADAM33 antibody RP3 successfully detected full-length recombinant ADAM33 by Western blot.

- Anti-ADAM33 antibody RP3 was shown to be effective against recombinant ADAM33 in whole cells by immunofluorescent staining.

- Commercially available anti-ADAM33 antibody RP3 specifically detected a number of proteins in primary airway fibroblasts. Due either to differential splicing or degradation of ADAM33.

Results Chapter 4

Results Chapter 4

Background

The biological function of ADAM33 is entirely unknown. In results chapter 2 a role for ADAM33 in TGFβ induced myofibroblast differentiation, similar to that played by ADAM12 in the differentiation of myoblasts(Lafuste et al., 2004), was hypothesised. The expression of ADAM12 increases during myoblast differentiation(Yagami-Hiromasa et al., 1995), so it was hypothesised that ADAM33 mRNA expression would increase upon TGFβ treatment of primary airway fibroblasts. However, within results chapter 2 it was established that ADAM33 mRNA expression globally, as well as expression of splice variants, was down regulated in a dose responsive manner by treatment with TGFβ in primary airway fibroblasts. Subsequent work in results chapter 3 identified an antibody preparation that is able to detect ADAM33-cytoplasmic tail, which is predicted by mRNA analysis to be present in any translated ADAM33 protein.

It remains crucial then to equate the expression of ADAM mRNA to the expression of protein. Using the reagents and protocols established in results chapter 3 I will therefore examine the expression of ADAM33 protein in primary airway fibroblasts upon treatment with TGFβ, firstly by immunofluorescent staining and subsequently by western blot analysis.

Results

ADAM33 protein expression in the differentiating primary airway fibroblast; immunofluorescence.

To visualise ADAM33 protein expression in the differentiating primary airway fibroblast, cells were cultured in the absence or presence of TGFβ (0.04nM) for 48 hours and ADAM33 detected by immunofluorescent staining (Fig 6.1).

In untreated fibroblast cultures, ADAM33 staining appeared diffuse and apparently located intracellularly. Occasional cells were stained more intensely, although these appeared to be cells of a particularly compact nature and thus the cytoplasm appeared more dense resulting in a more intensely stained cell. Following 48hours of TGFβ (0.04nM) treatment, ADAM33 staining was reduced to levels approaching the threshold of detection. Indeed control cells stained with isotype control immunoglobulins labelled in the same way showed a level of staining similar to that in the myofibroblasts. The finding that ADAM33 protein expression was reduced upon TGFβ treatment was consistent with the reduction in mRNA expression established in results chapter 2.

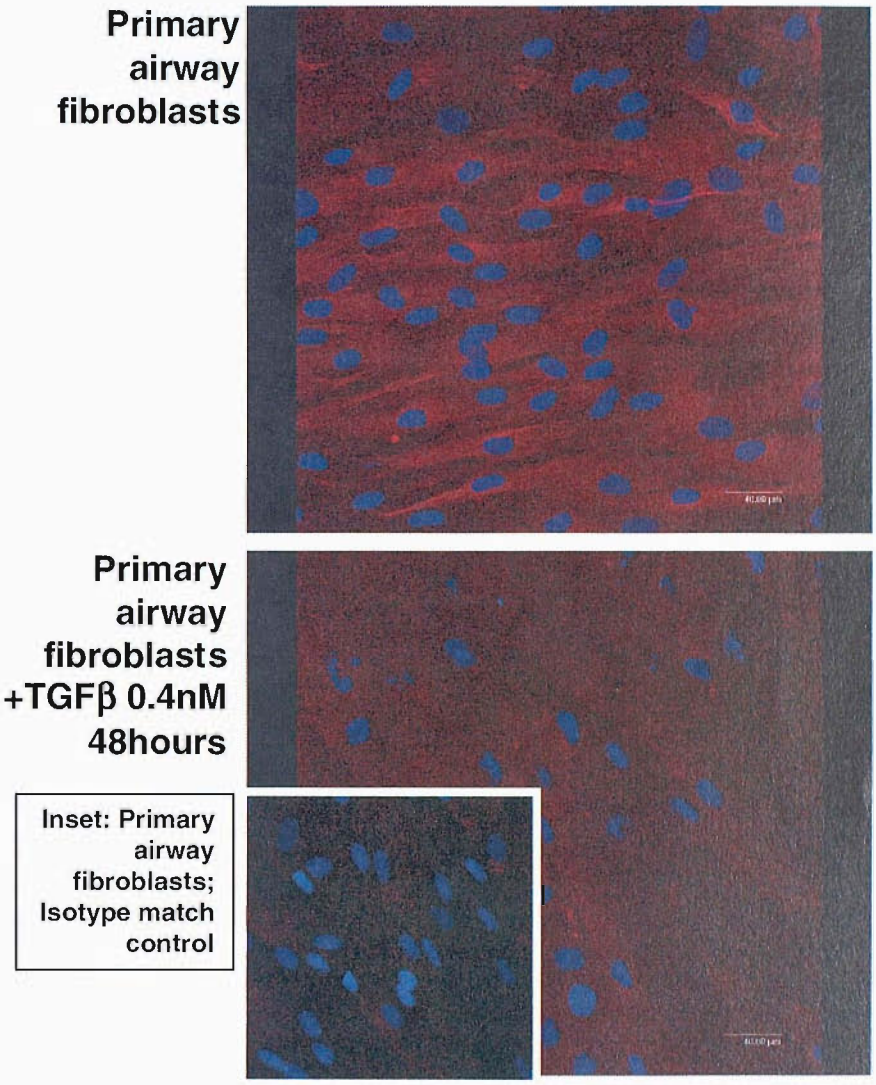


Fig 6.1 Immunohistochemical staining of primary airway fibroblasts untreated or treated with TGFβ 0.4nM for 48hours. Staining is with RP3 (anti-ADAM33 cytoplasmic tail antibody) or an isotype match control antibody at the same concentration. Nuclei are counter-stained blue.

ADAM33 protein expression in the differentiating primary airway fibroblast; western-blot.

To evaluate in more detail the effect of TGFβ on ADAM33 protein isoform expression, cell lysates were subjected to western blot analysis (Fig 6.2).
As described in results chapter 3, anti-ADAM33 cytoplasmic domain antibodies detected a number of bands in primary fibroblast lysate. However, upon treatment with TGFβ, the staining intensity of one band at around 17-19kDa increased considerably. The staining for this band was blocked completely by pre-adsorption of the antibody with the immunising peptide and was not detected by the isotype control immunoglobulin. Hence it is considered that this is an ADAM33 specific band.
There was a slight increase in the intensity of the expression of an ADAM33 band at ~35kDa. Staining for this band has been shown to be specific for ADAM33 in a recent publication(Powell et al., 2004) . The expression pattern of no other bands appeared to be altered by treatment with TGFβ.

ADAM33 protein expression in the differentiating primary airway fibroblast; western-blot, 10-day time course.

To examine the effect of prolonged exposure to TGFβ on ADAM33 protein isoform expression, primary fibroblast lysates were cultured for 10 days in the presence or absence of TGFβ then subjected to western blot analysis (Fig 6.3).
αSMA protein expression was also measured to show that TGFβ had caused myofibroblast differentiation.
On day two of TGFβ treatment, αSMA protein expression was significantly induced and continued to increase or remained high over the 10-day period of TGFβ stimulation. Likewise the expression of a 17-19kDa ADAM33 protein was increased by day two of TGFβ treatment. However the intensity of this protein band proceeded to diminish over the remainder of the 10-day time course until virtually undetectable.

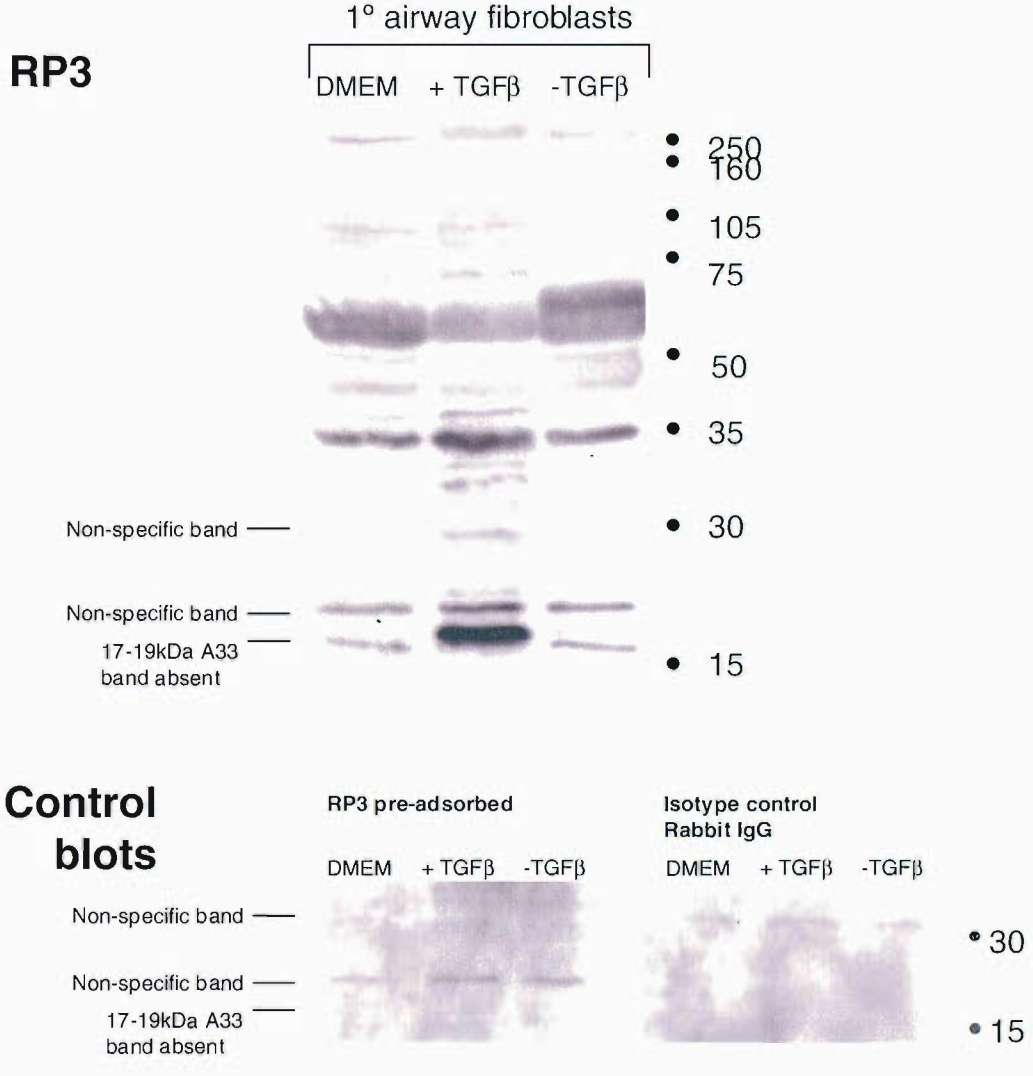


Fig 6.2 Identification of a TGFβ induced 17-19kDa ADAM33 protein by Western blot analysis. Primary airway fibroblasts were treated with DMEM or serum free medium with or without TGFβ 0.4nM for 48 hours. Membranes were probed with RP3 (anti-ADAM33 cytoplasmic domain antibody).

To confirm 17-19kDa band species as a specific antibody-protein interaction control blots were probed with RP3 pre-adsorbed with a molar excess of the immunising peptide or with rabbit IgG of the same concentration as the RP3 antibody.

(representative of four repeats with healthy control fibroblasts)

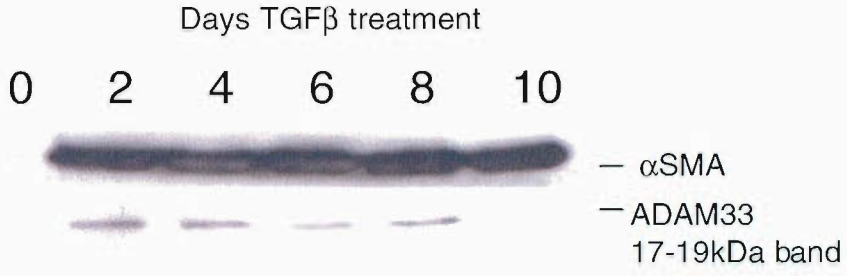


Fig 6.3 Western blot analysis. Primary airway fibroblasts were treated with TGFβ (0.4nM) every two days for a 10 day period. Lysates were collected at day 0 and every two days for the remainder of the time course. 17-19kDa ADAM33 band is very weak prior to TGFβ treatment. 48 hours of TGFβ exposure results in an accumulation of the 17-19kDa protein alongside an induction of αSMA. Whilst αSMA expression remains high over the remainder of the sustained TGFβ treatment time-course, ADAM33 17-19kDa protein band diminishes.Representative of four repeats with healthy control fibroblast.

ADAM33 expression in transfected Hek293 cell line: validated.

HEK293 cells stably transfected with recombinant ADAM33 were used as a tool to determine if the 17-19kDa ADAM33 protein detected in TGFβ treated fibroblasts was a product of degradation or a novel splice variant induced by TGFβ. If an identical band was present in the HEK293 at 17-19kDa it can be concluded that this is a product of degradation, as alternative splicing can not occur in the recombinant cell line.

Real-time qPCR analysis of the ADAM33 expressing cell line showed no native ADAM33 to be present in the transfected cell line or the untransfected parental cell-line (Fig. 6.4). Two real-time qPCR assays were used. The first detects the 3'UTR of ADAM33 and will therefore selectively detect endogenous ADAM33. The second targets the metalloprotease domain of ADAM33 and will therefore detect both endogenous and recombinant ADAM33. The absence of endogenous ADAM33 in either cell line either before or after TGFβ treatment (0.4nM 48hours) was confirmed by the absence of any signal from the assay targeting the 3'UTR. Assay efficiency was confirmed with fibroblast cDNA (data not shown)(Powell et al., 2004).
ADAM33 mRNA was detected in the ADAM33 transfected HEK293 as indicated by a signal from the assay detecting the metalloprotease domain of ADAM33. Therefore this signal derives from recombinant ADAM33. No metalloprotease domain mRNA was detected in untransfected parental control cells. No detection was present in either no-template control.

ADAM33 expression in transfected Hek293 cell line upon TGFβ treatment.

Western blot analysis of lysates of ADAM33 transfected HEK293 cells identified multiple bands similar to that seen in primary airway fibroblasts (Fig 6.5). Two bands were detected in the transfected cell line that were absent in the untransfected parental cell line at ~120 and ~100kDa. These are likely to represent the full-length form of ADAM33 and the processed form minus the pro-domain respectively. A large band of ~65kDa was detected in all cells including untransfected control cells. It is likely that this band represents albumin present in the cell lysates. Another band was detected at ~50kDa in the transfected HEK293 cells and the untransfected control HEK293s. As these cells are of epithelial origin it is likely that this band represents cytokeratins in the cells lysates. Both albumin and cytokeratin are likely to be present at high concentrations, so despite a low affinity of the antibody for them they are still detected.

A band of ~35kDa was detected in the ADAM33 transfected cells also. This band was absent from the untransfected control cells. It is likely that this band equates to the ~35kDa ADAM33 band detected in primary airway fibroblasts and hence it is implied that this band may be a degraded form of ADAM33 not a splice variant.

Of particular interest, the 17-19kDa protein of particular interest in differentiating fibroblasts was also present in the HEK293 line. Upon treatment with TGFβ the intensity of the 17-19kDa increased. These data indicate that the this protein is a product of the degradation of ADAM33 not a novel splice variant.

Morphological effects of TGFβ treatment on ADAM33 transfected HEK293 cell line

Untreated control HEK293 cells never reached 100% confluence. Small spaces remained between cells. ADAM33 transfected HEK293 cells were very different in

appearance however. They formed a highly confluent monolayer with an appearance similar to a population of cells with tight junction complexes between them. Treatment of the untransfected HEK293 cell line with TGFβ had no effect on cell morphology (Fig 6.6.). However, upon treatment with TGFβ the ADAM33 transfected HEK293 cells underwent a significant morphological change resulting in a much more mesenchymal-like appearance. Cells became more elongated and formed contractile-looking swathes similar to that seen in myofibroblast cultures. αSMA was constitutively expressed in the control cell population but was absent in the ADAM33 transfected cells until treated with TGFβ (Fig 6.6.).

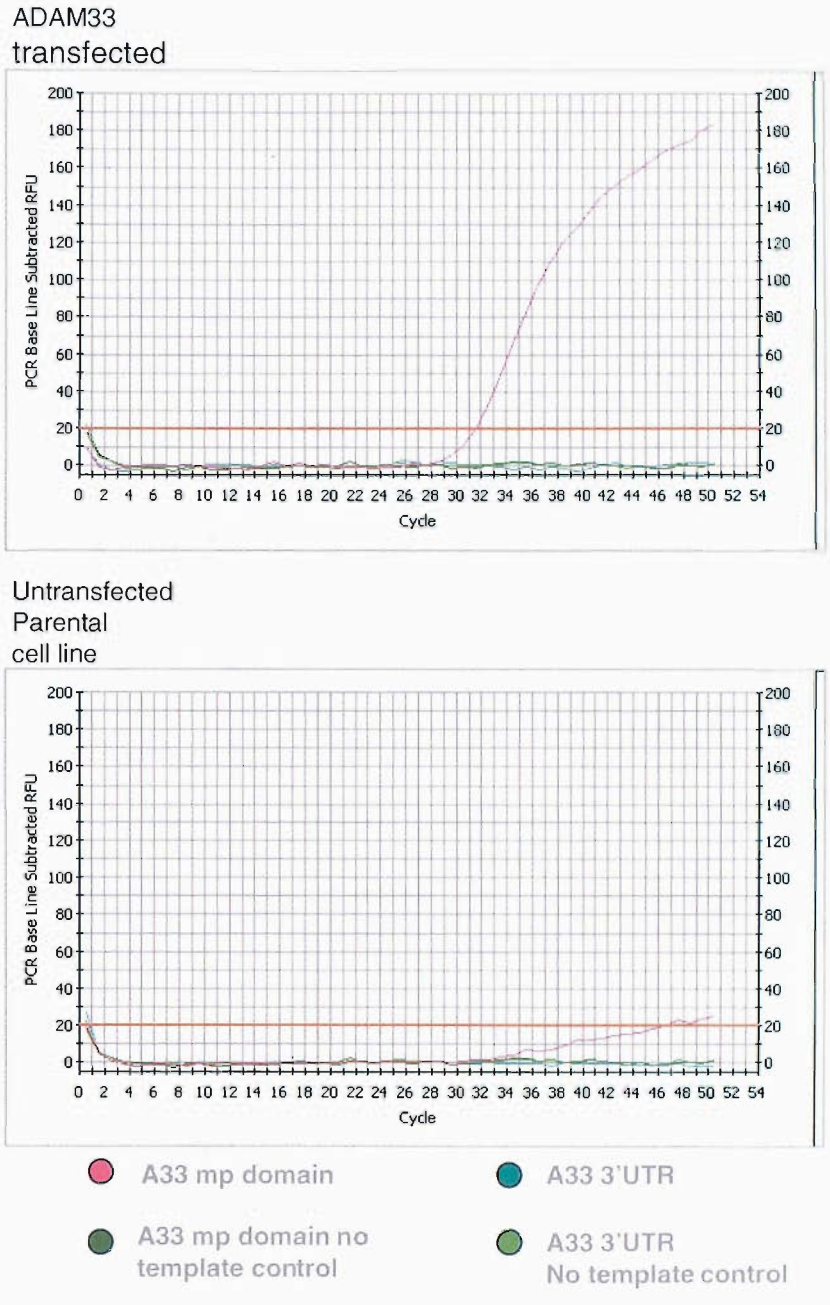


Fig 6.4 Real-time qPCR analysis of ADAM33 expression in ADAM33 transfected HEK293 stable cell line and untransfected parental control cells. An assay to detect endogenous ADAM33 via the 3'UTR is shown in jade. An assay to detect the metalloprotease domain of ADAM33 will detect both endogenous and recombinant ADAM33 is shown in pink. Control samples are shown in green. No endogenous ADAM33 is present in either cell line therefore mp-domain detection in transfected cells is purely from recombinant ADAM33. Fibroblast cDNA positive control was used (data not shown).

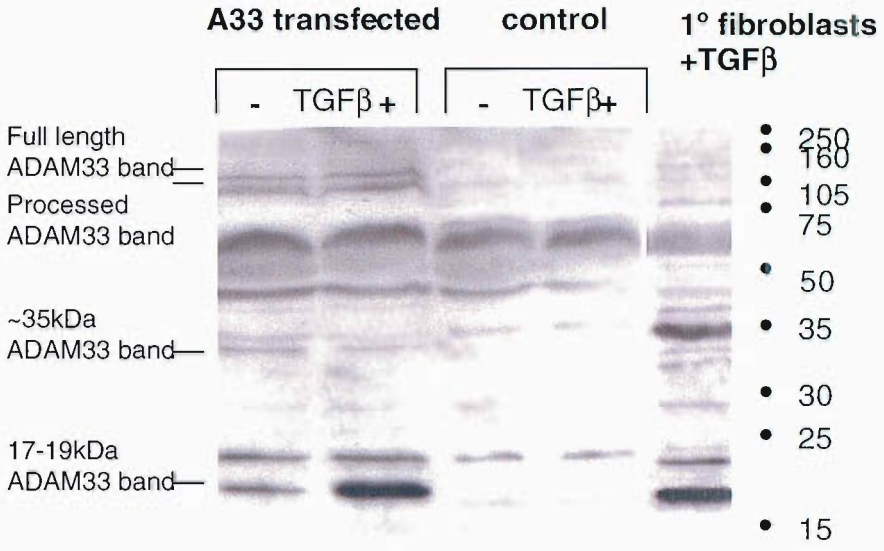


Fig 6.5 Western blot analysis of ADAM33 transfected HEK293 cells vs. untransfected parental line control cells vs. primary airways fibroblasts treated with TGFβ (0.4nM 48hours) HEK293s were grown in DMEM with or without TGFβ (0.4nM for 48 hours). Blots were probed with antibody against the cytoplasmic tail of ADAM33. Representative of 3 repeats.

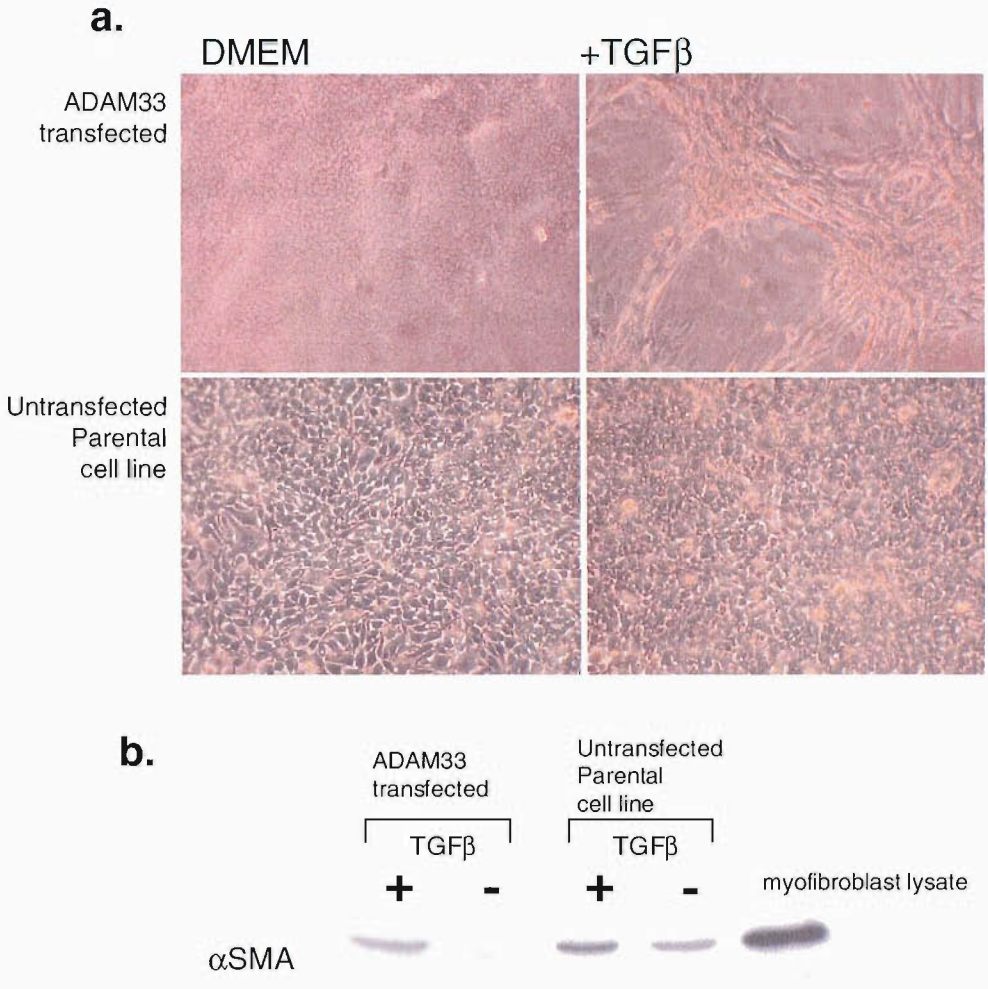


Fig 6.6 Characterisation of ADAM33 transfected HEK293 stable line's response to TGFβ treatment. Cells were grown to confluence then stimulated with or without TGFβ 0.04nM 48hrs. **a**. phase contrast images of cells. **b**. western blot for αSMA expression in lysates from photographed cells.

Accumulation of ADAM33 degradation product is arrested by treatment with protease inhibitors.

Having established that the 17-19kDa protein detected by western blot in primary airway fibroblasts treated with TGFβ is a product of degradation, protease inhibitors were used to establish if this degradation was protease dependent.

Recent micro-array analysis of differentiating embryonic fibroblasts identified serine protease 11 as a gene upregulated by stimulation with TGFβ(Chambers et al., 2003). Other work has shown Ubiquitin protease-activity to be induced by treating cultured cells with TGFβ(Izzi and Attisano, 2004, Fukasawa et al., 2004).

Hence mg132 an inhibitor of the ubiqutitin protease and a cocktail of protease inhibitors including an inhibitor of serine protease were tested.

Pre-treatment of primary airway fibroblasts with a cocktail of protease inhibitors led to the abolishment of the TGFβ induced accumulation of the 17-19kDa ADAM33 degradation product (Fig 6.7.). Similar pre-treatment with the ubiquitin protease inhibitor, mg132, reduced the accumulation of the 17-19kDa ADAM33 degradation product upon TGFβ treatment but did not abolish it. Treatment with either the cocktail of protease inhibitors or mg132 appeared to inhibit to some extent the TGFβ induced upregulation of αSMA expression compared to cells treated with DMSO alone.

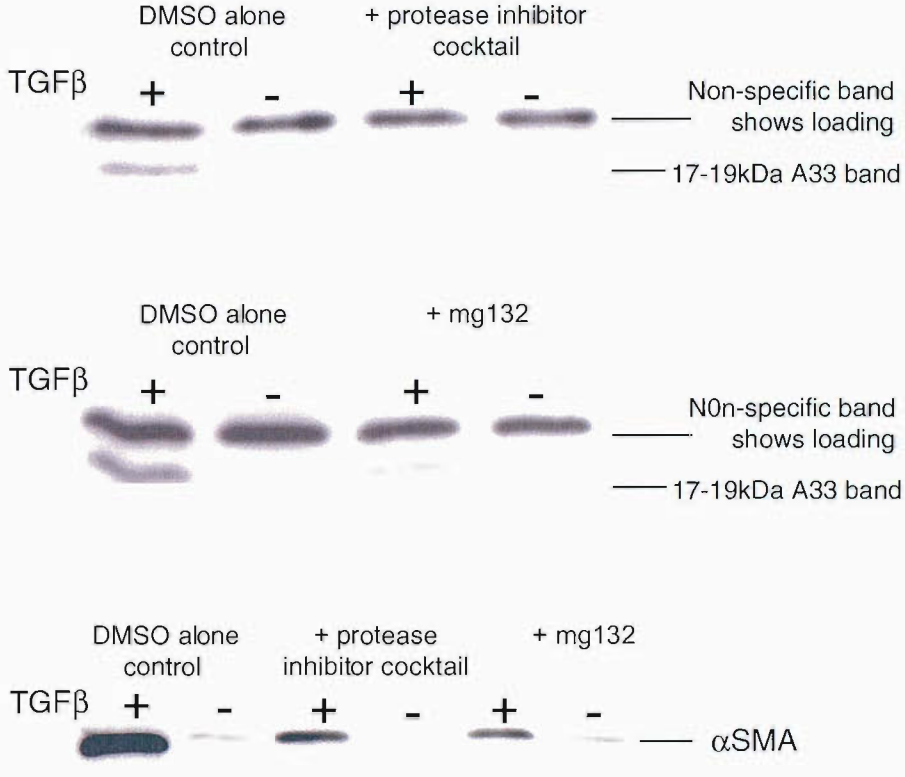


Fig 6. 7 Western blot data. Primary fibroblasts were treated with serum free medium containing DMSO (1:600 dilution), protease inhibitor cocktail (1:600 dilution) or mg132 (125ng/ml) for two hours. Media was then replaced with serum free media with or without TGFβ 0.4nM for 48 hours. Following SDS-page and transfer to nitro-cellulose membrane, blots were probed for ADAM33 cytoplasmic domain then stripped and re-probed for αSMA.

The presence of an unspecific band slightly larger than 17-19kDa shows an equal volume of protein was loaded in each well. (representative of three repeats with healthy control fibroblasts.)

Discussion

Immunofluorescent staining of primary airway fibroblasts.

Immunofluorescent staining of primary airways fibroblasts with the anti-ADAM33 cytoplasmic domain antibody apparently showed ADAM33 to be localised intra-cellularly. Studies into the trafficking and maturation of ADAMs 9 and 15 have shown the majority of ADAM protein to reside in the golgi apparatus as indicated by the co-localisation with golgi markers(Roghani et al., 1999, Lum et al., 1998). This may prove to be the case for ADAM33, although further studies into the cellular distribution and trafficking of ADAM33 via the co-staining of golgi markers would prove useful in determining this.

The staining for ADAM33 was weak and indicative of a rare protein species. As no full length ADAM33 was detected by western blot in these cultures it may be the case that the functional potency of ADAM33 is such that its expression has to be highly regulated. Alternatively under these conditions ADAM33 stability may be very low and turnover of protein is continuous. In either scenario, minimal protein would be present in the cell at a particular time-point thus making its detection under these circumstances very difficult.

As the ADAM33 molecule is predicted to contain a hydrophobic transmembranous region one might expect the molecule to be located at the cell surface. If a percentage of the ADAM33 protein was expressed at the cell surface, as prototypic of the ADAM family of proteins, it would be very difficult to localise in conditions such as these where the protein is so rare. Hence specific cell-surface expression of ADAM33 was not evident.

TGFβ treatment did, as hypothesised, down-regulate the overall expression of ADAM33 protein in primary fibroblast cultures. The pattern of staining in TGFβ treated cells was considerably less and compared closely to isotype control stained cells. Although subsequent western-blot analysis shows an ADAM33 peptide

containing the cytoplasmic tail to accumulate upon TGFβ treatment, this peptide is a degradation product and hence likely to be within the proteasome of the cell. Densely packaged degraded protein such as this is detectable in a denaturing environment such as that used in the western-blot analysis but inaccessible to the antibody during immunofluorescent staining of the whole cell.

ADAM33 protein expression in differentiating primary airway fibroblasts.

These data, considered alongside mRNA data from Results chapter 2, provide evidence for a controlled down-regulation of ADAM33 protein and mRNA in primary airway fibroblasts upon differentiation to a myofibroblast phenotype. Indicative of this down-regulation of ADAM33 protein is the accumulation of degraded ADAM33 protein in the cell, in particular a species of around 17-19kDa. It was shown that this protein was not an alternative splice form of ADAM33 as the same protein band was detected in a recombinant model of ADAM33 expression using an endogenous ADAM33 negative cell line (HEK293) (Fig 6.5) transfected with ADAM33. As splicing can not occur in such a model, degradation of the molecule is the only explanation for the presence of such a truncated form.

This protein must contain the cytoplasmic tail of ADAM33 as it was detected using the anti-cytoplasmic tail antibody. Based on molecular weight we can assume this protein to be the cytoplasmic tail with the transmembranous region of the molecule and a small section of the EGF-like domain. Attempts to immunoprecipitate the peptide for mass spectroscopy analyses were hampered by the similarity of the protein in size to the light chain of IgG (data not shown). Such a molecule could putatively have a functional role in cell fusion for example. However, given that the cells response to TGFβ is to down-regulate ADAM33 expression at both a mRNA (Fig 4.2) and protein level (Fig 6.1), and that the 17-19kDa protein diminishes rather than further accumulates upon persistent pressure with TGFβ (Fig 6.3), it is far more

likely that this species is an intermediate destined for complete degradation by the cell.

The degradation of ADAM33 during the process of TGFβ induced differentiation was shown to be protease dependent. The ubiquitin protease was a candidate for the unidentified processor of ADAM33, as it is a known mediator of the TGFβ/SMAD signalling pathway(Izzi and Attisano, 2004, Fukasawa et al., 2004). However inhibition of the ubiquitin protease by mg132 only partially inhibited the degradation of ADAM33 as indicated by the accumulation of the 17-19kDa product upon TGFβ treatment (Fig 6.7), whilst a cocktail of inhibitors did prevent the accumulation of the ADAM33 protein entirely. These data therefore are inconclusive in identifying which protease is responsible for the TGFβ induced degradation of ADAM33. Although higher doses of mg132 were shown to be toxic (data not shown) a slight increase in dose may inhibit the degradation of ADAM33 further without causing cell death. The cocktail of inhibitors used included inhibitors of serine proteases, aminopeptidases, cysteine proteases and acid proteases. Selective inhibitors of each of these families are available and systematic exclusion experiments could identify the class of protease responsible for the degradation of ADAM33 to a 17-19kDa species. qPCR data on the expression of such a protease family would identify particular proteases whose expression is upregulated by treatment with TGFβ.

Expression of ADAM33 recombinant protein.

Recombinant stably expressed ADAM33 in HEK293 cells appears to undergo the same process of degradation as native ADAM33 in primary airway fibroblasts. An ADAM33 protein identically sized (17-19kDa) to that seen in TGFβ treated fibroblasts is present in transfected and is absent from the control untransfected cells. The constitutive expression of this product is likely due to the cells response to the pressure of a viral promoter driven over-expression of the molecule. Given that the

expression of native ADAM33 appears highly regulated and consequently the native protein is rare. It is likely that the over-expression of the molecule results in a persistent degradation of the protein by the cell. Similarly to primary airway fibroblasts, ADAM33 transfected HEK293 cells, when treated with TGFβ, upregulate the degradation of ADAM33 suggesting a common mechanism of degradation between the two cell types.

The ADAM33 transfected HEK293 line does provide some clues that ADAM33 may play a role in differentiation. The untransfected HEK293 parental cell line express αSMA constitutively. Treatment with TGFβ has no effect on either αSMA expression, or cell morphology. Interestingly, ADAM33 transfected HEK293 cells do not express αSMA at baseline but αSMA protein is induced with TGFβ treatment. This induction of αSMA coincides with a radical change in cell morphology. The cells transform from an "epithelial-like" appearance into swathes of "mesenchymal-like" elongated cells (Fig 6.6). From these data it appears that ADAM33 expression in embryonic epithelial cells prevents the native expression of αSMA but enables the cell to become capable of a radical epithelial-mesenchymal transition.

The change in cell morphology caused by ADAM33 expression in HEK293 cells may also provide a hint as to the function of ADAM33. Full-length ADAM33 expresses an EGF-like domain with potential adhesion or fusion activity. This domain has been shown to be functional in other ADAMs(Gaultier et al., 2002, Zolkiewska, 1999). Of particular note, ADAM12 has been shown to promote membrane fusion in myoblasts(Yagami-Hiromasa et al., 1995). The morpholgy of the ADAM33 transfected HEK293 cells are similar to that of a culture of cells with increased adhesive potential. Cell-cell contact was complete and confluence was 100%. A recent study observed a similar phenotype in NIH3T3 cells transfected with ADAM15(Herren et al., 2001). This study implicated ADAM15 with a role in cell-cell adhesion and showed ADAM15 to be localised to the points of cell-cell contact in

these cultures. Furthermore ADAM15 has been shown to interact with α9β1 integrins in a non-RGD dependent manner similarly to ADAM33(Eto et al., 2000). The parallels between the role of ADAM15 in these studies and what has been observed in this study are interesting and may provide insight in to the biological role of ADAM33.

What relevance for asthma?

These data provide a novel insight into the potential significance of ADAM33 in the asthmatic airways. It appears that ADAM33 expression is downregulated at both a mRNA level and to a nearly undetectable level at a protein in primary airway fibroblasts treated with TGFβ. This observation may prove a significant one in terms of asthma. TGFβ is a potent cytokine whose levels are raised in the asthmatic lung and are raised further upon damage to the epithelium during an asthma attack. This finding is therefore a potentially crucial gene environment interaction. If this effect of TGFβ on mesenchymal cells translates into an *in vivo* scenario, it is possible that ADAM33 levels are reduced in the asthmatic lung. Polymorphisms within the molecule may also alter stability of ADAM33 protein or mRNA. Such polymorphisms have the potential therefore to increase or decrease the expression of ADAM33 in the lung. It is all the more crucial therefore to understand the biological role of ADAM33 in the lung in order to evaluate the potential for pharmaceutical intervention to alter the expression of the molecule. These data show the degradation of ADAM33 protein is be a protease driven event. Hence if it were found to be beneficial such a protease may prove a potential novel therapeutic target to prevent the degradation of ADAM33.

Summary of results and novel findings

- Commercially available anti-ADAM33 antibody RP3 was able to detect low levels ADAM33 protein in primary airway fibroblast cultures suggesting that native ADAM33 protein is not abundant.

- TGFβ treatment of primary airway fibroblasts induced the myofibroblast phenotype and a subsequent down-regulation of the expression of ADAM33 protein.

- TGFβ treatment of primary airway fibroblasts or recombinant ADAM33 expressing cells results in the accumulation of ADAM33 degradation products.

- The degradation of ADAM33 is protease dependent and can be inhibited by treatment with protease inhibitors.

- Recombinant ADAM33 expression in HEK293 cells increases cell-cell contact and primes the cell for epithelial-mesenchymal transition.

Final discussion

Summary of findings

This thesis concentrates on the effects of TGFβ on cultured primary airway fibroblasts and contributes further to the understanding of the importance of TGFβ in the asthmatic airway as a modulator of inflammation and remodelling(Duvernelle et al., 2003). Expression of TGFβ mRNA and protein is increased in the asthmatic airways. Likely sources of this TGFβ are inflammatory cells, fibroblasts themselves and the abnormal asthmatic epithelium. TGFβ's role in the remodelling of the asthmatic airways is yet to be fully understood. A role for TGFβ as a promoter of excess ECM deposition and cell proliferation is well established whilst the extent to which TGFβ may cause phenotype switching is only just beginning to be fully appreciated.

This thesis provides a number of novel findings surrounding the process of fibroblast differentiation to a myofibroblast phenotype by treatment with TGFβ. Results chapter 1 examined the potential for primary airway fibroblasts to differentiate towards a smooth muscle phenotype. Firstly a thorough study into the stability of normalising gene expression was carried out. Micro-array data has previously shown a vast array of genes to be regulated in airway fibroblasts in response to TGFβ stimulation. Data from this thesis showed a number of "house-keeping" genes that are commonly used to normalise quantitative PCR data had their expression stability altered by TGFβ treatment. Hence a multiplex assay of phospholipase A2 and ubiqutin C, which were shown to be stably expressed and practically useful, was selected to measure gene expression accurately during the process of myofibroblast differentiation.

Using this normalising assay the expression of a number of "smooth muscle marker" genes was shown to be upregulated in response to TGFβ stimulation. This included the expression of markers of a differentiated smooth muscle phenotype such as heavy chain myosin and desmin. The upregulation of these genes and protein was greater in asthmatic fibroblast cultures than in cultures of healthy control cells. Observing disease related phenomena such as this might prove crucial in fully

understanding the pathogenesis of asthma. Although all the smooth muscle marker genes measured were upregulated at a mRNA level in response to TGFβ stimulation, an induction of functional protein expression was not detectable in all cases. Hence it was postulated that TGFβ treatment of primary airway fibroblasts primes the cell for further differentiation to a smooth muscle phenotype by the induction of large pools of mRNA for smooth muscle genes, but further stimuli is required to translate these mRNA in to functional protein. These findings add weight to other *in vitro* work (Chambers et al., 2003) as well as animal based studies(Buoro et al., 1993, Jones and Jacobson, 2000) that have suggested that the fibroblast may have the potential to act as a smooth muscle precursor.

The remainder of the thesis was concerned with furthering our understanding of ADAM33 expression and function. ADAM12 is a closely related ADAM that has been shown to be instrumental in the differentiation of myoblasts in to skeletal muscle. ADAM12 expression is upregulated as these precursor cells differentiate. Hence, it was hypothesised that ADAM33 expression in primary airway fibroblasts would increase upon TGFβ stimulation as the cells become more smooth muscle-like. However ADAM33 was shown to be down-regulated in a dose dependent manner in response to TGFβ treatment. Likewise careful analysis of ADAM33 splice variant expression showed the major alternatively spliced forms of ADAM33 mRNA were regulated in their expression in a manner that mirrors total ADAM33 mRNA expression. Extracellular matrix composition was shown not to substantially affect myofibroblast differentiation but growth on laminin and fibronectin resulted in a lower expression of ADAM33 mRNA compared to cells grown on plastic or collagen. This observation is noteworthy in that both matrices have been shown to promote the maintenance of a contractile smooth-muscle phenotype in cultured cells (Zhang et al., 1999, Hayward et al., 1995). The findings of this thesis show TGFβ to induce a more smooth-muscle-like phenotype in airway fibroblasts and particularly those from

a diseased donor. Coinciding with this phenotypic change is a down regulation in the number of ADAM33 mRNA transcripts. It is consistent therefore that a matrix (fibronectin or laminin) that has been shown to maintain a smooth-muscle like phenotype in cultured cells could consequentially lead to a down-regulation of ADAM33 mRNA.

Data from results chapter 1 highlighted the importance of measuring protein expression alongside mRNA expression data, as an increase in mRNA did not necessarily result in an increase in protein expression. Hence a major component of this thesis was to generate useful antibody preparations with which to assay ADAM33 protein expression. Antibodies from ADAM33 peptide immunised rabbits and chickens were shown to be reactive against their respective peptide sequences. Disapointingly however, despite the large investment of time and effort, these preparations were unable to detect full-length ADAM33 in transfected cell lysates. A commercially available anti-ADAM33 antibody became available at his time and was carefully characterised in this study. Data generated in results chapter 3 demonstrated that this anti-cytoplasmic tail antibody was able to detect full-length ADAM33 by western-blot and immunofluorescent staining.

Using this reagent the expression of ADAM33 protein in differentiating primary airway fibroblasts was assayed. Treatment with TGFβ was shown to reduce the overall-levels of ADAM33 protein as indicated by immunofluorescent staining. Subsequent western blot analysis showed an accumulation of a small ADAM33 protein upon stimulation of fibroblasts with TGFβ. Comparisons of this with recombinantly expressed ADAM33 in an ADAM33 negative cell line, showed this small ADAM33 protein to be a product of TGFβ induced degradation. This degradation was subsequently shown to be protease dependent, as it was abolished by pre-treatment with a cocktail of protease inhibitors.

Finally, human embryonic kidney epithelial cells that were transfected with full-length ADAM33 were shown to be morphologically very different to untransfected control cells. The transfected cells formed a dense monolayer similar in appearance to a culture of cells displaying increased adhesion to one another. This appearance of increased cell-cell adherence was different from the appearance of untransfected cells, which had markedly less cell-cell contact and never achieved 100% confluence. In addition the transfected cells underwent a substantial morphological change towards a more mesenchymal appearance when treated with TGFβ, whereas untransfected cells were unaffected.

What relevance for asthma?

Many of the increases in smooth muscle marker gene mRNA expression observed in TGFβ treated fibroblast cultures are substantially more abundant in populations of asthmatic fibroblasts than healthy controls. Observing disease related phenomena such as this may contribute to our understanding of the pathogenesis of asthma. If the shift towards a smooth muscle-like phenotype is of significance to the pathogenesis asthma, it is logical that these disease-related observations may be particularly significant.

If a *bone fide* transformation of fibroblast to smooth muscle does occur *in vivo* these data would indicate that this has the potential to occur more readily in asthmatic individuals than in healthy subjects due to the presence of a more substantial pool of mRNA. Further studies are required to establish if such a transformation does occur and the mechanism by which it may occur. Inhibition of such a mechanism would provide an exciting novel target for therapeutic intervention in to the progression of asthma.

The identification of TGFβ as a regulator of ADAM33 expression is a novel finding. This is the first work to identify any soluble factor that can regulate the expression of this asthma susceptibility gene. Although the changes in global expression of

ADAM33 in TGFβ treated fibroblasts appear small they may still be highly significant to the translation of what appears to be a relatively rarely expressed protein(Garlisi et al., 2003). TGFβ levels are known to be elevated in the asthmatic airways compared to the airways of healthy people. Hence these data might suggest that airway ADAM33 levels in asthmatics will be reduced. However, studies by investigators within our laboratory have shown ADAM33 levels to be similar in asthmatic bronchial biopsies compared to healthy control biopsies(Haitchi et al., 2005). The fact that both ADAM33 mRNA and protein expression are reduced during the process of TGFβ induced myofibroblast differentiation raises the hypothesis that ADAM33 may somehow inhibit myofibroblast differentiation, and that ADAM33 expression must first be reduced to allow the process to take place. In an attempt to test this hypothesis efforts have been made to generate an ADAM33 over-expressing fibroblast cell line. If the hypothesis were accurate such a line should not differentiate with TGFβ treatment. MRC5 fibroblasts were transfected with an ADAM33 full-length clone. G418 resistant cells clones were generated although for unknown reasons ADAM33 expression was not detectable in these clones. Subsequent similar experiments with alternative mesenchymal cell lines by other investigators within our laboratory have yielded the same result. These data are frustrating and difficult to interpret. The same ADAM33 clone was used to transfect the HEK293 cells used in this study. It appears then that cells of a mesenchymal origin cannot tolerate ADAM33 over-expression. A sensible approach to investigate this further would be to transfect the cells of choice with an ADAM33 clone driven by an inducible promoter. Thus any toxic or pro-apoptotic effects of ADAM33 expression could be studied in controlled conditions. Alternatively strategies to entirely block ADAM33 expression such as a siRNA approach could be used to investigate the effects of a complete absence of ADAM33 on myofibroblast differentiation.

The metalloprotease domain of ADAM33 has been shown to be catalytically active *in vitro* (Zou et al., 2003). Purified recombinant ADAM33 has been shown to cleave a number of synthetic peptides. Favourable experimental conditions and some substrate specificity data has been collected(Zou et al., 2005) but the native ligand of ADAM33 is still unknown. In asthma altered ADAM33 activity may effect processing of cytokines or growth factors. ADAMs have been shown to play such a role in other diseases. In cardiac hypertrophy for example, ADAM12's processing of HB-EGF is believed to drive the disease progression(Asakura et al., 2002). Inhibition of ADAM12 via a dominant negative construct or chemical inhibition inhibits the enzymatic cleavage of HB-EGF, preventing hypertrophy.

Data from this thesis confirm the recent published finding that ~95% of all ADAM33 transcripts found in primary airway fibroblasts do not contain the metalloprotease domain(Powell et al., 2004). These data are surprising in that the metalloprotease domain of ADAM33 *has* been shown to be catalytically active *in vitro*. Evolutionarily it would be very unusual for a functional domain such as this to remain but have no biological relevance. Of course ADAM33 may have distinct patterns of splicing and therefore play a distinct role in other tissue types that includes proteolytic activity. Alternatively the metalloprotease domain may be a potent enzyme that is highly regulated by the cell and hence is selectively spliced out in the majority of transcripts. Another possibility is that SNPs in an asthmatic may affect the splicing of the metalloprotease domain and therefore alter the abundance of these rare transcripts. However data from this thesis and other findings thus far have indicated that splicing is similar between asthmatics and healthy control cells. The absence of the metalloprotease domain from the vast majority of ADAM33 transcripts indeed raises some interesting questions about the biology of ADAM33. One conclusion we can draw from these data however is that the other domains of ADAM33 are in such relative abundance that they are likely to be important to the biology of ADAM33 in primary airway fibroblasts.

A recent study showing SNPs within ADAM33 to be associated with accelerated decline in lung function is the first data to implicate ADAM33 in the progression of asthma rather than just susceptibility to the disease(Simpson et al., 2005). This would indicate that altered ADAM33 function could affect fibrosis in the asthmatic lung, which is associated with decreased lung function(Chetta et al., 1997). Increased TGFβ expression in the asthmatic airways(Redington et al., 1997) is likely to be responsible for the increase in fibrosis(Kenyon et al., 2003). This thickening of the *lamina reticularis* results in a reduced elasticity of the airways and therefore reduced lung function in asthma. The myofibroblast is considered responsible for the thickening of the subepithelial basement membrane(Brewster et al., 1990). Data from this thesis shows ADAM33 expression to be reduced upon induction of the myofibroblast phenotype by TGFβ and hence suggests ADAM33 may play a protective role by the prevention of fibrosis. Altered ADAM33 expression or function in asthma may therefore lead to an increase in fibrotic activity.

Recently published data has shown that SNPs within ADAM33 can be predictive of impaired lung function in young children(Simpson et al., 2005). Carriers of the rare F+1 SNP (located in the metalloprotease domain) for example were shown to have reduced lung function at three years, whilst four SNPs (F+1, M+1, T1 and T2) were associated with reduced FEV1 at age five. These data suggest a role for ADAM33 in asthma development in early life or perhaps prenataly. Recent studies of ADAM33 expression in human embryonic lung have shown ADAM33 to be expressed predominantly in the undifferentiated mesenchyme but with some co-localisation with the differentiated smooth muscle in the developing airway wall(Haitchi et al., 2005). These data suggest a role for ADAM33 in the differentiation of mesenchymal cells in the developing lung and fits the model whereby altered ADAM33 function might lead to altered "modelling" of the airway wall. Such data is complimentary to data from this thesis which suggests a role for ADAM33 in mesenchymal differentiation. Both data are consistent in substantiating the link between ADAM33 and bronchial hyperresponsiveness.

Other data from this thesis draw distinct parallels with work on other ADAM proteins. NIH3T3 cells transfected to over express ADAM15, show a marked increase in cell-cell interaction and a morphology highly reminiscent of the HEK293 cells transfected with ADAM33 in this thesis(Herren et al., 2001). Furthermore this increase in cell-cell interaction has been shown to be due to interactions between ADAM15 and $\alpha 9\beta 1$ integrins via an RGD independent mechanism(Eto et al., 2000). To date the only integrin complex ADAM33 has been shown to interact is $\alpha 9\beta 1$ and is likely to occur via an identical RGD independent mechanism(Bridges et al., 2004). These findings therefore suggest a role for ADAM33 in cell-cell interaction, perhaps in the interaction between smooth muscle precursor cells prior to their fusion to become functional contractile smooth muscle. Such a scenario would be homologous to the role of ADAM12 in skeletal muscle. ADAM12 in myoblasts is central to the alignment and fusion of myoblasts to become mature skeletal muscle(Yagami-Hiromasa et al., 1995). This too is driven by ADAM12 interaction with $\alpha 9\beta 1$ integrin via an RGD independent mechanism(Lafuste et al., 2004).

ADAM33 via its EGF-like and cysteine-rich domains has the potential to adhere to ECM proteins. This thesis has demonstrated circumstances whereby ADAM33 expression is regulated by particular ECM components. The signalling mechanism underlying this regulation for example may be propagated by ADAM33 itself. Alternatively by these domains ADAM33 could play a role in cell migration. Fibroblasts are well known to migrate to sites of tissue damage and recently airway smooth muscle cells have been shown to have the capacity for migration. A molecule central to mesenchymal cell migration may therefore be involved in airway remodelling.

When considering the possible function of ADAM33 it is important to consider the potential role of the 5' end of the molecule. The cytoplasmic tail is predicted to be present in the majority of ADAM33 mRNA transcripts(Powell et al., 2004), and data from this thesis has shown it to be present in native ADAM33 in primary airway fibroblasts. The ADAM33 cytoplasmic tail is relatively short compared to its nearest homologues but still contains several motifs with potential function. The tail is rich in prolines and has a putative SH3 binding site that that may affect function. There is also a MAPK consensus sequence and casein kinase F/II phosphorylation site. Such motifs have the potential to alter ADAM33 function by phosphorylation via interaction with neighbouring membrane-anchored proteins or to propagate extracellular signals inside the cell.

Future work

Data from this thesis did not conclude that a *bone fide* transition from fibroblast to smooth muscle was possible, although the data was suggestive of such a possibility. Further studies to identify possible stimuli's for further differentiation is required to further this aspect of the study. Such stimuli may include other cytokines and chemokines although such studies have not previously shown a greater propensity to differentiate than TGFβ(Gailit et al., 2001, Richter et al., 2001). Alternative stimuli to try may include mechanical stimulation of the cells, which has previously been shown to be stimulus for differentiation in mesenchymal cell cultures(Yang et al., 2000). Mechanical stimulation of mesenchymal cells in culture has been shown to alter the cellular distribution of SRF factor and in particular an alternatively spliced form of SRF known as SRFΔ5(Yang et al., 2000). It is also believed that serum deprivation results in an upregulation of nuclear SRF expression which stimulates smooth muscle gene expression(Camoretti-Mercado et al., 2000). It would be interesting therefore to investigate the effects of prolonged serum deprivation on cultures of fibroblasts both untreated and pre-stimulated with TGFβ. Data from results chapters

1 and 2 showed no change in smooth muscle gene expression but an increase in ADAM33 gene expression respectively in cells deprived of serum for 48 hours. Classically a much longer (~30 days) period of serum deprivation is required to achieve a highly differentiated phenotype in smooth muscle cultures. It is possible that the slight increase in ADAM33 shown in serum free conditions is indicative of a change in cellular differentiation that would become more apparent in a longer experiment.

As discussed the phenotype observed in HEK293 cells transfected with ADAM33 is very similar to that of NIH3T3 cells transfected to over express ADAM15. This phenotype has been shown to be dependent on the interaction of ADAM15 with $\alpha 9\beta 1$ integrin. As $\alpha 9\beta 1$ is the only integrin shown to interact with ADAM33, it is possible that such an interaction is present in the phenotypically altered HEK293 cells. Disruption of this interaction may be possible by treating cultures of ADAM33 transfected HEK293 cells with anti-$\alpha 9\beta 1$ antibodies. Reversal to a wild-type phenotype would be a useful functional read out by which to assay the interaction of ADAM33 and $\alpha 9\beta 1$.

Preliminary studies by myself using a bronchial smooth muscle line (data not shown) have repeated the published observation that deprivation of smooth muscle cells in culture causes an upregulation of smooth muscle genes(Camoretti-Mercado et al., 2000). Alongside this ADAM33 mRNA expression increased ~2 fold. This is analogous to the upregulation of ADAM15 during the differentiation of myocytes(Lafuste et al., 2004). The authors of this observation reported an increase in $\alpha 9$ alongside the upregulation of ADAM15. Therefore it would be interesting to measure $\alpha 9$ mRNA expression in this model of differentiation as well as in the serum free and TGFβ treated fibroblast cultures. If these studies were able to prove a role for ADAM33 in the cell-cell adhesion of fibroblasts or smooth muscle cells prior to

fusion and the formation of functional smooth muscle bundles, this could be a function for ADAM33 that was altered/upregulated in a diseased state.

Novel therapeutic targets

The focus of this study is on the effects of TGFβ on cultured primary airway fibroblasts. Data generated shows asthmatic fibroblasts to differentiate upon $TGF\beta_2$ stimulation toward a smooth muscle phenotype to a greater extent than healthy control cells. If this is shown to be significant in the progression of remodelling in asthma, is TGFβ a viable target for therapeutic intervention? Cambridge Antibody Technology has trialled anti-$TGF\beta_1$ antibodies in humans as a treatment for Scleroderma. Locally administering TGFβ to the lungs is achievable by inhalation so such therapy could be considered in asthma. However, the functions of TGFβ are far reaching, a fact well illustrated by micro-array analysis which showed TGFβ to upregulate 146 genes by >2 fold in airway fibroblasts alone(Chambers et al., 2003). Inhibition of such a potent cytokine is likely to have as yet unseen effects. Such side-effects may be acceptable in life-threatening diseases such as scleroderma, but are unlikely to be accepted in a common disease such as asthma where mortality rates are relatively low. Furthermore TGFβ has been shown to have anti-inflammatory effects and thus inhibition of this effect in the airways may cause exacerbations of asthma rather than improving the condition. An alternative and probably more useful strategy may be the administration of IL-1β to the airway. IL-1β has been shown to cause a dose-dependent increase in apoptosis in rat lung fibroblasts(Zhang et al., 1997). These observations have also been observed in smooth muscle cell populations(Trinkle et al., 1992, Beasley et al., 1989). An upregulation of fibroblast and/or smooth muscle apoptosis, if well regulated, could have beneficial effects on the damaging aspects of airway remodelling seen in asthma such as sub epithelial fibrosis and increased smooth muscle mass. However it should not be ignored that

IL-1β is a potent pro-inflammatory cytokine that may exacerbate asthma via the induction of inflammatory pathways(Watkins et al., 1999).

This thesis provides detailed novel data about the expression of ADAM33 in primary airway fibroblasts and some data suggesting a role for ADAM33 in adhesion. To consider ADAM33 as a therapeutic target a greater understanding of the functional biology of the molecule is required. Also detailed analysis of the effects of SNP's within the molecule may yield crucial evidence surrounding the altered ADAM33 activity in diseased patients. Without such analysis it is not possible to determine if a gain or loss of function is responsible for the association of ADAM33 with asthma. And hence it is not known if inhibition or upregulation of the molecule is the therapeutic approach to consider. Whichever scenario is proven to be the case, data from this thesis may prove useful in targeting the regulation of ADAM33 expression. ADAM33 was shown to be degraded by an as yet unidentified protease. Pharmaceutical inhibition of such a protease might maintain ADAM33 levels if it were considered to be beneficial. Alternatively pharmaceutical intervention to upregulate protease activity could reduce ADAM33 levels.

Conclusions

Since the identification of ADAM33 as an asthma susceptibility gene(Van Eerdewegh et al., 2002) no individual SNP, has emerged as a candidate for the cause of ADAM33 dysfunction in asthma(Blakey et al., 2005). Such data may suggest that further study of the genetics of ADAM33 may not be the key to our understanding the role of ADAM33 in asthma. Functional studies are becoming more and more important. This thesis finds evidence of ADAM33 regulation during the process of myofibroblast differentiation by TGFβ. These findings complement recent work that shows ADAM33 expression in the developing embryonic mesenchyme(Haitchi et al., 2005) and data showing ADAM33 expression to be limited to mesenchymal and neuronal cells of the airway(Van Eerdewegh et al., 2002), in pointing towards a role

for ADAM33 in the abnormal remodelling of the airways in asthma. All of these data also complement the genetic findings that link ADAM33 to a greater extent to bronchial hyperresponsiveness than asthma alone.

There is still much to understand about this novel and exciting molecule and it's relationship with airway dysfunction in asthma, and in turn there is still much to understand about asthma as a whole. Our understanding of the structural "remodelling" changes to the airways in asthma is likely to become more and more important to the future of asthma treatment. Data from this thesis and recent literature would suggest that ADAM33 might play a role in these fundamental abnormalities. The association of ADAM33 with asthma, as well as a number of other genes in recent times, indeed heralds a new era in our comprehension of this complex disease and holds promise of an exciting and potentially beneficial new understanding of asthma.

Appendix

Clinical details of subjects used in this thesis

	Code	Age at Bronchoscopy	PC20 (mg/ml)	FEV1 (l)	FEV1 (%predicted)	Inhaled Steroid	SA β2 agonist	LA β2 agonist	Other treatment
Healthy Control subjects	1	21	>32	4.8	104	N	N	N	N
	2	20	>32	5.9	118	N	N	N	N
	3	21	>32	4.2	93	N	N	N	N
	4	20	>32	3.15	97	N	N	N	N
	5	21	>32	3.5	97	N	N	N	N
	6	21	>32	4.14	95	N	N	N	N
	7	30	>8	3.6	105	N	N	N	N
	8	53	>8	2.9	116	N	N	N	N
	9	37	>8	3.5	120	N	N	N	N
Asthmatic Subjects	1	20	5.7	2.9	83	N	Y	N	N
	2	20	1.49	3.05	75	N	Y	N	N
	3	30	1.47	3.1	90	N	Y	N	N
	4	42	0.53	2.24	76	N	Y	N	N
	5	21	0.5	2.4	70	Y	Y	N	N
	6	36	1.2	2.75	69	Y	Y	N	N
	7	34	0.16	2	70	Y	Y	N	N
	8	21	0.62	3.6	76	Y	Y	N	N
	9	46	0.04	2.7	67	Y	Y	Y	N

Buffers and Solutions

SDS page and Western blotting:

Seperation Gel Stock:

	7.5%	**12.5%**
30% (w/v) acrylamide/		
0.8% (w/v) bis acrylamide	22.5ml	30ml
1.5M Tris-HCl, pH 8.8	22.5ml	22.5ml
dH_20	44.6ml	37.1ml
20% (w/v) SDS	0.45ml	0.45ml

Stacking gel stock

30% (w/v) acrylamide/	
0.8% (w/v) bis acrylamide	12.5ml
0.5M Tris-HCl, pH6.8	25.0ml
dH_20	62.0ml
20% (w/v) SDS	0.50ml

Running Buffer

0.025M Tris	15.15g
0.192M glycine	72.0g
0.1% (w/v) SDS	25ml;20%SDS
pH 8.3 with HCL	
Made up to 5l with dH_20.	

5 x sample buffer

0.3125M Tris-HCl pH6.8	10.41ml;1.5M
50% glycerol	25ml
25% 2-mercaptoethanol	12.5ml
10% SDS	5g
0.01% bromophenol blue	5mg

Coomassie Brilliant Blue R-250

0.00125%(w/v) Coomassie Brilliant Blue R-250
45%(v/v) methanol
45%(v/v) dH_2O
10%(v/v) glacial acetic acid

Gel Destain

25%(v/v) methanol
10%(v/v) glacial acetic acid
65%(v/v) dH_2O

Western Blot Stripping Buffer

100mM 2-mercaptoethanol
2% (w/v) SDS
62.5mM Tris pH 6.7
Made up to 100ml in dH_2O

Western Blot Wash buffer

1 x PBS
0.5ml Tween 20/L
2% (w/v) dried low fat skimmed milk (Marvel)
pH 7.4 with HCl

Western Blot Blocking Buffer

1x PBS
0.5% (v/v) Tween 20
s% (w/v) bovine serum albumin
+ Inhibitors (Roche)

ELISA:

ELISA Coupling Buffer

Na_2CO_3	1.59g/l
$NaHCO_3$	2.93g/l
NaN_3	0.2g/l

ELISA wash buffer

1 x PBS
0.5ml Tween 20/L
pH 7.4 with HCl

ELISA blocking buffer 10x

dH_20	100ml
NaCl	8.0g
$Na_2HPO_4.2H_2O$	1.42g
KH_2PO_4	0.2g
KCl	0.2g
BSA Frac. V	5.0g

ELISA assay buffer

X1 blocking buffer	100ml
0.1% Tween-20	100µl

ELISA Chromagen diluent

Sodium acetate.$3H_20$	13.6g
dH_2O	1L
pH 6.0	

TMB stock solution

Tetra-methyl benzidine	30mg
Dimethyl sulphoxide	5ml

Chromagen solution

Chromagen diluent	12ml

TMB stock solution	200µl
30%H_2O_2	1.2µl

Buffers for ammonium sulphate precipitation

TE8 0.2M, pH 8.0

dH_2O	1L
Tris	24.2g
1M HCl	100ml
$Na_2EDTA.2H_2O$	3.72g

PE7.3 0.3M, pH 7.3

Na_2HPO_4	32g
KH_2PO_4	10.2g
$Na_2EDTA.2H_2O$	3.72g

Saturated ammonium sulphate, pH

TE8 0.2M	1L
$(NH_4)_2SO_4$	770g

Shaken and filtered.

Reagents used in this thesis and their source

Reagent	Supplier
7-AAD	Sigma Aldrich, Poole, UK
2-mercaptoethanol	Sigma Aldrich, Poole, UK
Acetic acid (Glacial)	Sigma Aldrich, Poole, UK
Acetone (Dry)	Sigma Aldrich, Poole, UK
Acrylamide	Bio-Rad, Herts, UK
Agarose	Sigma Aldrich, Poole, UK
Ammonium persulphate	Sigma Aldrich, Poole, UK
Ammonium sulphate	Sigma Aldrich, Poole, UK
anti-ADAM33 Cyto tail antibody	Triple biologica, UK
anti-ADAM33 MP antibody	Triple biologica, UK
anti-ADAM33 pro domain antibody	Triple biologica, UK
anti-αSMA antibody	Sigma Aldrich, Poole, UK
anti-Calponin antibody	Sigma Aldrich, Poole, UK
anti-Chicken HRP antibody	Sigma Aldrich, Poole, UK
anti-Desmin antibody	Sigma Aldrich, Poole, UK
anti-HCM antibody	Sigma Aldrich, Poole, UK
anti-mouse HRP antibody	Dako, Denmark
anti-rabbit HRP antibody	Dako, Denmark
Bis- acrylamide	Bio-Rad, Herts, UK
Bovine Serum Albumin	Sigma Aldrich, Poole, UK
Bromophenol blue	Sigma Aldrich, Poole, UK
Chloroform	Sigma Aldrich, Poole, UK
Coomassie brilliant blue R-250	Sigma Aldrich, Poole, UK
DAB substrate/buffer tablets	Sigma Aldrich, Poole, UK
DMEM	Sigma Aldrich, Poole, UK
DMSO	Sigma Aldrich, Poole, UK
DNase	Ambion, Austin, US
DNase inactivation slurry	Ambion, Austin, US
dNTP	Invitrogen, Paisley, UK
DPX	Sigma Aldrich, Poole, UK
ECL plus	Amersham, Buckinghamshire, UK
EDTA	Sigma Aldrich, Poole, UK
Effectene	Sigma Aldrich, Poole, UK
Ethanol	Sigma Aldrich, Poole, UK
Fibronectin	Sigma Aldrich, Poole, UK
Foetal Bovine Serum	Sigma Aldrich, Poole, UK
Fotospeed FX20 rapid fixer	Jay House Ltd, UK
Fotospeed PD5 print developer	Jay House Ltd, UK
Freund's complete adjuvent	Sigma Aldrich, Poole, UK
G418	Sigma Aldrich, Poole, UK
Glutamine	Sigma Aldrich, Poole, UK
Glycerol	Sigma Aldrich, Poole, UK
Glycine	Sigma Aldrich, Poole, UK
HBSS	Sigma Aldrich, Poole, UK

HCl	Sigma Aldrich, Poole, UK
Hyperfilm ECL	Amersham, Buckinghamshire, UK
Immunising peptides	Southampton PolyPeptides Ltd, Southampton, UK
Isopropanol	Sigma Aldrich, Poole, UK
Laminin	Sigma Aldrich, Poole, UK
Low fat dried skimmed milk	Marvel, UK
Mayer's Haemalum	Sigma Aldrich, Poole, UK
MEM	Sigma Aldrich, Poole, UK
Methanol	Sigma Aldrich, Poole, UK
Methanol	Sigma Aldrich, Poole, UK
MG132	Sigma Aldrich, Poole, UK
Nitrocellulose membrane	Amersham, Buckinghamshire, UK
Non-essential amino acids	Sigma Aldrich, Poole, UK
Normalising gene TAQman assays	EuroGentech, Belgium
Paraformaldehyde	Sigma Aldrich, Poole, UK
PBS	Sigma Aldrich, Poole, UK
pEGFP-n1 vector	ClonTech, CA, US
Penicillin	Sigma Aldrich, Poole, UK
Protease inhibitor cocktail	Sigma Aldrich, Poole, UK
Rainbow Molecular weight marker	Amersham, Buckinghamshire, UK
Random hexamer	MWG Biotech, Covent Garden, UK
Reverse transcriptase (MMLV)	Promega, Southampton, UK
SDS	Sigma Aldrich, Poole, UK
Sodium pyruvate	Sigma Aldrich, Poole, UK
Streptomycin	Sigma Aldrich, Poole, UK
SYBR Green	Oswell, Southampton, UK
TAQman Mastermix	EuroGentech, Belgium
TAQman primers	MWG Biotech, Covent Garden, UK
TAQman probes	Oswell, Southampton, UK
TEMED	Sigma Aldrich, Poole, UK
TGFβ2	Sigma Aldrich, Poole, UK
TO-pro3 blue	Molecular probes, Invitrogen, UK
Tris	Sigma Aldrich, Poole, UK
Tris-Borate-EDTA-buffer	Sigma Aldrich, Poole, UK
Tris-HCl	Sigma Aldrich, Poole, UK
Triton-X	Sigma Aldrich, Poole, UK
Trizol	Sigma Aldrich, Poole, UK
Trypsin	Sigma Aldrich, Poole, UK
Tween	Sigma Aldrich, Poole, UK
Ultraculture	Sigma Aldrich, Poole, UK
Vectashield	Vector Laboratories Inc. CA, US
Vitrogen 100	Nutacon, UK
Zenon alexafluor label	Molecular probes, Invitrogen, UK

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