The role of WNT5a in asthmatic airway epithelial repair

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By

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Abstract

The role of WNT5a in airway remodelling in asthmatic airway epithelial repair

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Asthma is heterogeneous disease characterised by distinct tissue molecular phenotypes (Choy *et al*, 2015). However, the process of repair and remodelling remains ambiguous in this context. Although previous reports have shown elevated protein and mRNA expression of WNT5a, there is limited evidence in the literature to the major source of and function of WNT5a in asthma. Furthermore, WNT5a acting through the non-canonical axis exhibits functional cross talk with TGF-β1, which may influence repair and remodelling in asthma.

We sought to evaluate protein expression of WNT5a and TGF-β1 in bronchial biopsies from a previously described cohort of subjects (9 healthy and 23 asthmatics) in whom aggregate gene signature profiles for Th2 and Th17 activity from tissue homogenates were available. After observing co-localised protein expression of both WNT5a and TGF-β1 in the epithelium, further investigations in BEAS-2B cells as a basal cell wound repair model were undertaken.

We observed increased WNT5a protein expression pattern *in vivo* in asthma. WNT5a protein expression was significantly elevated in the epithelium in Th17 gene expression high asthmatic biopsies and the lamina propria, but not the airway smooth muscle bundle. We found a significant correlation and co-localisation of protein expression between TGF-β1 and WNT5a immunostaining in the epithelium suggestive of crosstalk. Further evidence supportive of cross talk was that both TGF-β1 and WNT5a were shown to induce SMAD2/3 nuclear translocation, which was inhibited by BOX-5 (a WNT5a inhibitor). Furthermore, WNT5a increased $[Ca^{2+}]_I$ suggestive of non-canonical pathway engagement. Lastly, both WNT5a and TGF-β1 dual stimulation increased wound closure in BEAS-2B cells. Finally, stimulation of BEAS-2B cells with either TGF-β1 or WNT5a increased the expression of epithelial-mesenchymal transition (EMT) markers.

WNT5a protein is increased in the airway epithelium in patients with asthma displaying a mucosal Th17-dependent gene signature. Additionally, we show potential *in vitro* evidence of TGF-β1-WNT5a cross talk via the SMAD2/3 axis, promoting EMT and epithelial wound closure. This study potentially highlights a novel crosstalk pathway between WNT5a-TGF-β1 as a possible epithelial repair mechanism employed in asthma that warrants further molecular and functional characterisation.

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Abstracts and Presentations arising from this thesis

The role of WNT5a in Th17 asthma

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The role of WNT5A in airway remodelling in asthma

Daud T, Amrani Y, Bradding P, Siddiqui S

Infection, Immunity and Inflammation postgraduate conference, University of Leicester, 2014 2015 and 2016.

The role of WNT5A-TGF-β1 crosstalk in epithelial repair in asthma

Daud T, Amrani Y, Bradding P, Siddiqui S

Belfast, Leicester and Hull (BLH) Scientific Research Collaborative meeting, 2016

The role of WNT5A in airway remodelling in asthma

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Statement of work performed

Professor Salman Siddiqui, Professor Peter Bradding and the Respiratory Biomedical Research unit nurse team conducted patient recruitment, clinical assessment and bronchoscopies. Furthermore, Dr Matthew Richardson assisted in the extraction and analysis of Th-status and log ratio values for specific genes. Airway smooth muscle cells were harvested and characterised from bronchial biopsies by Dr Amanda Sutcliffe.

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List of Abbreviations

AHR	Airway Hyper Responsiveness
EMT	Epithelial Mesenchymal Transition
HBEC	Human Bronchial Epithelial cells
HASM	Human Airway Smooth Muscle
ECM	Extracellular matrix
ASM	Airway smooth muscle
IHC	Immunohistochemistry
IF	Immunofluorescence
TGF-β1	Transforming growth factor-β1
GMA	Glycol methacrylate
IL-13	Interlukin-13
IL-17A	Interlukin-17A
IL-5	Interlukin-5
FEV_1	Force expiratory volume in one second
FVC	Force vital capacity
ICS	Inhaled corticosteroids
GWAS	Genome wide association studies
BALF	Bronchoalveolar lavage fluid

DALY's	Disability adjusted life year's
SQS	Semi-quantitative score
PBMC	Peripheral blood mononuclear cells
PCP	Planar cell Polarity
ALI	Air-liquid interface
WIF	WNT inhibiting Factor
sFRP	Secreted frizzled related protein
LEF 1	Lymphoid enhancer-binding factor 1
TCF	T-cell factor
TAK-1	TGF-β1 activated kinase
SNP	Single nucleotide polymorphism

Chapter 1:

Introduction

1.1 A Brief History of asthma

The lungs are constantly exposed to the external environment and as a result they are susceptible to injury. Chronic non-communicable diseases have become the biggest cause of global mortality accounting for 60% of the deaths (Meetoo, 2008; Unwin and Alberti, 2006).

Asthma is one of the most common chronic respiratory conditions and affects approximately 5.5 million in the UK (300 million people worldwide) and is responsible for 1200 deaths per year in the UK (Levy, 2015).

Mentioned as early as 1550 BC in the Ebers papyrus in ancient Egypt, asthma has always perplexed clinicians in its heterogeneity and has historically been recognised as a condition associated with breathlessness and wheeze. The term asthma was first reported by Hippocrates (at around 400BC) (Keeney, 1964), and is derived from the Greek word 'ασθμα' meaning a short-drawn breath or panting.

Therapeutics linked to specific mechanisms responsible for airways dysfunction for asthma only became apparent in the beginning of the 20th century where John Auer and Paul A. Lewis (Auer and Lewis, 1910) described the bronchial smooth muscle as the phenomenon responsible for anaphylaxis, paving the way for muscle relaxing β_2-adrenergic drugs in the 1960's (Lands *et al*, 1967). Building on this, the subsequent discovery of glucocorticoids in the 1930's and their mechanism of action dampening the inflammatory response, made them efficacious in the treatment and management of asthma (Ellul-Micallef, Borthwick and McHardy, 1974; Brown,

Storey and George, 1972a). Nevertheless, over the next 50 years prevalence of asthma has continually soared and therapeutic intervention has largely remained unchanged with Inhaled corticosteroids (ICS) in conjunction with short/long acting beta-agonist (LABA) have become the cornerstone of asthma therapy in both national and international guidelines.

1.1.1 Current definition of Asthma

Building from the definition of Dr Henry Hyde Salter in the 19th century (Sakula, 1985), where he describes it as a "Paroxysmal dyspnoea of a peculiar character, generally periodic with intervals of healthy respiration between the attacks" with a "perverted nervous action". The current and evolving definition of asthma is updated annually with evidence-based and practice recommendations according to the global initiative for asthma (GINA), an organisation formed in 1989 to reduce asthma prevalence and consequently mortality and morbidity is;

"Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation." (Global Initiative for Asthma, GINA, 2018)

However, due to the incomplete understanding of the causes and mechanisms of asthma, the definition has switched from a nominalist to an essentialist definition. The nominalist referring to a cause of or component of the disease and is short, while the essentialist results to using words in an abstract fashion, which according to some (Scadding, 1996) has no place in

science (Hargreave and Nair, 2009). This is because the authors recognised that the essentialist definition recognised asthma as a separate disease entity and failed to identify primary defining characteristics of asthma. While the nominalist definition takes advantage of describing asthma as an abnormality of the airway function which may vary between and within patients taking into account the variation in airflow limitations and airway inflammation highlighting a precedent for subtyping asthma.

1.2 Asthma epidemiology and aetiology

The high prevalence of asthma sets the stage for its own awareness. To gauge an understanding on what is occurring in the organ, lung transplant studies have shown when asthmatic lungs are transferred to an individual without asthma, they can eventually develop asthma. Conversely, when asthmatic patients receive healthy lungs, they do not develop asthma even after 3 years of follow up since the transplantation (Corris and Dark, 1993). This suggests asthmatic lungs have an intrinsic property coupled with a strong genetic component, which manifests in a hazardous environment leading to a 'local' disease termed asthma.

1.2.1 Social and Economic burden of asthma

Nevertheless, Asthma is expected to increase in prevalence by 50% every decade (Braman, 2006) with an additional 100 million individuals afflicted by the disease in 2025 (Global Initiative for Asthma, GINA, 2004). The prevalence of asthma varies worldwide (Figure 2); this is supported by the ISAAC study that also showed a great variation of self-reported asthma

symptoms worldwide ('Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC)', 1998).

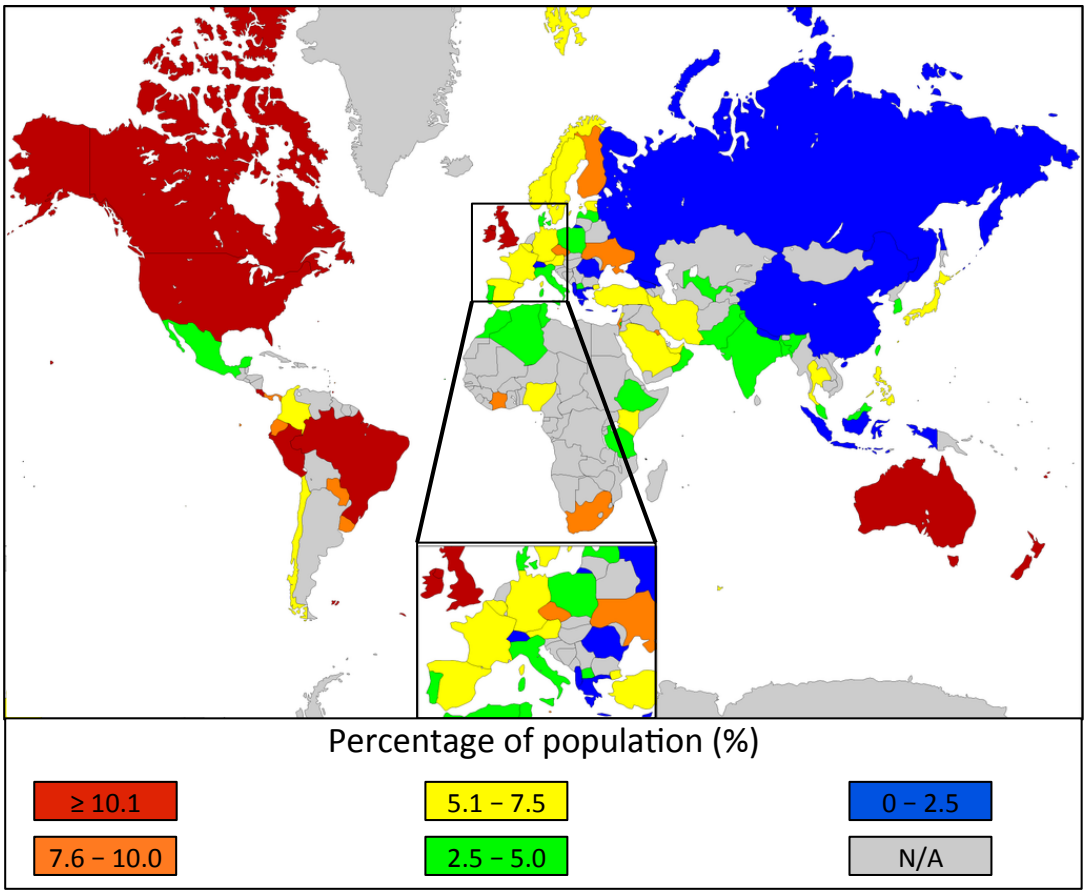


Figure 1: Global prevalence of asthma in individual countries depicted by percentage of population. Original image edited with permission from Author Armond under the Creative Commons CC0 1.0 Universal Public Domain Dedication.

Currently asthma is ranked 28th worldwide for leading cause of disability adjusted life years (DALY's), a measurement for the indirect cost of asthma. This accounted for 1% of DALY's (24 million years), which is higher than

breast cancer and chronic kidney disease (Murray et al, 2012). Even though there was a percentage change of 4.6% between 1990 and 2010 DALY's, the costs are exceeding into billions and can be disproportionately distributed on varying severity (Braman, 2006; Masoli et al, 2004; Levy et al, 2009; Barnes, Jonsson and Klim, 1996). In 2012 data showed 1160 deaths were a result of asthma, in addition costs were estimated at £1.1 billion for the NHS, with the major cost being attributed to the treatment of an asthmatic exacerbation (Mukherjee et al, 2016). This is also apparent in other developed countries such as the USA with a similar prevalence of asthma (proportion of population), where costs exceed $56 billion (Barnett and Nurmagambetov, 2011).

1.2.2 Risk factors for Asthma

Longitudinal studies interpreting the aetiology of asthma suggest it occurs early in life (Barbee et al, 1985; 'Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC)', 1998). Duffy and co-workers looking at pairs of twins from Australia, which of note has a high prevalence of asthma (figure 2), estimated that over 50% of cause for asthma was heritable (Duffy et al, 1990). Further observations revealed that individuals suffering from atopic diseases i.e. allergic rhinitis or atopic dermatitis would be more likely to have asthma. After the completion of the human genome project in 2003, a GWAS study was conducted for asthma and identified multiple markers on chromosome 17q21 associated with asthma (Moffatt et al, 2007). Further studies involving GWAS have provided novel data linking to the developmental causes of

asthma (Akhabir and Sandford, 2011; Moffatt et al, 2010). Particularly IL-33 which has recently been linked to Airway hyperresponsiveness and allergic inflammation, in part via the stimulation of innate lymphoid cells 2 (ILC2) (Barlow et al, 2013; Lund, Walford and Doherty, 2013). Identification of ADAM33 a gene encoding a disintegrin and metalloproteinase glycoprotein has also found to be associated with bronchial hyperresponsiveness (Van Eerdewegh *et al*, 2002), additionally, a combination of SNP's in the ADAM33 gene can result in a higher risk of asthma. ADAM33 can mediate the effects of airway wall thickening by increasing angiogenesis when in a soluble form but also disrupt cytokine and growth factor release (Cakebread *et al*, 2004; Mahesh, 2013).

On the other hand, in animal models (infant rhesus monkeys) it has been shown that exposure to an allergen or an environmental toxicant such as house dust mite or ozone can cause remodelling of the airway wall, once the cascade has been initiated, cessation of the toxicant exposure does not revert or protect against further damage (Plopper et al, 2007). There is also clear evidence that lower respiratory infections (LRI) occurring early in life can promote susceptibility to asthma (Welliver and Duffy, 1993). Furthermore, Hilty and co-workers looking at the microbiome of adult lungs showed striking results, not only the bronchial tree is covered with microbiota but also when compared with asthmatics this microbiota diverges with increases in proteobacteria (Hilty et al, 2010).

Likewise, increased use of household chemicals during pregnancy was associated with persistent wheezing and abnormal lung function in non-atopic

children (which does not say it cannot occur in atopic children) (Henderson et al, 2008). Interestingly this association was independent of the fact there was a likely increase in hygiene that can also be a candidate for asthma aetiology (Ramsey and Celedon, 2005). Additionally, studies investigating pollution and lung function in utero suggest that exposure during pregnancy can affect lung function but also hinder foetal growth (Hansen, Barnett and Pritchard, 2008; Latzin et al, 2009).

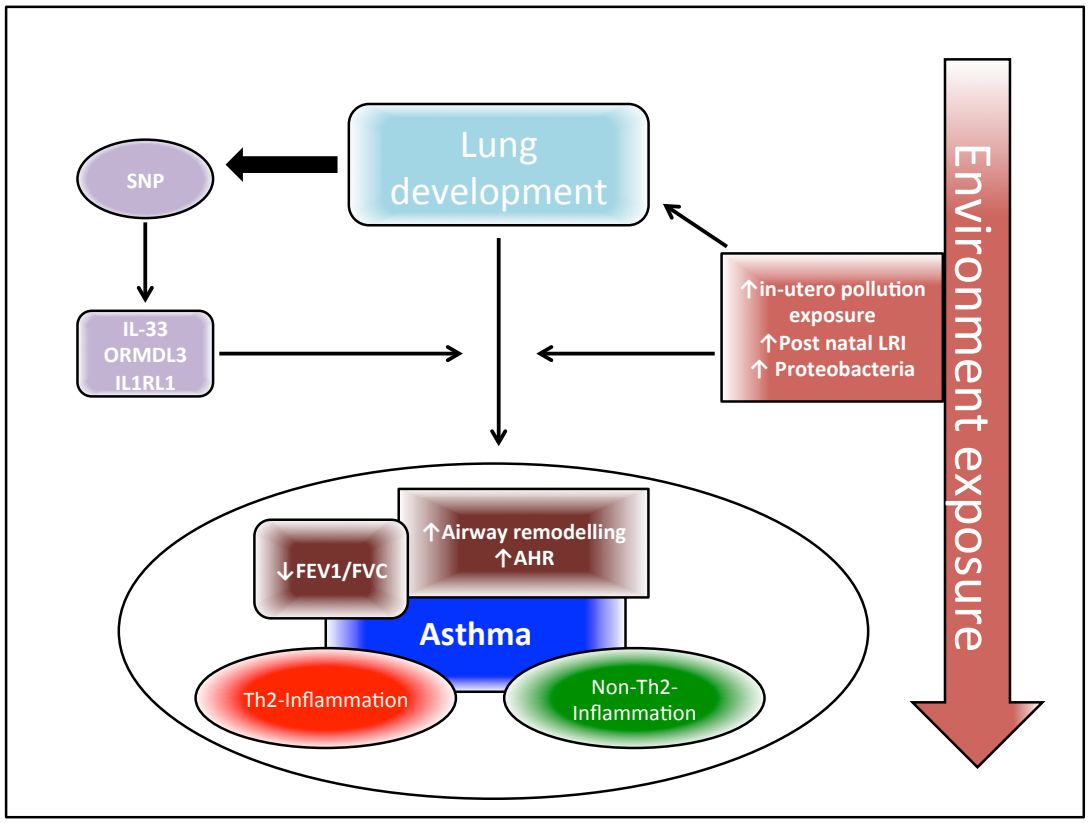


Figure 2: A diagram depicting the various risk factors from lung development, including genetic components and continuous environmental exposure that synergistically can lead to asthma.

In conclusion of the studies mentioned, it could be collectively deduced that asthma is a multifactorial disease where the pathogenesis has been attributed

to multiple gene-environment interactions (Schwartz, 2011) and SNP (Single nucleotide polymorphisms) in IL-33, ORMDL3 and ILR1 can skew normal lung development. Additional exposure from hazardous compounds from the environment can contribute to the pathogenesis of asthma (figure 3).

1.3 Asthma heterogeneity and sub-phenotypes

Asthma with an inflammatory pathophysiology gained pace in the late 18th and 19th century from the work of Paul Ehrlich describing aniline stains for eosinophils (Vyas and Krishnaswamy, 2006) and Sir William Osler who concluded clinical observations of airflow obstruction with a pathophysiological basis i.e. contractions of bronchial smooth muscle (Osler, 1895). The introduction of Endobronchial biopsies supported with observations from Bronchoalveolar lavage fluid (BALF), also contributed majorly to this paradigm shift to an inflammatory state (Holgate, Wilson and Howarth, 1992).

it was well appreciated since the mid-20th century that asthma wasn't just an allergic-inflammatory disease; rather it had an intrinsic component as well as the typical extrinsic (environmental) component (RACKEMANN, 1947). Exploring this heterogeneity, subsequent studies started to decipher that there isn't a difference in the mRNA levels of Th2 cytokines IL-4 and IL-5 in intrinsic and extrinsic asthma (Humbert *et al*, 1996) and as a consequence it could still not be concluded the diversity of pathophysiology displayed by asthmatic.

The discovery of mast cell activation through allergen exposure and subsequent recruitment of eosinophils through chemical mediators (Austen, 1974) provided a mechanistic evidence for asthma therapeutics. Using bronchodilators (to alleviate the bronchospasm) and glucocorticosteriods to reduce inflammation and subsequently improve lung function was the approach adopted for treating asthma, “a one size fits all” per se. Further progression in asthma heterogeneity came during the end of the 20th century, data in the literature started emerging that in the presence of inhaled corticosteroid (ICS) treatment, neutrophilic inflammation still persisted (Wenzel *et al*, 1997), but also glucocorticosteriods lacked efficacy in improving lung function (Pavord *et al*, 1999), particularly in 5-10% of the sufferers who have ‘refractory’ asthma (Holgate and Polosa, 2006), also referred to as steroid insensitive. This idea of asthma phenotypes otherwise, “observable properties of an organism that are produced by the interactions of the genotype and the environment” started to become established at the start of the 21st century and the true heterogeneity of asthma become more widely known (Wenzel, 2012).

Nevertheless, the current paradigm of asthma pathogenesis and physiology centres on the continuous exposure of pathogens and allergens to the external milieu, which can result in inhalation of various noxious agents. The airway epithelium a physical barrier is the first line of defence against the external milieu, with evidence of damage/shedding in asthmatics (Sumi and Hamid, 2007; Laitinen et al, 1985), most notably with loss of tight junctions (Xiao et al, 2011; Puddicombe et al, 2000) and increased susceptibility to

apoptosis (Trautmann et al, 2002; Bucchieri et al, 2002), the epithelium can facilitate the entry of pathogens and or allergic antigens initiating the inflammatory processes (figure 1).

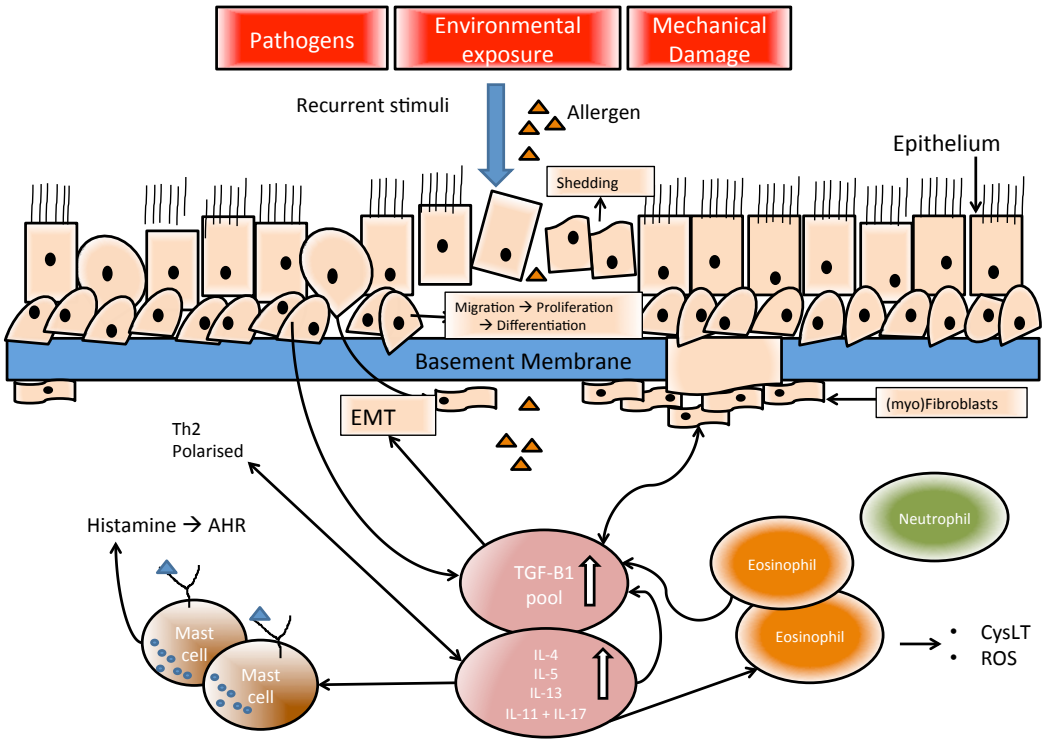


Figure 3: Depicting the inflammatory process initiated in the large airways after subsequent epithelial damage, resulting from an asthma exacerbating stimuli.

This is evident in asthmatic airways with a notable presence of inflammatory cells including but not limited to T-lymphocytes, eosinophil's and mast cells (Hamid *et al*, 2003; Brightling *et al*, 2002) releasing a plethora of cytokines (Chung and Barnes, 1999; Hamid *et al*, 1991; Robinson *et al*, 1992; Wills-Karp *et al*, 1998). It was this inflammatory cascade with multiple cells and mediators to blame the airway remodelling processes in asthma. Evidence of eosinophils and CD4+ T cells has shown to be increased after allergen

challenge in Bronchoalveolar lavage (BAL) with increased IL-4 mRNA expression. Furthermore, eosinophils correlated with a fall in FEV_1 (Robinson *et al*, 1993). This is supported by (Wardlaw *et al*, 1988) who previously showed a significant increase in eosinophils and major basic protein a substrate for eosinophils increased in symptomatic asthmatic BAL patients.

Further classification of asthma heterogeneity came by identifying patterns of granulocyte infiltration in induced sputum of asthma patients which showed a degree of variation, particularly eosinophilic, neutrophilic, mixed granulocytic and paucigranulocytic asthma. Interestingly 50% of asthmatic patients displayed eosinophilic while the latter represented a mix of granulocyte infiltration (Gibson, 2009). Collectively these data started to emerge subtypes of asthma with individuals displaying unique inflammatory cell-infiltration and how these could be utilised to evaluate clinical outcomes with treatment (Berthon *et al*, 2017).

Statistical methods started to help distinguish subgroups of asthma where Haldar and co-workers used cluster analysis on asthmatics in primary and secondary care. The classification of clinical phenotypes of asthma was done numerically, which eliminated any bias knowledge we have of the disease. What they found was similar to other reports (Moore *et al*, 2010) where age of onset was a key differentiating factor and early onset tended to be atopic, while late onset was mostly females and obese individuals (Haldar *et al*, 2008). Supporting this view of clinical heterogeneity (Moore *et al*, 2010) also showed 5 distinct clinical asthma phenotypes using cluster analysis. Similar to Haldar and co-workers there was a unique group of older obese females

with non-atopic asthma, but early-onset atopic asthma was differentiated by patients who either had increased medication use and subsequent health care utilisation or vice versa.

To see if similar to clinical heterogeneity, asthmatics at a molecular level also displayed heterogeneity Woodruff and colleagues in 2009 identified sub phenotypes of asthma, where the inflammatory mediators involved in asthma could be used to distinguish between different pathophysiology.

1.3.1 Th2-high asthma

Woodruff and his colleagues in 2007 found a novel method of identifying specific sub phenotypes of asthma. They conducted a high-throughput microarray analysis of asthmatic and healthy individual's genes in airway epithelial cells activated by a typical type 2-cytokine interlukin-13. Analysing the changes in gene expression, the authors established a gene signature of Periostin, CLCA1 and SerpinB2 as a surrogate for the effect of TH2 cytokines (Woodruff *et al*, 2007). In addition, they found analysing epithelial airway brushings from asthmatics and controls similarly, 50% of asthmatics displayed Th2-high inflammation, while the other 50% (Th2-low) were indistinguishable similar to controls. Furthermore, using bronchial biopsies they confirmed the Th2-high group was more characteristic of atopic disease with high airway eosinophilia and steroid sensitive, suggesting a Th2-high and Th2-low phenotype (Woodruff *et al*, 2009). The former phenotype has also been previously reported by (Wenzel *et al*, 1999), with both cases showing corticosteroid efficacy in atopic groups.

1.3.2 Th2-low asthma

Asthmatics with a high Th2-inflammatory phenotype account for 50% of the asthmatics and respond to current treatments. However not much is understood about asthmatics with a Th2-low inflammatory phenotype, besides having the typical diagnostic traits of an asthmatic and in addition high neutrophilic inflammation, being late onset and patients could also display steroid insensitivity (McGrath *et al*, 2012; Woodruff *et al*, 2009; Choy *et al*, 2011a; Fahy, 2015). From this Th2-low asthma phenotype a new asthma inflammatory phenotype has arisen with a Th-17 inflammatory profile. Deriving from naïve CD4+ T-cell and differentiated due to the presence of TGF-β1, IL-6 and IL-23 (Korn *et al*, 2009) cytokines present in asthmatics, Th-17 cells can secrete IL-17A, IL-22 and more in asthma (Ricciardolo *et al*, 2016). As a consequence Th-17 cells can promote neutrophilic inflammation through secretion of CXCL8 chemokine by epithelial cells, mucus cell metaplasia (Newcomb and Peebles, 2013), correlate with airway hyperresponsiveness (Barczyk, Pierzchala and Sozanska, 2003) and induce glucocotricosteriod insensitivity as witnessed in IL-17A pre-treated airway epithelial cells (Zijlstra *et al*, 2012).

Interestingly, using a similar approach as (Woodruff *et al*, 2009) and colleagues to identify a gene expression signature, (Choy *et al*, 2015) stimulated normal bronchial epithelial cells suspended in an air-liquid interface with 10ng/ml of IL-13 or 10ng/ml of IL-17A. In line with Woodruff et al., observation, they confirmed retrospectively that IL-13 in their cohort also induces the same Th2-gene expression signature of Periostin, CLCA1 and

sepineB2. In addition, stimulation with IL-17A induced a unique expression of neutrophil related chemokine's CSF3, CXCL1, 2, 3 and IL8, which was more pronounced in the presence of TNF-α. Extending this observation in previously reported gene expression microarrays of bronchial biopsies (Shikotra *et al*, 2012), the authors found that patient samples with detectable IL-13 or IL-17A mRNA by qPCR discriminated between Th2 and Th17 gene scores respectively.

1.3.3 T-helper inflammatory Th2 vs. Th17 asthma phenotypes

The following studies highlight that current asthma phenotypes can be categorised into a Th2-high or non-Th2 driven inflammation (i.e. Th17) (Woodruff *et al*, 2009; Choy *et al*, 2015), based on the composite of gene expression signature derived from mRNA of mucosal bronchial biopsies (Table 1).

Moreover, current evidence supports these Th-inflammatory gene signatures

Asthma Phenotype	Th2	Th0	Th17
Genes expressed	CLCA1, Periostin and SerpinB2	Unknown/no gene expression signature	CXCL1, CXCL2, CXCL3, IL-8 and CSF-3

Table 1: Genes associated with asthmatic phenotypes.

to be mutually exclusive and reciprocally regulated. This is supported by (Choy *et al*, 2015), where the authors show genes compromising either the Th17 or Th2-inflammatory profile score were negatively correlated and no

patients displayed overlap between the two gene signature scores or IL-13 and IL17A mRNA expression. Supporting the following observations, IL-22 a Th-17 cell cytokine can promote IL-17A production and neutralization of IL-22 by an antibody can enhance IL-13 production and subsequent eosinophil recruitment. Furthermore, IL-22 can also inhibit IL-13-mediated expression of cytokines such as IL-25 in lung epithelial cell line MLE-15 (Takahashi *et al*, 2011). Conversely, Th-17 cells have shown to express IL-13 receptor and when stimulated with 10ng/ml of IL-13 it can attenuate IL-17A production (Newcomb *et al*, 2011) .

The following data suggests therapies targeted towards a Th2-high inflammatory profile could in part be responsible for the emergence of a Th17-high inflammatory profile. This can occur in part by pro-type 2 cytokine IL-25, as blockade can inhibit neutrophil recruitment (Beale *et al*, 2014). Moreover, the neutrophils can increase IL-17A and IL-17F production when stimulated with Th17-cell derived cytokines (Halwani *et al*, 2017), this positive feedback loop can promote Th-17-phenotype that displays high neutrophils and increased steroid insensitivity as corticosteroids can enhance neutrophil survival *in vitro* by reducing apoptosis (Sampson, 2000) However, further studies are needed to show *in vitro* the emergence of a Th-17 inflammatory profile from a Th2-high state and if it is reversible, raising the question which side of the scale should the treatments favour for asthmatics?

1.3.3 Diagnosis and treatments of asthma

The symptoms of asthma typically consist of shortness of breath, the chest feeling tight, coughing and wheezing. These symptoms can have various

triggers, either by exercise (mechanical), irritant in a workplace (occupational) or by allergens and pathogens.

To effectively diagnose asthma a physician requires the history of the patient. This is because as mentioned earlier asthma is a heterogeneous disease resulting in a complex natural history and can vary demographically.

According to the NICE guidelines typical symptoms of wheeze, cough or breathlessness will be checked and any triggers that exacerbate these symptoms. If asthma is suspected, then clinical assessment is conducted based on objective measurements. This is because symptoms are non-specific and as highlighted by (Hargreave and Nair, 2009) quoting Lord Kelvin in 1891,

“When you can measure what you are speaking about and express it in numbers, you know something about it;”

Therefore, to assess asthma from a qualitative to a quantitative approach, the spirometer can measure static mechanics such as force vital capacity (FVC), where an individual is encouraged to breathe in (Inspiratory Capacity, IC) then asked to forcefully breathe out until the lung volume reaches the RV (residual volume), from which it cannot go any lower as the expiratory muscle force is insufficient to reduce transpulmonary pressure. Measuring dynamic mechanics involve FEV_1, which is the amount of volume of air that can be exhaled forcefully in one second.

Expressing FEV_1 as a percentage of FVC (FEV_1/FVC), a normal individual can force out >70% of the air inhaled in one second. A small FEV_1/FVC can be an indicative of an obstruction. However, in a restrictive disease as the

lung is less compliant (more stiff) and not easily distended, the FVC and FEV_1 decrease equally, appearing to be normal and in these cases absolute values must be obtained for the latter and other parameters need to be observed i.e. FRC, RV and TLC (Berne et al, 2008; Davies and Moores, 2010).

Asthma severity can be categorized by objective measurements (Table 2). This involves measuring the FEV_1/FVC ratio, a percentage of <70% indicates airflow obstruction. Reversibility of this obstruction can be confirmed using a bronchodilator and comparing spirometric parameters before and after. If the FEV_1 increased by ≥12% (or ≥200ml) this is considered significant reversibility. Further sensitive techniques can be employed for the assessment of bronchial hyper-reactivity; this involves measuring FEV_1 after inhalation of metacholine or histamine. If a concentration of <8mg/ml (PC_{20}) causes a fall of 20% in FEV_1, this confirms diagnosis of AHR.

Severity of asthma	Lung function	Symptomology
Intermittent	FEV1 ≥ 80% predicted FEV1 Variability <20%	Brief exacerbations Symptoms less than a week
Mild persistent	FEV1 ≥ 80% predicted FEV1 Variability <20 - 30%	Symptoms more than a week Exacerbations, sleep affected

Moderate persistent	FEV1 60 - 80% predicted FEV1 Variability >30%	Symptoms daily Exacerbations, sleep affected
Severe persistent	FEV1 ≤ 60% predicted FEV1 Variability >30%	Symptoms daily Frequent exacerbations Limited physical activities

Table 2: Listing the varying severities of asthma based on lung function and symptomology. Created from (Bateman et al, 2008).

Understanding the pathobiology of asthma has assisted in delineating the phenotype of asthma most responsive to treatment. With Th2 inflammation affecting 50% of asthmatics (Woodruff et al, 2009), diagnostic tests have been evolved for measuring eosinophilic inflammation. This is confirmed with >2% sputum eosinophil or forced exhaled nitric oxide (FE_{NO}) concentration of >25ppb at 50ml/sec, this is validated in 80% of untreated asthmatics (American Thoracic Society and European Respiratory Society, 2005; Pavord et al, 1997) and serves an important tool for clinicians to validate the efficacy of the treatment provided. Figure 4 can summarize the British thoracic guidelines on the management of asthma.

1.3.4 Current and future direction of Th-2 Asthma therapies

In 1948 Raymond P. Ahlquist displayed two different classes of adrenergic receptors (α and β) and created the foundation for the bronchodilators widely

used today. The inflammatory concept of asthma gave rise to the use of glucocorticosteriods, used as early as 1950's systemically, until refined and in 1972 when the first inhaled glucocorticosteriods were established for asthma control (Brown, Storey and George, 1972b; Clark, 1972). However, it is now widely accepted that glucocorticosteriods are most effective with eosinophilic inflammation as noted in Th2-High asthma (Woodruff et al, 2009; Haldar et al, 2008; Wenzel et al, 1999; Bhakta and Woodruff, 2011). This apparent when patients with asthma are treated based on sputum eosinophil count. A study manging treatments using the BTS guidelines or sputum management group (where anti-inflammatory treatment was increased or decreased based on eosinophil sputum count %) showed that severe exacerbations were reduced significantly (35 vs 109) and use of oral corticosteroids, however, no difference was observed in asthma quality of life questionnaire (AQLQ) lung function or β_2-agnonists over 12 months (Green *et al*, 2002). This is supported by (Jayaram *et al*, 2006) who also witnessed a reduction in eosinophilic exacerbations but not non-eosinophilic exacerbations. Interestingly the cumulative dose of corticosteroid was similar in both groups and benefited moderate-to-severe asthmatics. The difference in ICS treatment strategy by reducing sputum eosinophil count promoted the reduction of exacerbations (Chlumsky *et al*, 2006). However, ICS lack the efficacy to reduce airway remodelling, particular aberrant deposition of extracellular matrix (ECM) proteins.

A study by (de Kluijver et al, 2005) looked at mild asthmatics exposed to low-dose allergen with ICS or placebo. The results showed an increase in reticular

basement thickening in asthmatics vs. controls and that ICS treatment had no effect, further the steroids had the ability to alter expression of proteoglycans (↑biglycan and versican) but have no effect on fibronectin, lumican and decorin. In line with this (Bergeron et al, 2005) conducted a study on mild-moderate asthmatics before and after a 6-week course of treatment with inhaled steroids. The results also supportive showed that ICS could not modulate the deposition of collagen or TGF-β1 expression. (Boulet et al, 2000) described a similar observation where ICS had no effect on collagen I and collagen III deposition. Additionally, investigating airway wall thickness using a helical computed tomography (CT) and measuring sputum TGF-β1 by ELISA; (Yamaguchi et al, 2008) showed in asthmatics treated with ICS, there was still elevated TGF-β1 in sputum, a thicker wall and more importantly, TGF-β1 positively correlated with wall thickness and negatively correlate with FEV_1.

As outlined current treatments do not display efficacy against fibrotic remodelling features of asthma (Jeffery et al, 1992a). Furthermore, individual with the more severe form of asthma ('refractory asthma') can be glucocorticosteriods insensitive (Corrigan and Loke, 2007). Recent appreciation of asthma heterogeneity has helped addressed this issue and given rise to new anti-biologic therapeutics.

Mepolizumab is an anti-IL-5 monoclonal antibody that can prevent the activation of eosinophils by effectively mopping up IL-5. This is a more attractive option for individuals insensitive to glucocorticosteriods. Nevertheless, recent trails have elucidated that IL-5 cannot affect the

thickness and density of pro-collagen III and lumican; however, it can reduce the levels of profibrotic cytokine TGF-β1 (Flood-Page et al, 2003a). Furthermore, as eosinophils are the major source of TGF-β1, treatment with Mepolizumab reduces eosinophils in sputum, and subsequent exacerbations, however it cannot affect lung function even after cessation of treatment (Haldar et al, 2014). Other monoclonal antibodies such as omalizumab an anti-IgE showed enhanced efficacy in reducing seasonal exacerbations and reduced ICS use to control asthma, however again it had no effect on lung function (Busse et al, 2011). Conversely, clinical trials investigating benralizumab an anti-IL5R binds to the IL-5 receptor and blocks eosinophil proliferation and activation showed improvement in FEV_1 over 52 weeks but not in trails with a duration of 28 weeks. Furthermore, it can reduce oral corticosteroid intake, reduce exacerbation and most importantly reduce blood eosinophils, but further studies are required to determine the effects of benralizumab on structural airway remodelling (Pelaia *et al*, 2018).

Together these findings suggest current therapies targeting inflammation are not sufficient to reduce remodelling features observed in Th2-high asthma moreover a reliable biomarker does not exist to evaluate the remodelling features in question. This is possibly because, even though remodelling features are instigated by chronic inflammation, once initiated they occur in parallel to inflammation. This is clear when anti-inflammatory agents i.e. anti-IL-5 and anti-IgE can reduce the population of inflammatory cells but not the features these cells can initiate.

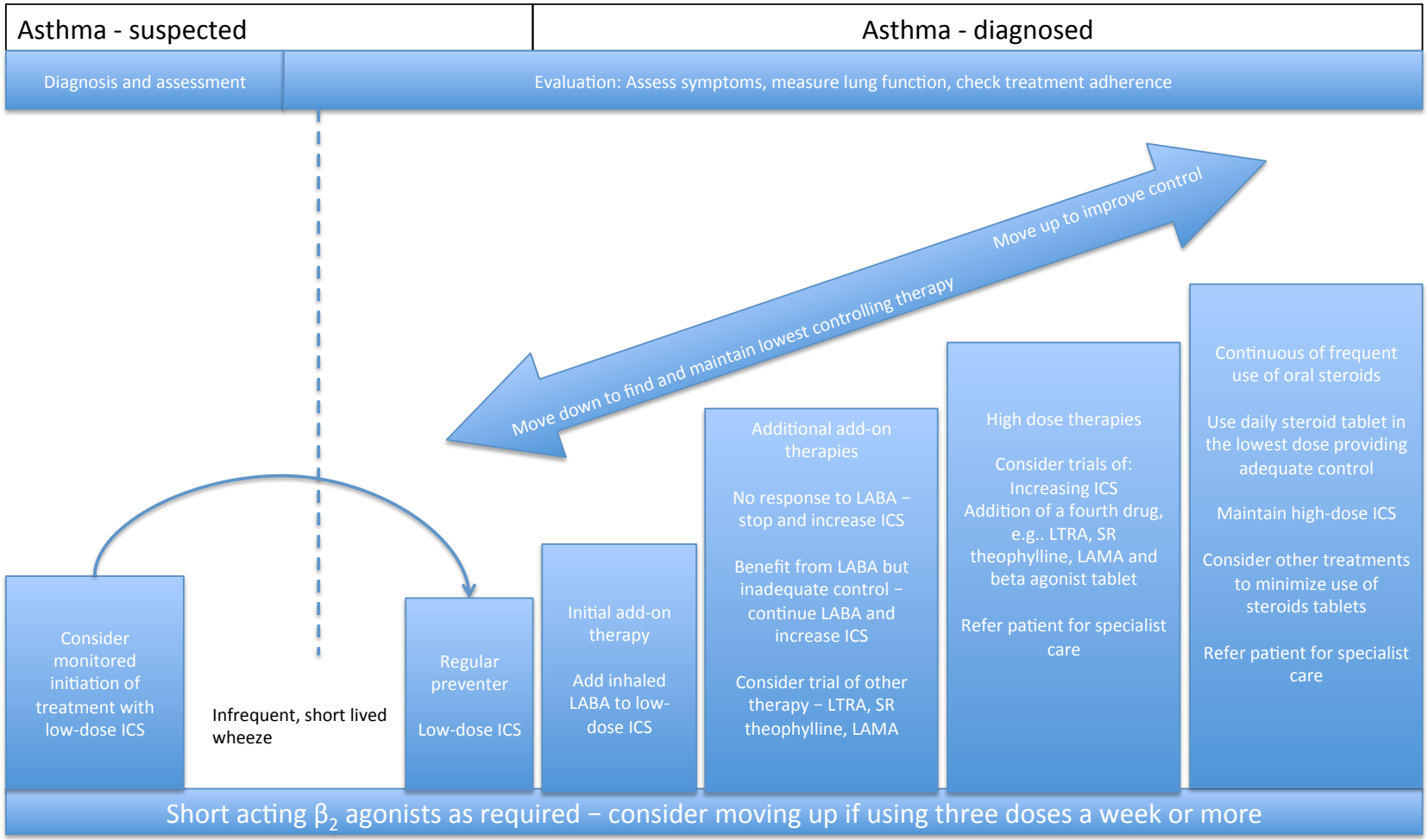


Figure 4: Diagram depicting the process of treating and managing asthma following the BTS guidelines 2016.

1.4 Airway remodelling in asthma

Airway wall remodelling is a well-established phenomenon in asthma and varies greatly between individuals suffering from the disease. Huber and Koessler first described airway wall remodelling in 1922 in 21 cases of fatal asthma (individuals who died due to asthma). Airway remodelling is a term that encompasses the alterations in the structural cells and tissues from the norm and occurs disproportionately throughout the lungs. Looking at the anatomy of the lungs the diversity in remodelling can be attributed to the variety of the lung composition as depicted by figure 5.

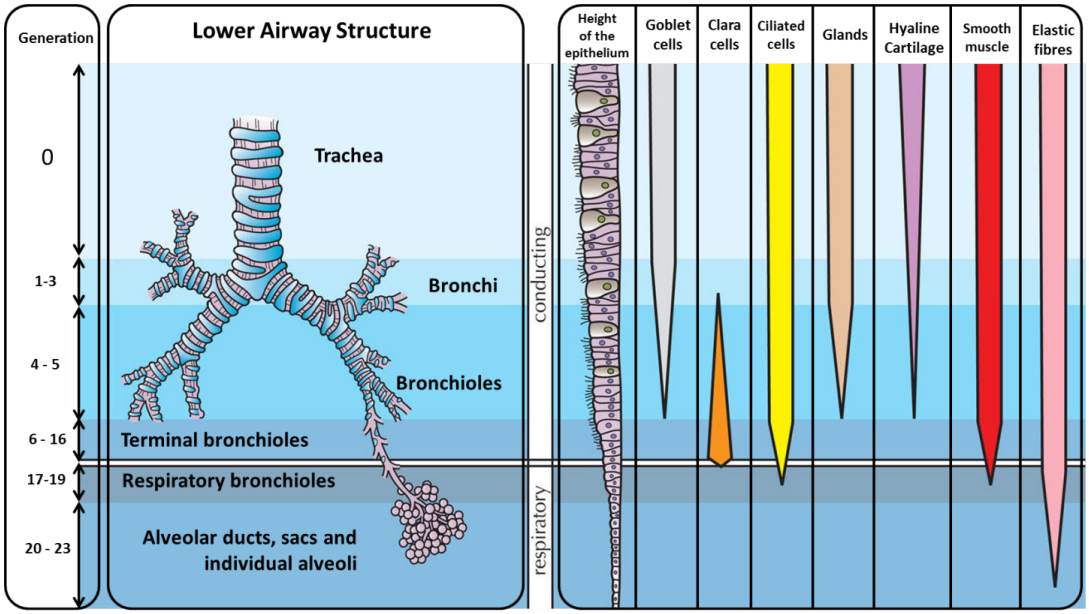


Figure 5: Depicting the different generation of airways and subsequent changes in composition of cellular elements. Original image edited with the permission from Author Holly Fischer under the http://creativecommons.org/licenses/by/3.0/ licence.

Anatomically speaking the airways are distributed into the upper airways and lower airways, with the later containing the lungs. Ssubsequently, the lungs can be further divided into large/central and small/peripheral airways of which successive generations of the smaller airways (generation ≥6) have a diameter of ≤2mm. As a consequence, this has made investigating small airways difficult as no safe invasive techniques currently exist to obtain biopsy from patients. Nevertheless, small airways have shown to play an important role in asthma, with increased levels of IL-5 and IL-4 cytokine mRNA and increase in eosinophil's and T-cells, interestingly asthmatic small airways of <2mm diameter also contain more activated eosinophil's than larger airways of >2mm diameter (Tulic, Christodoulopoulos and Hamid, 2001). Furthermore considered a 'quiet zone' of the lungs by (Mead, Takishima and Leith, 1970) as it only accounted for <10% of total airflow resistance, recent data (Usmani *et al*, 2016) highlights small airways can contribute to airway hyper-responsiveness through methacholine challenge and as a consequence influence airway resistance (Wagner *et al*, 1990).

On the other hand, the discovery of flexible bronchoscopies has resulted in well-characterised large airways, showing stark differences between asthmatics compared to healthy control. This large wealth of data on large/proximal airways has allowed us to compartmentalise the remodelling features observed as discussed below.

1.4.1 Epithelial remodelling

Constantly exposed to the external environment the epithelium is the first point of contact to noxious stimuli. Forming a pseudo-stratified layer (as each

epithelial cell is actually tethered to the basement membrane), the epithelium can be categorised with an apical and basal layer figure 6.

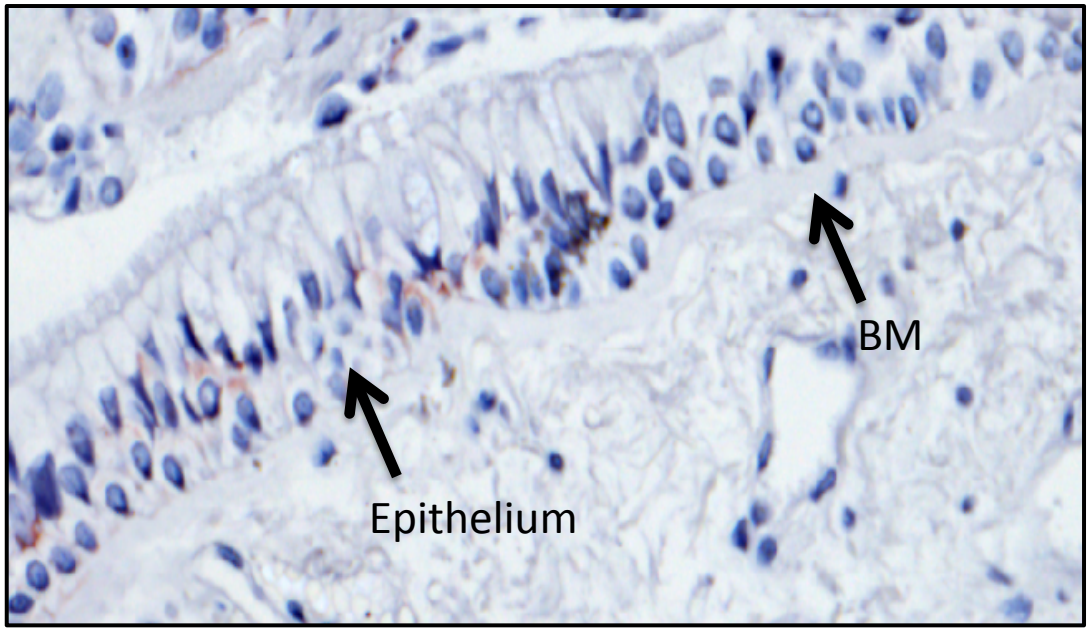


Figure 6: Pseudo-stratified epithelial layer stained with cytokeratin-5 (red) at X400 magnification, arrows indicate the epithelium and basement membrane.

The ciliated epithelial cells covered with a mucus layer can help trap various micro particles and pathogens and the co-ordinated beating of the cilia can as a consequence keep the airways clean. Furthermore, the airway can replenish itself from basal cells that act as a niche of stem cells, which can differentiate (Borthwick *et al*, 2001).

However in asthmatics there is evidence of damage/shedding in asthmatics (Sumi and Hamid, 2007; Laitinen *et al*, 1985) most notably with loss of tight junctions (Xiao *et al*, 2011; Puddicombe *et al*, 2000) and increased susceptibility to apoptosis (Trautmann *et al*, 2002; Bucchieri *et al*, 2002) the

epithelium can facilitate the entry of pathogens and or allergic antigens (Figure 1) initiating the inflammatory processes.

Repair involves various stages, leading from basal cell phenotypic changes to directional migration and lastly, restoring basal-apical polarity (Figure 7).

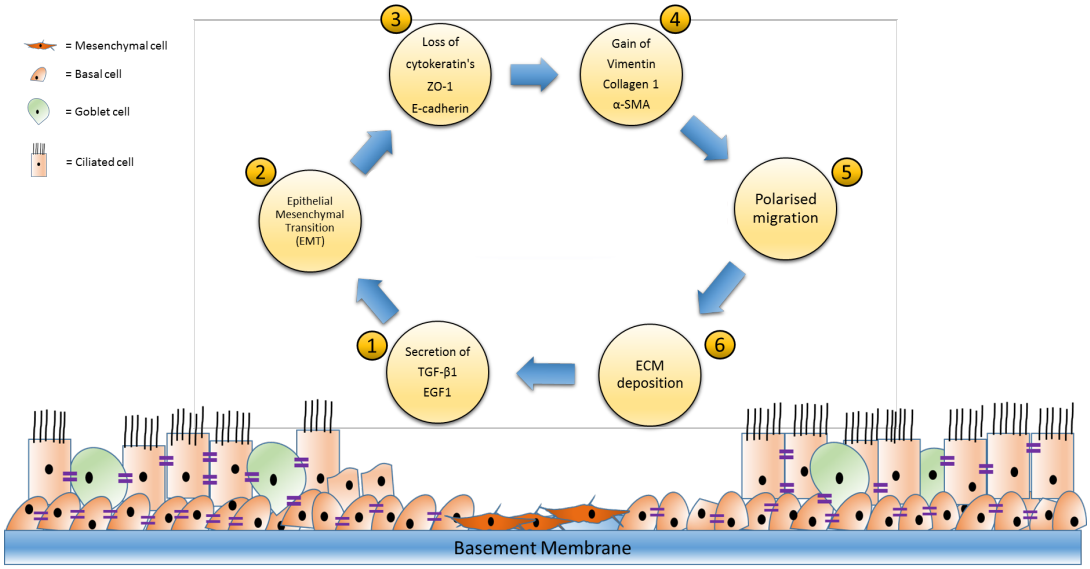


Figure 7: Depicting the epithelial restitution process. Briefly, (1) damage to the epithelium results in secretion of growth factors (i.e. TGF-β1), (2) initiating epithelial to mesenchymal transition (EMT). This can result in (3) a loss of epithelial markers and (4) gain of mesenchymal markers. (5) The differentiated cell migrates into the wound area and consequently (6) deposits extracellular matrix proteins (ECM).

For the epithelial repair program to occur, growth factors such as TGF-β1 increased in asthmatics are secreted (Flood-Page *et al*, 2003b; Duvernelle, Freund and Frossard, 2003; Howell and McAnulty, 2006) leading to changes such as epithelial-mesenchymal transition (EMT) in basal cells (Hackett *et al*, 2009; Halwani *et al*, 2011; Magnan *et al*, 1994; Jetten, Shirley and Stoner,

1986; Redington *et al*, 1998) This results in the gain of various fibrotic-related cell markers within 48 hours, such as α-smooth muscle actin, collagen and vimentin and loss of typical epithelial markers such as cytokeratin and tight junction cadherin's. Following EMT, the epithelial cell is primed for migration, subsequent wound closure and ECM deposition and WNT5a investigated in this project may facilitate this.

1.4.2 Extracellular matrix remodelling

Evidence of sub epithelial fibrosis has been shown in the large and small airway (James et al, 2002) and consequently affects the composition of the airway wall shown in figure 8. Many studies investigating Endobronchial biopsies in asthmatics have witnessed thickening of the lamina reticularis (basement membrane) (Sobonya, 1984; Wilson and Li, 1997; Hoshino, Nakamura and Sim, 1998; Benayoun et al, 2003; Chu et al, 1998; Cho et al, 1996; Tomkowicz et al, 2008; Chetta et al, 1997) . The composition consists of collagen type I and III, laminin, tenascin, and fibronectin, of which all are increased in asthmatics (Chakir et al, 2003; Hoshino et al, 1998; Jeffery et al, 1992b) as shown in table 3.

Source	Type of Remodelling	Asthma vs. Healthy/Control
(Sobonya, 1984)	Increased Bronchial Basement membrane thickness ($p<0.01$)	Asthmatics vs. Control
(Roche *et al*, 1989)	Increase in interstitial collagen beneath the Basement membrane	8 Asthmatics vs. Control
(Hoshino *et al*, 1998)	Increased deposition of Collagen III, V and tenascin in reticular basement membrane	25 Asthmatics vs. 10 Healthy
(Wilson and Li, 1997)	Increased RBM thickness and increased collagen III and V in the submucosa	15 Asthmatics vs. 13 Healthy
(Hoshino, Nakamura and Sim, 1998)	The true basement membrane was preserved (basal lamina). Increased thickness in the lamina reticularis (RBM)	21 Asthmatics vs. 8 Healthy

(Benayoun *et al*, 2003)	Subepithelial basement membrane thickness increased in all categories of asthma. Collagen III deposition only increased significantly in severe asthma.	10 Control vs. 10 Intermittent Asthma 15 Mild-moderate asthma 15 Severe asthma 10 COPD
(Chu *et al*, 1998)	No significant difference observed in subepithelial basement membrane thickening measured by collective collagen, collagen I and III. Overall group of Asthmatics (n=33) had a higher SBM than control. No significant difference in total submucosa collagen deposition.	8 Control vs. 7 Mild asthmatics 9 Moderate asthmatics 17 Severe asthmatics
(Cho *et al*, 1996)	Basement membrane thickness significantly different in moderate and severe asthmatics. No difference between mild asthmatics and controls	4 Controls 4 Mild asthmatics 4 Moderate Asthmatics

		5 Severe Asthmatics
(Jeffery *et al*, 1992b)	Increased basement membrane reticular collagen (lamina reticularis) in both asthma groups (P<0.01)	12 Healthy 11 Mild 'atopic' asthma 10 Severe asthma
(Huang *et al*, 1999)	Increase in proteoglycan deposition in the subepithelial layer, which includes versican (P 0.06), Lumican (P<0.05) and biglycan (P<0.05).	7 Asthmatic vs. 6 Control
(Tomkowicz *et al*, 2008)	Total basement membrane (TBM) thickness increases with asthma duration. No correlation between TBM thickness and collagen III. No significant subepithelial collagen III expression between the groups	13 Control vs. 18 Recently Diagnosed Asthma (untreated) 16 Long standing asthma with ICS treatment

(Chetta *et al*, 1997)	Increased subepithelial layer thickening vs. controls, the degree of thickening correlated with disease severity and have a negative correlation with lung function. No difference regarding the length of asthmatic history or atopy.	8 Healthy vs. 14 Mild asthmatic 14 Moderate asthmatic 6 Severe Asthmatic

Table 3: Highlighting multiple studies investigating ECM remodelling in asthma.

Deposition is also seen in the lamina propria (Huang et al, 1999; Minshall et al, 2000; Roche et al, 1989; Wilson and Li, 1997) and around the airway smooth muscle (Araujo et al, 2008; Dekkers et al, 2009; Pini et al, 2007) .

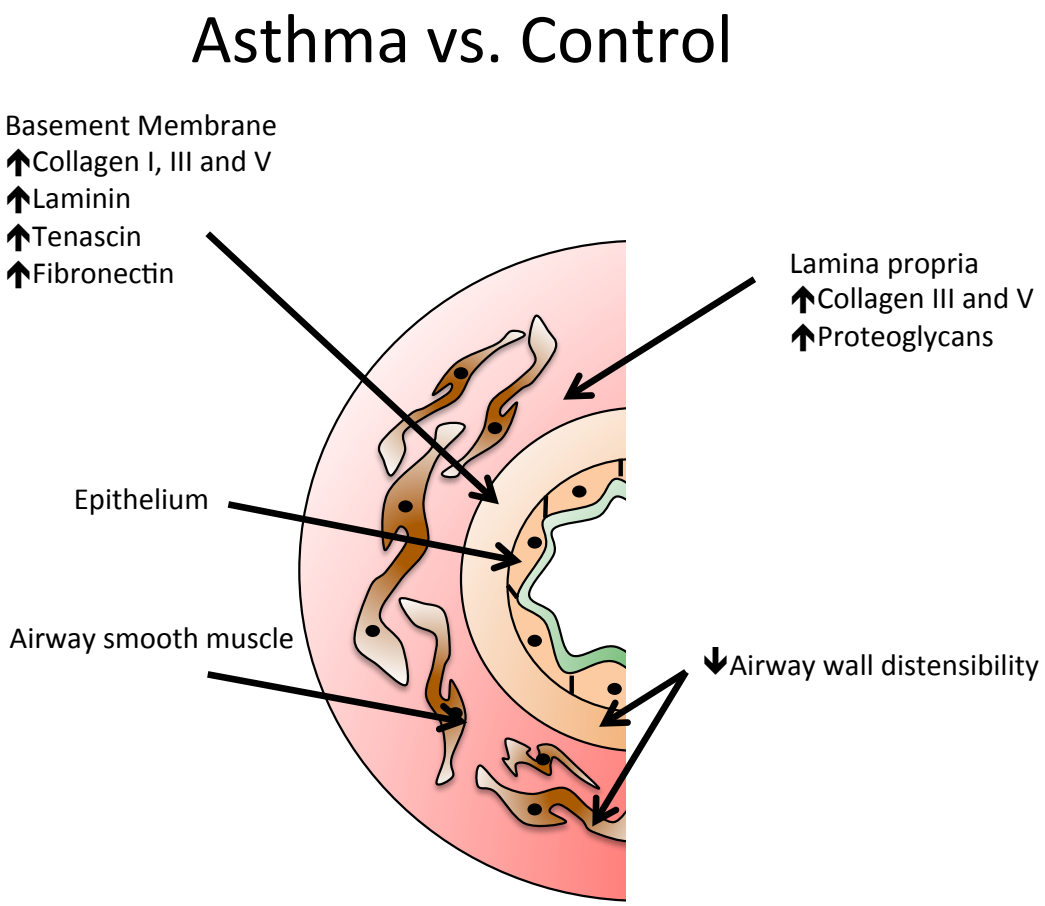


Figure 8: A diagram highlighting the structures of the airway wall where excessive deposition of ECM occurs.

Excessive ECM deposition tends to be associated with disease severity (Cho et al, 1996; Chetta et al, 1997; Benayoun et al, 2003) and particularly much higher with the atopic Th2-high eosinophilic inflammation phenotype (Amin *et al*, 2000; Woodruff *et al*, 2009; Wenzel *et al*, 1999). Various hypothesis suggest different functional relevance of airway wall thickening by ECM, (1)

can cause fixed airflow obstruction and subsequent loss of reversibility (2) negatively correlated with lung function (3) increase airway resistance resulting in reduce airway calibre (4) reduce lung compliance and subsequent distensibility to fight against the hyper contractile ASM.

The candidates for ECM deposition by structural cells are airway smooth muscle (Kumawat *et al*, 2013), fibroblasts and epithelial cells (Yasukawa *et al*, 2013) as depicted in figure 1. The pro-fibrotic signalling molecule implicated is TGF-β1. Increased in Th2 asthmatics, TGF-β1 activity can be enhanced in the presence of IL-11 and IL-17, also increased in asthmatic airways (Minshall *et al*, 2000; Molet *et al*, 2001). TGF-β1 can increase fibrotic remodelling by inducing EMT in epithelial cells (Hackett *et al*, 2009) and cause ASM to increase production of ECM proteins (Kumawat *et al*, 2013). Unfortunately therapeutics for anti-TGF-β1 is not viable as this profibrotic cytokine can have a pleotropic effect, most notably in asthma (Howell and McAnulty, 2006). It can induce production of resolvins for resolution of inflammation, have immunosuppressive effects on Th2 proliferation (Akhurst and Hata, 2012), but also inhibition can lead to cancer (Roarty *et al*, 2009).

Nevertheless, the true nature of excessive ECM deposition whether beneficial or detrimental and the mechanism behind it remains poorly characterised.

1.4.3 Inflammation

Initiated by inhalation of noxious stimuli, epithelial cells can trigger the release of cytokines including but not limited to IL-25, IL-33 and TSLP (Shikotra *et al*, 2012) recruiting inflammatory cells such CD4+ T-cells (Lloyd and Hessel,

2010) and ILC2 cells (McKenzie, 2014). Which in turn can amplify the inflammatory response and recruit other cells.

1.4.3.1 Mast cells

Mast cells are granulated immune cells as shown in figure 9 that can secrete various biological mediators ranging from proteases, histamine cytokines and more. Responsible for a myriad of functions of them the most notable is binding of IgE immunoglobulins to the high affinity FcεRI receptor located on mast cells.

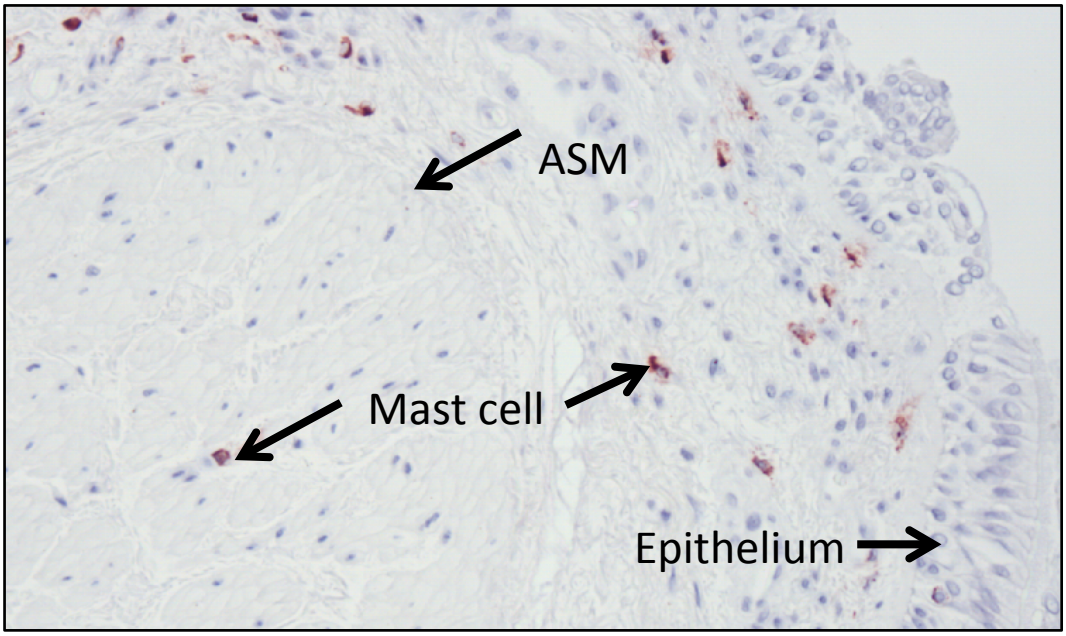


Figure 9: Mast cell stained with anti-tryptase (red) at x400 magnification, arrows indicate positive stained mast cells in ASM, epithelium and lamina propria.

The IgE primed mast cells can be triggered to secrete its mediators once the IgE molecules antigen recognising surface binds to an allergen. In asthma we find mast cells located in airway smooth muscles as a defining feature

(Bradding and Brightling, 2007; Brightling *et al*, 2002). Expressing CXCR3 receptor mast cells can migrate into the muscle bundle through activation of CXCL10 secreted by ASM (Brightling *et al*, 2005). In addition to mast cells being located in ASM, it is the only remodelling feature associated strongly with AHR (Siddiqui *et al*, 2008a), as histamine is a potent agonist for ASM contraction.

1.4.3.2 Eosinophil's

Eosinophils are increased in asthma particularly in individuals with a Th2 phenotype. Sourced from peripheral blood, eosinophil's can migrate into the lung by the release of chemokine eotaxin (Ponath *et al*, 1996). Once in the lungs the Th2-high environment can activate eosinophils with IL-5 (Yamaguchi *et al*, 1988). These activated eosinophil's expressing MBP shown in figure 10, can increase oxidative stress, promoting apoptosis but also used as a diagnostic tool for exhaled nitric oxide. Eosinophils also release various cytokines and leukotrienes (Hogan *et al*, 2008). Furthermore, eosinophils are a major source of growth factors particularly TGF-β1 in asthmatics (Flood-Page *et al*, 2003a).

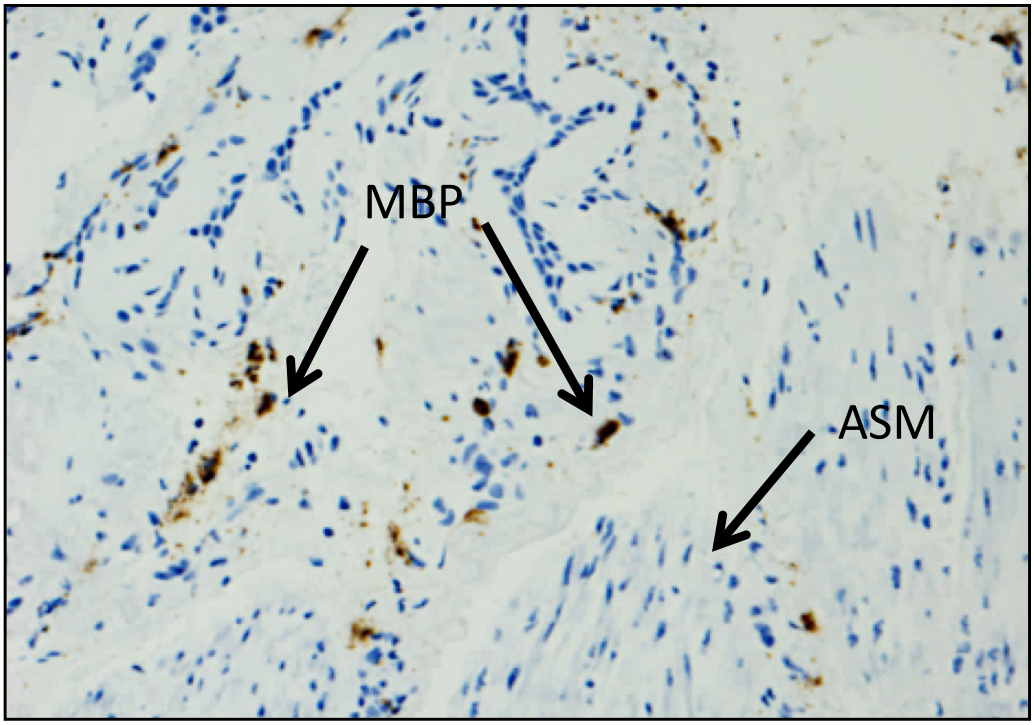


Figure 10: Eosinophils stained with major basic protein (MBP, borwn) at X400 magnification, arrows indicate positively stained eosinophils in ASM and lamina propria.

1.4.3.3 Neutrophils

Neutrophils also localised in peripheral blood are increased in asthma particularly severe asthma and Th-17 phenotype (Wenzel *et al*, 1997; Choy *et al*, 2015). Similar to eosinophils they can migrate to the lung by chemotaxis by the chemo-attractant IL-8. Once at the site of inflammation neutrophils can ingest and dissolve the noxious agent that are coated with opsonins. Neutrophils as shown in figure 11 can also release an array of cytokines, proteases and ROS to combat foreign agents and amplify the inflammatory response (Macdowell and Peters, 2007).

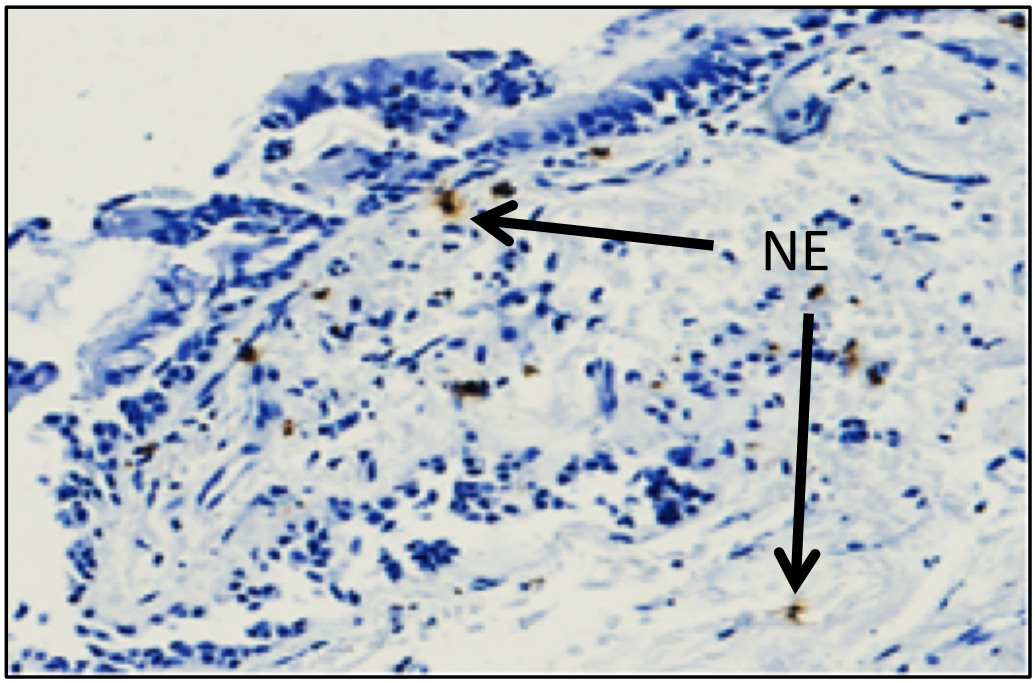


Figure 11: Neutrophils stained with neutrophil elastase (NE, brown) at X400 magnification, arrows indicate positively stained neutrophils in lamina propria.

Furthermore, neutrophils are considered to be the primary cause of viral-induced asthma exacerbations, with infected individuals presenting increased sputum neutrophils (Wark *et al*, 2002). In addition, asthmatic individuals and virus infected bronchial epithelial cells express more IL-25 and blockade of the IL-25 receptor can inhibit neutrophil recruitment (Beale *et al*, 2014). On the other hand stimulating neutrophils derived from peripheral blood; with Th17-cell cytokines such as IL-21, can increase IL-17A and IL-17F cytokines and thus provide a positive feedback mechanism that maintains Th17-inflammation (Halwani *et al*, 2017).

1.4.4 Airway smooth muscle remodelling

The airway smooth muscle in the airway shown in figure 12 is the first culprit for airway contraction, and as a result has been targeted by therapeutics that inhibit muscle contraction. Asthmatics have a larger smooth muscle bundle than healthy (James *et al*, 2009), however it is still not clear if this is due to hypertrophy (increased muscle cell size) or hyperplasia (increase in muscle cell number) as both have shown to contribute to the large smooth muscle bundle (James *et al*, 2012).

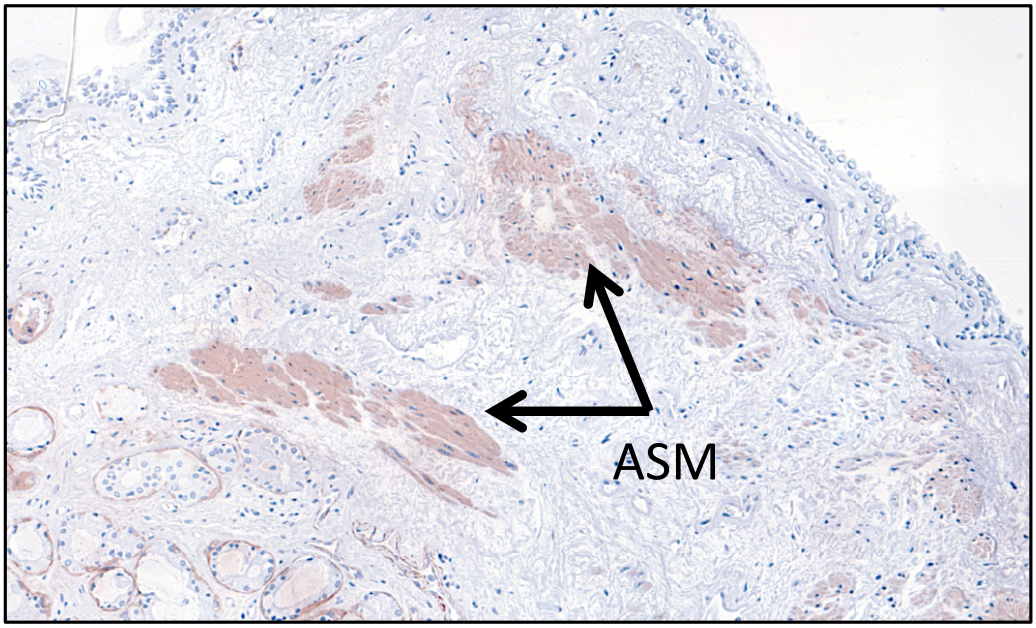


Figure 12: Airway smooth muscle stained with α-smooth muscle actin (red) at X200 magnification, arrows indicate airway smooth muscle bundle positively stained for α-smooth muscle actin.

This aberrant growth of smooth muscle is supported by the release of growth factors from immune cells. For instance, a unique of feature of asthma of mast cells located in airway smooth muscle can promote TGF-β1 secretion. Co-

culturing of ASM and mast cells increased TGF-β1 secretion from ASM and as a result also increased α-smooth muscle actin expression associated with increased muscle contraction, interestingly effects on contraction and actin expression were diminished once TGF-β1 was neutralised (Woodman *et al*, 2008). Additionally, fibrocytes from peripheral blood and in ASM bundles could also contribute to the larger ASM bundle as fibrocytes can also differentiate into ASM cell after exposure to TGF-β1 (Saunders *et al*, 2009). Lastly ASM cells once provoked by inflammation can continue to preserve this response by secreting various cytokines such as IL-5, IL-13 and eotaxin (Doeing and Solway, 2013).

1.4.5 Structural and inflammatory airway remodelling are not equal

Remodelling of airway structural cells and infiltration by immune cells is well appreciated in asthma; in addition, localised inflammation can instigate and promote structural airway remodelling. However once initiated by inflammation in asthma the two remodelling feats are no longer dependent. This can be demonstrated by the current and future therapeutics targeting the inflammatory pathway (de Kluijver *et al*, 2005; Flood-Page *et al*, 2003b), however they have minimal effect on ECM remodelling (Boulet *et al*, 2000) as they do not affect growth factors such as TGF-β (Yamaguchi *et al*, 2008; Bergeron *et al*, 2005) and do not improve overall lung function (Haldar *et al*, 2014; Busse *et al*, 2011) but rather reduce exacerbations.

Moreover, structural remodelling can occur from a young age before asthma diagnosis is made (Baena-Cagnani, Rossi and Canonica, 2007), becoming aberrant in the presence of inflammation and consequently remodelling

become separate entities that occur in parallel. Furthermore, once inflammation is resolved it does not recapitulate normal structural remodelling. Evidence for this has been shown by (Saglani *et al*, 2009) in an allergic mice model, where inflammation and AHR subsided after 4 weeks of without allergen challenge, but collagen deposition and RBMT persisted. This observation is also seen in asthmatics after an allergen challenge, where cellular inflammation returned to baseline after 7 days but expression of collagen (I and III) remained elevated (Kariyawasam *et al*, 2007). Lastly (Plopper *et al*, 2007) exposing infant rhesus monkeys with allergen or house dust mite exposure found typical asthmatic remodelling features described, interestingly, early exposure to the post-natal lung resulted in continual airway wall remodelling even after cessation of environmental factors contributing to asthma. In conclusion, inflammation is the primary event in asthma, which can lead to an epiphenomenon of airway remodelling. Treatments try to resolve inflammation, but asthmatics airways still have some form of disordered repair (Berair and Brightling, 2014). With a better understanding of asthma molecular inflammatory phenotypes, airway remodelling has not been evaluated in this context and this molecular stratification of asthmatics could highlight new molecular players involved.

1.5 Learning from lung development - WNT signalling

The first sign of lung organogenesis begins at ~4 weeks post gestation and carries on postnatally, until overall body size ceases to increase (Hislop *et al*, 1972). Development of the prenatal lung involves various stages, starting as a laryngotracheal bud derived from the foregut. Following this the buds extend

into the surrounding mesenchyme and begin the process of branching morphogenesis (i.e. forming the bronchial tree). The molecular mechanisms underlying this process require fibroblast growth factor-10 (FGF-10). As knockout models in mice who are FGF-10 deficient have demonstrated the inability for the lungs to form branches and display a 'lungless phenotype' (Min *et al*, 1998). In addition, (Li *et al*, 2002) showed targeted knockout of WNT5A (-/-), a glycoprotein ligand for the WNT signalling pathway can increase the expression of FGF-10, cause over branching of the distal airways and inhibit maturation of the saccular structures into alveoli.

In addition, prenatal animal models constitutively expressing canonical WNT signalling show undifferentiated epithelium, reducing the size of the alveoli and consequently the lung (Okubo and Hogan, 2004). Furthermore WNT signalling can also dictate the distal branching of the lung, where mice died of respiratory failure after birth due to the lack of the peripheral/small airways (Mucenski *et al*, 2003) The results from these developmental studies evaluating both canonical and non-canonical WNT ligands knockout in mice point towards critical role in branching morphogenesis of the airways, alveolar development and an important role in the lineage commitment of cells in the lung. Aberrant expression in adult could disrupt the homeostasis resulting in the abnormal pathology.

Supporting this view (Morrisey and Hogan, 2010) reviewing the lung development processes, elaborate on a plethora of signalling cascades all connected by feedback loops. But the 'cross-talk' of these developmental signal pathways is not completely deduced in pathological states. It is

important to understand these developmental signals as highlighted by (Demayo *et al*, 2002) who suggest that developmental processes which are the primary method of forming the lung are also subsequently responsible for maintaining homeostasis. This is because, during injury and repair the lung recapitulates these developmental motifs involved during lung development to restore and repair the disruption of adult homeostasis. However, unlike neonatal lung development the presence of an active immune system postnatally can disorder the development signalling cascades observed leading to chronic lung diseases (Torday and Rehan, 2007; Holgate *et al*, 2004). Consequently, understanding developmental signals can shed light on the biological cascades required to maintain normal lung homeostasis.

1.6 Diversity of WNT signalling

Conserved in all animal species WNTs represent an ancient signalling pathway utilised from development. The quest for the discovery of WNT signalling began in the second half of the 20th century. The aim was to identify host genes that are activated by insertion of mouse mammary tumour viruses (MMTV) leading to cancer (LYONS and MOORE, 1962) using a proviral tagging method still widely used (Copeland and Jenkins, 2010). The discovery made by (Nusse and Varmus, 1982) was a host gene that could be transcriptionally activated by MMTV proviruses, which they called Int1 (integration site 1). Interestingly Int1, a difficult gene to study is highly conserved and as a result had the same homologue of the fly gene Wg (wingless) and hence by combination of the two names it was known as WNT1.

It took over a decade since the revelation of WNT1 to identify its receptor (Bhanot *et al*, 1996). After three decades of effort made at deciphering the downstream signalling mechanism reviewed by (Klaus and Birchmeier, 2008b; Nusse and Varmus, 2012), the number of possible permutations of WNT ligand receptor and co-receptor interaction is bewildering. Today there are nineteen different reported WNT ligands that can bind to ten membrane bound frizzled (FZD1-10) receptors (7TMD) coupled to co-receptors (LRP 5/6 or RYK, ROR) shown in table 4. Unlike the nomenclature of the receptors WNT ligands even though having different coding regions, isoforms and cellular functions, share high amino acid sequence homology and as a result have similar names (Katoh, 2001; Bergstein *et al*, 1997). Furthermore, even though WNT ligands have high amino acid sequence identity and signal through the same WNT signalling pathway (i.e. canonical) they can mediate different effects (David, Canti and Herreros, 2010).

WNT Ligand	WNT signalling	Receptor interaction
WNT1	Canonical	FZD1, FZD3, FZD4
WNT2	Canonical	FZD1, FZD5, FZD9
WNT2b	Canonical	FZD7
WNT3	Canonical	FZD1
WNT3a	Canonical	FZD1
WNT4	Canonical and Non-canonical	Unknown

WNT5a	Non-canonical	FZD2, FZD4, FZD5, FZD7, FZD8, ROR1/2
WNT5b	Non-canonical	RYK
WNT6		FZD7
WNT7a	Canonical	FZD5
WNT7b	Canonical	FZD1, FZD10
WNT8a	Canonical	FZD1, FZD3, FZD4, FZD5, FZD7, FZD8
WNT8b	Canonical	FZD1, FZD3, FZD4, FZD5
WNT9a	Canonical	FZD4, FZD7, FZD9
WNT9b	Canonical	Unknown
WNT10a	Canonical	Unknown
WNT10b	Canonical	FZD5
WNT11	Non-canonical	FZD5, FZD7
WNT16	Unknown	Unknown

Table 4: List of WNT ligands and their interaction with receptors

In conclusion, the signalling cascade can be split into canonical (i.e. β-catenin dependent) or non-canonical (i.e. β-catenin independent) and more importantly is dictated by cell type and receptor context (van Amerongen, 2012), as to date there is no evidence of a side-by side comparison of all 19 different ligands/receptor interactions.

1.6.1 Canonical WNT signalling

Being the first discovered of the two, canonical signalling when inactive allows the free roaming of glycogen synthase kinase-3β (GSK-3β), axin a scaffold protein and adenomatous polypsis coli APC) destruction complex in the cytosol, resulting in the constitutive phosphorylation of β-catenin. The phosphorylated β-catenin is subsequently degraded by ubiquitination and as a consequence reducing β-catenin in the cytoplasm (Aberle *et al*, 1997). On the other hand, when a WNT canonical ligand (table) binds to a Frizzled (FZD) receptor and the co-receptor LRP5/6, the receptor complex can phosphorylate and recruit Dishevelled (Dvl) at the PDZ domain and bind to the C-terminal region of the FZD receptor (Wong *et al*, 2003). The auto-phosphorylation cascade can recruit casein kinase 1 (CK1) and the destruction complex to the receptor complex. This in turn deactivates GSK-3β by phosphorylation, allowing β-catenin to translocate into the nucleus and bind to the TCF/LEF transcription complex and induce cell proliferation and cell survival as depicted by figure 14.

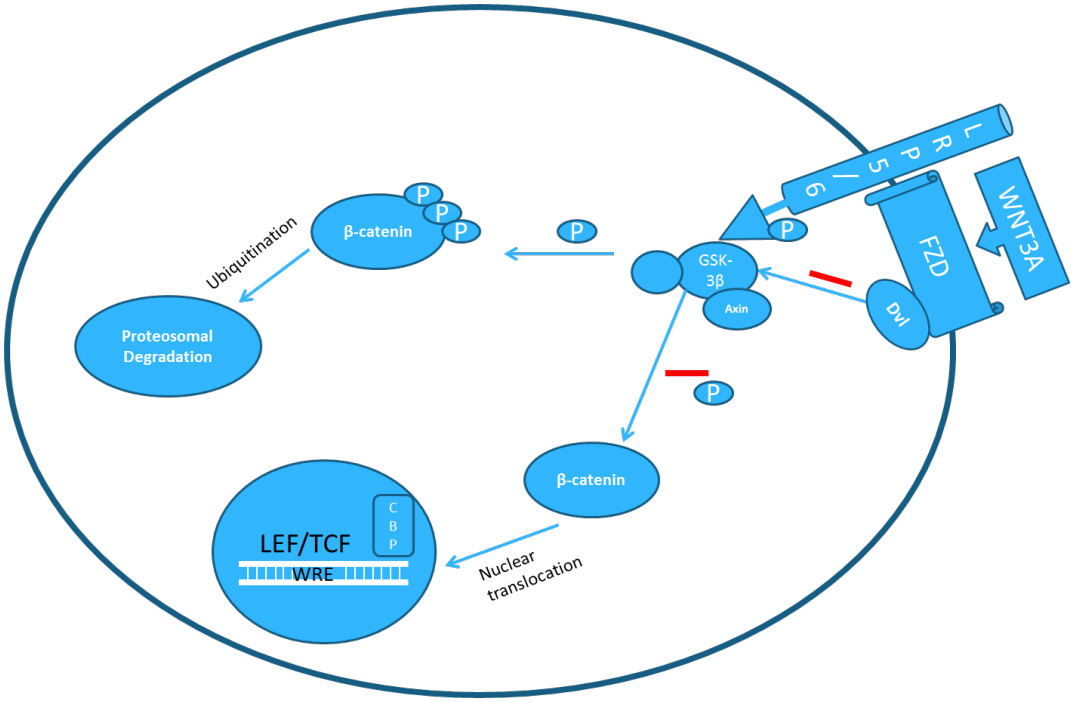


Figure 13: WNT canonical β-catenin-dependent signalling pathway.

1.6.2 Non-canonical Planar cell polarity/Ca^{2+} WNT signalling

With the discovery of more WNT ligands and receptors, it was evident that the diversity of the WNT signalling cascade could not be concluded in a 'canonical' fashion. Hence the name non-canonical was coined to describe the variable responses displayed. To add to these complexity WNT non-canonical ligands could either stimulate cytoskeletal rearrangement or calcium release, causing non-canonical WNT signalling to be synonymous with planar cell polarity/ Ca^{2+} WNT signalling, as they are β-catenin independent as shown in figure 14.

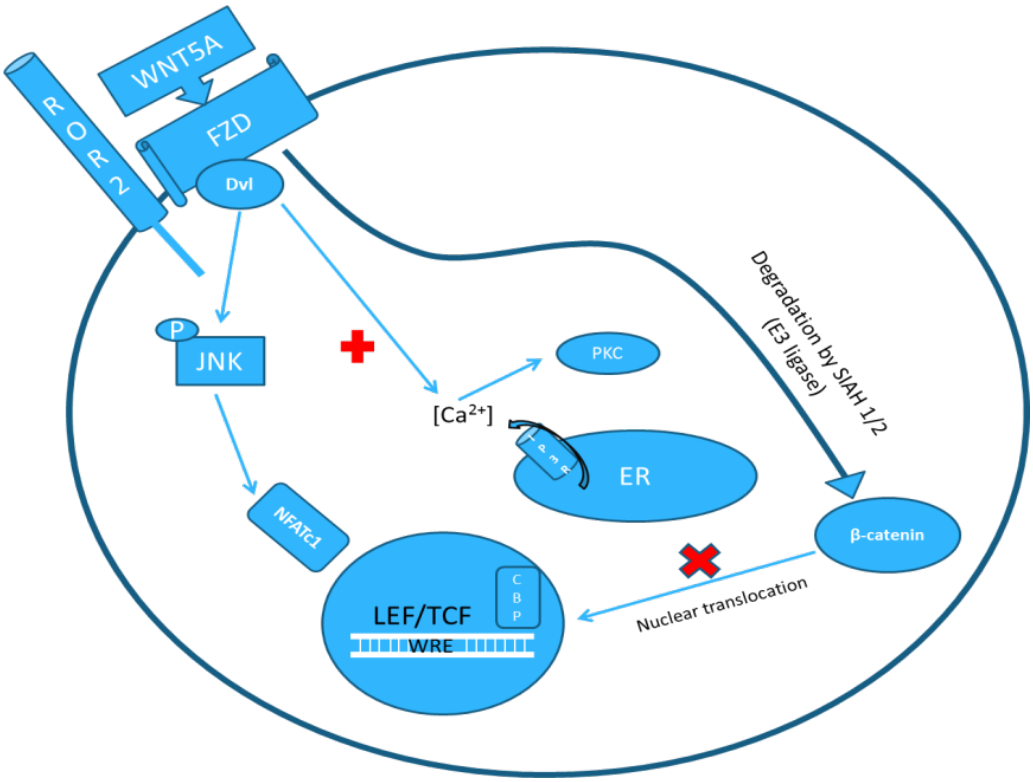


Figure 14: WNT non-canonical β-catenin-independent signalling pathway.

Similar to canonical WNT signalling, non-canonical WNT ligands can bind to a receptor and phosphorylate Dvl albeit different to canonical WNT signalling (Yan *et al*, 2001) attributed to the ligand-receptor complex conformation. In addition non-canonical ligands can bind to ROR/RYK alone or in combination with frizzled receptors to transduce their signal (Nishita *et al*, 2010; Ho *et al*, 2012). With the addition of kinase receptors and recruitment of Dvl, auto-phosphorylation can activate the planar cell polarity pathway, recruiting small GTPases, Rac1 and RhoA kinase resulting in cytoskeletal rearrangement. In addition auto-phosphorylation can activate the JNK pathway and subsequently allow NFATc1 to translocate into the nucleus to induce gene transcription of cell survival (Marchetti and Pluchino, 2013; Pongracz and

Stockley, 2006). Conversely, FZD receptors are from the super family of GPCR and have the capacity to interact with g-proteins αi, q and s (Nichols *et al*, 2013). These following interactions can allow the activation of phospholipase C (PLC) and amplify the production of IP_3 for release of intracellular calcium stores and various responses mediated by GPCR's.

1.6.3 Reciprocal regulation of WNT signalling

As we study the WNT signalling pathway more we start to appreciate the diversity it proposes from development to adult homeostasis and current WNT classification can be interrupted as an oversimplification of its diversity. For instance, a specific mouse model overexpressing non-canonical WNT5a ligand found increased activity of β-catenin activity in bone development, in contrast, overexpression of WNT5a in the skin inhibited the β-catenin/TCF signalling (van Amerongen *et al*, 2012a).

The ability for WNT ligands to activate canonical and non-canonical signalling pathway can be dictated by the phosphorylation of their respective co-receptors LRP5/6 or ROR1/2 that couple with FZD receptors. In addition, WNT5a can antagonise WNT3a-FZD interaction and co-receptor phosphorylation (Grumolato *et al*, 2010). Evidence of reciprocal regulation of canonical and non-canonical WNT signalling has also been shown downstream, where WNT5a can up regulate siah2 (E3 ubiquitin ligase), this effect was independent of GSK-3β a natural method of β-catenin degradation as WNT5a could still inhibit β-catenin activity even in the presence of LiCl a GSK-3β inhibitor (Topol *et al*, 2003a). Supporting this, (Nemeth *et al*, 2007) also showed a specific reporter gene induced in the presence of wnt3a was

significantly inhibited in the presence of wnt5a in hematopoietic stem cells, this observation was also complimented with decreased β-catenin protein expression. This was also shown in synapse formation, where WNT5a reduced activated β-catenin levels (Davis, Zou and Ghosh, 2008). These differing reciprocal effects could be manipulated by the co-receptors associated during receptor-ligand interaction (Yuan *et al*, 2011; Minami *et al*, 2010).

Nevertheless, WNT signalling can also be regulated by a whole host of native secreted biological inhibitors such as DKK-s, sFRP and WIF, which can contain cysteine rich domains similar to WNT ligands and competitively antagonise WNT signalling (Kawano and Kypta, 2003).

1.6.4 Expression and consequence of WNTs in adults

A wide range of WNT signalling ligands and receptors can be expressed in lungs as shown in primary airway structural and immune cells (Winn *et al*, 2005; Kumawat *et al*, 2013; Konigshoff and Eickelberg, 2010) and like many organ systems WNT components tend to express abundantly in stem cell niches (Miyoshi *et al*, 2012; Roarty and Serra, 2007), which can be found abundantly in the epithelial basal cell layer (Borthwick *et al*, 2001) of the lungs.

In addition, WNT signalling can modulate the inflammatory pathway. Evidence of this is shown in T-cells that express high levels TCF/LEF1 transcription factors which can bind to WNT/β-catenin pathway, inducing GATA-3 expression and promote IL-4 production, which is important for initiating Th2 cell differentiationand thus a potential role for WNT as a proximal initiator of Type-2 signalling (Yu *et al*, 2009; Xue and Zhao, 2012). Moreover,

knockout of TCF-1 transcription factor in mice can prevent β-catenin binding and enhance T-cells to differentiate into Th-17 cells and increase Th-17 cytokines (Ma *et al*, 2011) suggesting WNT's could play a role in inflammatory switching from Th2 to Th17 disease seen in asthmatics. WNT ligands such as WNT5a can also promote T-cell migration by sustaining CXCR4 expression via protein kinase C, allowing CXCL12 a strong chemo attractant of T-cells to bind to its receptor (Ghosh *et al*, 2009), this may be important to recruit the initial T-cell in asthma. Furthermore, WNT5a can also promote neutrophil migration and chemokine production of CXCL8 and CCL2 via phospholipase C a downstream effector of G-coupled protein receptor (Jung *et al*, 2013). This is also supported by studies in rheumatoid arthritis were WNT5a can promote IL8 production (Sen *et al*, 2000), suggesting a role for WNT in driving CS insensitive non-type2 neutrophilic inflammation.

Besides promotion and dictation of inflammation, WNT's as per their discovery can play notable roles in various cancers (Klaus and Birchmeier, 2008a) particularly breast cancer where WNT5a is activated in over 50% of cases (Zhan, Rindtorff and Boutros, 2017).

WNT5a signalling can also influence fibrosis in the lungs affected by interstial lung diseases (ILD) by promoting MMP expression to allow migration of fibroblasts (Pongracz and Stockley, 2006; Newman *et al*, 2016a) and potentiate the effects of growth factors such as TGF-β1-induced ECM deposition via cross talk mechanisms (Miyoshi *et al*, 2012; Kumawat *et al*, 2014). Better understanding of the role of WNT axis in promoting and modifying type 2 inflammation and influencing asthma inflammatory

phenotypes might lead to novel therapeutic approaches for asthma as they could be the developmental motifs for recapitulating lung homeostasis.

1.6.5 WNT5a structure and function

WNT5a is a non-transforming ligand of the WNT signalling pathway, being the most extensively studied (Kikuchi *et al*, 2012). Cloned from mouse fetal tissue during the early 90's, the WNT5a gene is mapped to chromosome 3p14-p21 (Clark *et al*, 1993). It is highly conserved sharing 87% amino acid identity to WNT5B and 99% sequence homology with mouse WNT5a. Alternative splicing can give rise to two sizes of WNT5a, (1) 380 amino acids (isoform 1 ~42kDa) and (2) 365 amino acids (isoform 2 ~40kDa). The main difference is isoform 2 lacks the first 15 amino acid sequence (Katoh and Katoh, 2009a; Bauer *et al*, 2013a). Regarding signalling, the two isoforms have recently shown different effects, where WNT5a-L the first larger isoform can inhibit proliferation of cancer, WNT5a-S the second isoform can promote cancer cell line growth. Interestingly both isoforms expression are altered in breast and cervix carcinomas, with WNT5a-L down regulated and WNT5a-S up regulated and consequently promoting the malignancy (Bauer *et al*, 2013b).

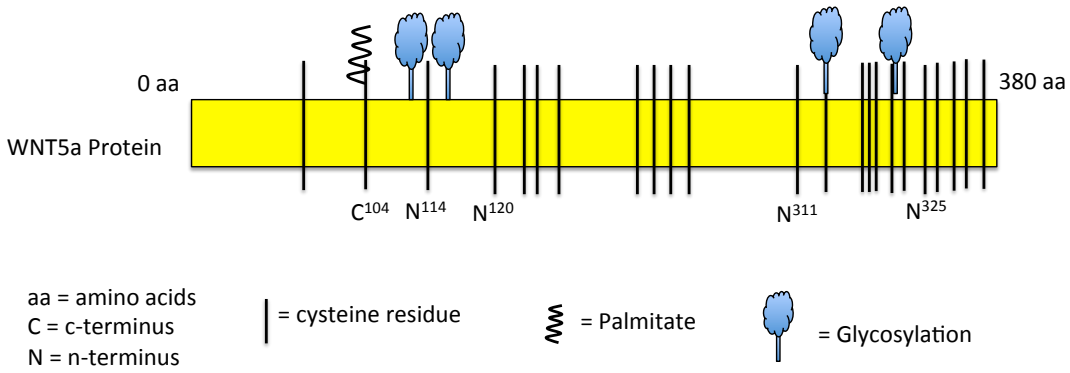


Figure 15: Representing the structure of WNT5a and important post-translational modifications.

Up-regulation of WNT5a gene can occur by multiple cytokines and growth factors that can allow transcription factors such as NF-κB, STAT3, SMAD-complex and FOX to bind to the target gene (Kumawat and Gosens, 2016; Katoh and Katoh, 2009b; Katoh and Katoh, 2007). Conversely, WNT5a transcription can be inhibited by amino acid limitation, as shown in cancer cell line SW480, where amino acid starvation lead to ERK1/2 phosphorylation and subsequent down regulation of WNT5a mRNA. Inhibition of ERK1/2 phosphorylation during amino acid starvation rescued WNT5a mRNA levels (Wang and Chen, 2009). In addition, the ELAV-like protein 1 can inhibit translation of WNT5a mRNA by stabilising it, this is achieved by WNT5a mRNA containing AU-rich motifs, allowing ELAV-like protein 1 which contains 3 RNA-binding domains to bind to the WNT5a gene and supress its translation (Leandersson, Riesbeck and Andersson, 2006).

Nevertheless, WNT5a is a highly modified and conserved protein and it's only in the past decade it has been appreciated that WNT5a is highly glycosylated

(N-linked) at residues 114, 120, 312 and 326. Further post-translational modifications occur at cys-104 and Ser-244 (UniProt, 2014; Kikuchi *et al*, 2012). Glycosylation is required for the secretion of the molecule, while palmitoylation is required for binding to its receptor and proper cell trafficking (figure 15). WNT5a signals via FZD receptor coupled to a co-receptor RYK or ROR1/2, activating the non-canonical signalling pathway. However the receptor and subsequent downstream signalling cascade is highly dependent on cell type and as a consequence requires further investigation most notably in pathological states (Baarsma, Konigshoff and Gosens, 2013).

1.7 The role of WNT signalling in Asthma

With the apparent diversity of WNT signalling we need the ability to refine which WNT could be the effector in asthma. Building upon this (Choy *et al*, 2011a) split a group of asthmatics (mild-moderate) into Th2-high and Th2-low category using the signature described previously. They made sure the subjects were under no ICS treatment to prevent any effect on gene expression and conducted a whole-genome microarray analysis in bronchial biopsies. Interestingly, they found that asthmatics were able to differentially express WNT signalling components. Further the Th2-high group had expected elevation of IL-13, but also TGF-β1 notorious for its fibrotic effects secreted from various sources and WNT5a the most highly and differentially expressed WNT gene. This study was the first to display WNT signalling may be important in asthma classified by molecular inflammatory phenotypes.

However, to date very little evidence exist with regards to the role of WNT signalling in lung inflammatory diseases and may reveal important pathways involved in airway remodelling repair. For instance, GWAS studies have identified limited genes in asthma susceptibility, however categorising these genes into biological pathways with a gene-set enrichment analysis, (which essentially categorises genes into groups that may over represent large set of genes) shows two biological processes, one of which includes WNT signalling was significantly enriched in asthma (Barreto-Luis *et al*, 2017). Building on this, microarray analysis of airway epithelial cells whose expression correlated with FeNO a biomarker of asthma identified 5 clusters with distinct characteristics, looking at the gene differences between these clusters or as the authors refer to as subject clusters, they found WNT pathways was an important biological characteristic to distinguish the individuality of subject clusters (Modena *et al*, 2014). Furthermore, (Sharma *et al*, 2010) wanted to identify which genes during human lung development are differentially expressed in utero and as a consequence could affect lung function in asthmatics. Analysis of gene expression during the pseudo glandular and canalicular stage found thirteen SNP's in three WNT genes that were associated with lung function. Taken together these studies reveal WNTs may play an important role in asthma that has not been extensively delineated. This raises the question, is loss of lung function caused by aberrant activation of these pathways from the beginning or manifesting in adulthood?

Further studies have revealed that WNT5a expression is increased in asthmatic airway smooth muscle (Kumawat *et al*, 2013) and can promote ECM deposition (Kumawat *et al*, 2014) when induced by TGF- β1. Co-culture of ASM with asthmatic eosinophils for 24h, which have a higher affinity for adhesion to ASM, can also exacerbate WNT5a and TGF-β1 gene expression, while eosinophils from healthy subjects can only augment TGF-β1 expression (Januskevicius *et al*, 2016). With AHR being centre of attention for asthma exacerbations and symptoms, WNT5a has shown to increase contractility of ASM by regulating TGF-β1-induced α-SMA expression and increased in airway myocytes the ASMC responsible for contraction. As such WNT5a promoting actin polymerisation but not increasing intracellular calcium can increase maximum isometric tension (contractile force) of bovine tracheal smooth muscle strips (Koopmans *et al*, 2016).

Additionally, a report looking at dysregulated repair of the epithelium in asthmatics found, that fibronectin secretion is important. More importantly, they conducted global analysis of gene expression in epithelial cells from atopic asthmatics or healthy non-atopic individuals. The results showed >1.5 fold increase in WNT5a expression in asthmatics and a FDR (false discovery rate) of <0.01 (Kicic *et al*, 2010). On the other hand TGF-β1 a known cytokine for inducing EMT in epithelial cells and fibronectin expression (Hackett *et al*, 2009) was down regulated by 2-fold (with a FDR value of 0.106). Peripheral blood mononuclear cells (PBMC's) from healthy donors have also shown to increase WNT5a expression in the presence of Th2-cytokines IL-13 and IL-4,

interestingly anti-IL-13 antibody supressed WNT5a mRNA expression (Syed *et al*, 2007).

Likewise, asthmatic rats displaying typical histological features of asthma had increased WNT5a mRNA and p-JNK protein levels in lung tissue and peripheral blood (Han *et al*, 2015). Furthermore, an asthmatic mouse model displaying Th17 phenotype and subsequent IL-17A production correlated with airway remodelling and *in vitro* mouse fibroblasts stimulated with purified IL-17A increased TGF- β1 secretion and collagen transcription that was significantly enhanced in the presence of WNT5a co-stimulation (Peters *et al*, 2016). Complimenting this observation, a mouse model sensitised to house dust mite (HDM), found increased levels of WNT5a in the epithelium, interestingly this was particular in adult mice. The same pattern was also apparent in adult human nasal polyps, which represent the upper airways. *In vitro* analysis of normal human bronchial epithelial cells present in the lower airways displayed increased WNT5a secretion in the presence of IL-4 but not TGF- β1 (Dietz *et al*, 2017) as previously shown in ASM.

As a conclusion this evidence highlights that structural cells (epithelial and airway smooth muscle) have the capacity to induce WNT5a expression, the most differentially expressed WNT ligand in Th2-high asthma. However, its role particularly during airway remodelling in asthma phenotypes remains undefined.

1.7.2 Proposed role of WNT5a in asthma airway remodelling

Increased at a gene expression level in asthmatics (Choy *et al*, 2011b; Kicic *et al*, 2010; Kumawat *et al*, 2013; Syed *et al*, 2007) WNT5a is notoriously known for its migratory role in malignancies (Endo *et al*, 2015; Dissanayake *et al*, 2007; Nishita *et al*, 2006; Nomachi *et al*, 2008; Pourreyron *et al*, 2012; Witze *et al*, 2008) and spatial polarisation/patterning during development (Attisano and Labbe, 2004; van Amerongen *et al*, 2012b; Roarty and Serra, 2007). Mediating its effects through the planar cell polarity and Ca^{2+} pathway (Endo *et al*, 2015; Bhatt and Malgor, 2014) WNT5a can promote directional migration and apical basal cell polarity as witnessed by (Yamamoto *et al*, 2015; Witze *et al*, 2008; Gon *et al*, 2013). Interestingly, WNT5a is increased in fibroblasts from UIP compared to healthy increasing their proliferation and resistance to H_2O_2 induced apoptosis (Vuga *et al*, 2009). In addition, WNT5a expression in IPF lungs is expressed in multiple compartments including the epithelium, the ASM and fibroblastic foci and is enhanced in *in vitro* when stimulated with TGF-β1 (Newman *et al*, 2016b). The following data indicate that WNT5a can play multiple roles in airway remodelling promoting migration and subsequent ECM deposition, however the role in asthma even though increased at an mRNA level has not been delineated.

1.8 WNT5a recapitulates developmental cross-talk

Evidence from lung development suggests cross talk mechanism is essential and is the complex mechanism the lung utilises to reach homeostasis. TGF-β1 signalling can be split into SMAD dependent and independent (Derynck

and Zhang, 2003), however current targets have been ineffective due to the pleotropic nature of the signalling pathway (Massague, 2000; Akhurst and Hata, 2012). Nevertheless, The role of TGF-β1 in the airway epithelium is well documented in the literature (Hackett *et al*, 2009; Halwani *et al*, 2011; Magnan *et al*, 1994; Jetten, Shirley and Stoner, 1986; Redington *et al*, 1998) correlating with basement membrane thickness (Vignola *et al*, 1997; Hoshino, Nakamura and Sim, 1998).

Cross talk mechanism of WNT5a with growth factors is apparent from development and recent evidence highlights non-canonical WNT5A ligand can cross talks with TGF-β1 shown in figure 16. Promoting transcription factors to bind and up-regulate WNT5a gene expression (Katoh and Katoh, 2009a).

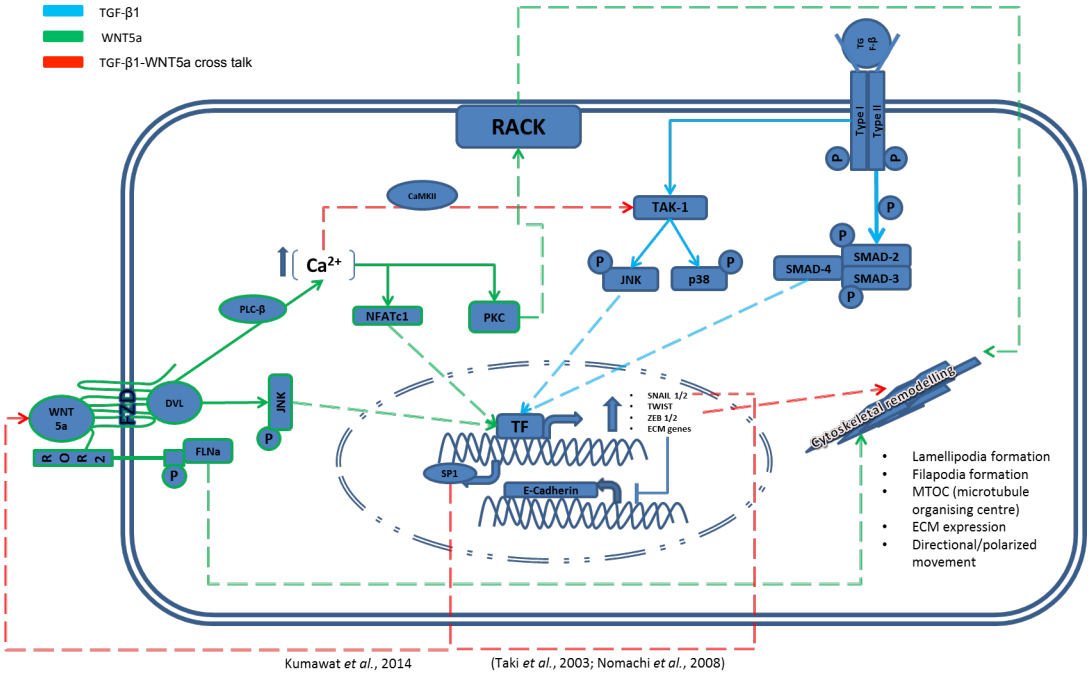


Figure 16: Depicting the cross talk pathway between WNT5a and TGF-β1 signalling pathways.

In mammary epithelium, attenuation of TGF-β1 or WNT5a signalling can induce the WNT-β-catenin axis; this reciprocal regulation between canonical and non-canonical is evident in other cell types (Grumolato *et al*, 2010; Roarty *et al*, 2009; Topol *et al*, 2003b). Furthermore, evidence exists for downstream messengers of both TGF-β1 and WNT5a pathways can interact (Hocevar *et al*, 2003; Dao *et al*, 2007; Gao and Chen, 2010). More recently, (Kumawat *et al*, 2013) showed that TGF-β1 can differentially induce the expression of WNT ligands and receptors in ASM, most notably WNT5a (non-canonical ligands) by TGF-β1 activated kinase 1 (TAK-1) via sp1 transcription factor (Kumawat *et al*, 2014) and Frizzled 8. Inhibition of WNT5a or frizzled 8 by siRNA's diminished TGF-β-induced ECM gene and protein expression in ASM. Complimenting this, inhibition of TGF-β1 type 1 receptor can also lead to

WNT5a mRNA suppression in human melanoma cells promoting melanoma adhesion and migration (Jenei *et al*, 2009).

Likewise in an epithelial injury model *in vivo*, undifferentiated epithelial cells are required to cover the wound bed and these non-proliferative cells over express WNT5a mRNA. WNT5a knockout resulted in abnormal wound channels and *in vitro* investigation showed TGF-β1 and WNT5a can induce serpine 1 expression and enhance smad3 phosphorylation and nuclear translocation. Conversely, in the WNT5a knockout mice model this was associated with loss of smad3 phosphorylation and nuclear translocation (Miyoshi *et al*, 2012).

The evidence highlighted suggests that lung structural cells particularly the ASM and epithelial cells can express WNT5a. However to date very little investigation has carried out on the mechanism of WNT5a signalling particularly in the context of asthma phenotypes. Particularly with interesting observations showing canonical WNT/β-catenin pathway (activated by WNT1) can attenuate the effects of allergic airway disease, by suppressing the activation of dendritic cells, a critical Antigen presenting cell for initiating the T-cell response (Reuter *et al*, 2014).

1.8.1 Defining cross-talk signalling

Signalling pathways of a giving system are generically always thought to be static or in other words canonical. However, if two different signalling pathways engage with either of their downstream systems this gives rise to the notion of cross talk signalling. For example WNT5a-induced intracellular calcium release via PKC, where Gq-coupled GPCR's are typically associated

with this static signalling network. Evidence shows that when WNT5a is inhibited by BOX5, WNT5a-induced intracellular calcium is attenuated, however addition of carbachol activating the Gq-coupled pathway can restore intracellular calcium release (Prgomet *et al*, 2015). Suggesting that even though WNT5a utilises a downstream cascade of Gq-coupled receptor it is not potentiated, amplified or regulated by Gq-coupled GPCR agonists.

Evidence against cross talk suggests unlike the signalling patterns in worms and fly, mammalian cells do not actually have static signalling models rather a subset of increasingly interconnected signalling models that may be operational in specific cell-time context (Noselli and Perrimon, 2000). This is inherently apparent in biased agonism/functional selectivity signalling, where a ligand can manipulate a diverse cell response based on receptor-ligand conformation states (Kenakin, 2011; Rang and Dale, 2011).

Nevertheless, in the context of this study we have adopted the conservative approach where cross talk signalling is defined as the ability of one or more components of signal transduction to affect another signalling cascade, achieved at a protein or transcriptional level. It is important to note that this can occur primarily in a unidirectional rather bidirectional environment.

As witnessed by (Coster *et al*, 2017) TGF-β3 can cross talk with BMP4 via SMAD4 but not vice versa, furthermore the authors show that TGF-β3 has no cross-talk potential via canonical WNT signalling. Similar to Miyoshi *et al*, 2012 who demonstrated that WNT5a can potentiate smad3 nuclear translocation similar to TGF-β1 but TGF-β1 could not activate WNT5a downstream messengers. Additionally, Kumawat *et al*, 2013 demonstrate

WNT5a can augment TGF-β1-induce ECM in ASM, However, TGF-β1 cannot augment WNT5a downstream processes, rather increase WNT5a and its receptor FZD8 mRNA expression, suggesting cross talk pathways can help augment and or potentiate other signalling systems.

As such it is important to highlight that cross talk does not refer to two signalling pathways dependent on each other. Rather they are independent and the apparent complexity of a subset of discovered signalling networks may function during a given biological context. This overlapping of downstream signalling messengers by two different signalling pathways, if augmented or potentiate the final effect can be considered to be cross talk.

Therefore, as a conclusion WNT5a can drive non-canonical signalling and inhibit canonical signalling by reciprocal regulation, and over expression of WNT5a and subsequent fibrotic effects can be potentiated by TGF-β1. Exploring WNT5a offers the following benefits to translational research in Asthma research:

1. Provide an alternative therapeutic target to reduce ECM deposition and subsequent airway wall remodelling.
2. A biomarker for treatments targeting Th2-high asthma and or ECM deposition
3. Highlight an unknown repair mechanism employed in asthma epithelial repair.

1.9 Aims and Hypothesis

The Th2 inflammatory profile in asthma is well-established causing airway remodelling (Woodruff *et al*, 2009). However the molecular mechanism of remodelling remains elusive (Elias *et al*, 1999), particularly in steroid insensitive patients, which display no therapeutic efficacy against fibrosis. With ever more evidence for the role of WNT signalling in fibrotic disorders, the lack of functional studies in asthma warrants a need to explore WNT signalling causing airway remodelling.

1.9.1 Hypotheses

1. WNT5A and TGF-β1 are involved in epithelial airway remodelling in asthma and its inflammatory sub-phenotypes.
2. Epithelial basal cell repair in asthma is associated with WNT5A-TGF-β1 cross talk at a molecular level.

1.9.2 Aims and objective

To test the hypotheses above using a combination of *in vivo* (bronchial biopsies) and *in vitro* approaches:

1. Examine the expression of WNT5a and TGF-β1 in airway structural cells using a combination of IHC, IF and western blotting
    - Examine differential expression of WNT5a and TGF-β1
    - Examine correlation with disease severity and Th-inflammatory genes expression signatures
2. Investigate the TGF-β1 – WNT5a signalling axis *in vitro*

- Functional studies in primary and cell line airway structural cells exposed to TGF-β1 ± WNT5a
- Outputs in 2.1 will include – Ca^{2+} signalling, wound repair, proliferation, migration and ECM deposition.

Chapter 2:

Methods and

Materials

2.1 Fibre-optic bronchoscopy and clinical characterisation

Bronchoscopy was conducted by Dr Salman Siddiqui and Professor Peter Bradding following the British thoracic society guidelines (British Thoracic Society Bronchoscopy Guidelines Committee, a Subcommittee of Standards of Care Committee of British Thoracic Society, 2001) as described previously by (Shikotra *et al*, 2012; Siddiqui *et al*, 2008b). Mucosal biopsies and bronchial brushings were taken from the right middle lobe and lower lobe carinae. Biopsy specimens were immediately fixed in acetone for embedding in glycol methacrylate and or placed in RNA preservative (RNAlater, Ambion) for gene expression analysis, bronchial brushings were placed in BEGM (Lonza) for tissue culture.

Asthma severity was classified using GINA guidelines as shown previously by (Siddiqui *et al*, 2008b; Shikotra *et al*, 2012) and control subjects had normal lung function with no history of respiratory diseases. Recruitment and further characterization of clinical parameters was conducted by members of staff at; Institute of Lung Health, Leicester Glenfield Hospital. Written informed consent was gained for all participants recruited for the bronchoscopy procedure and the Leicestershire Research Ethics committee approved the study. Determination of Th-status of asthmatic patients was based on a composite of gene expression signature derived from mRNA of mucosal bronchial biopsies (table 5) as described previously (Choy *et al*, 2015; Woodruff *et al*, 2007).

Asthma Phenotype	Th2	Th0/Thx	Th17
Genes expressed	CLCA1, Periostin and SerpinB2	Unknown/no gene expression signature	CXCL1, CXCL2, CXCL3, IL-8 and CSF-3

Table 5: Genes associated with Asthmatic phenotypes.

2.2 Immunohistochemistry (IHC)

Immunohistochemistry can be used to identify native protein expression in *in* vivo tissue sections using targeted antibodies. The technique allows cross-sectional evaluation of protein of interest by binding to a primary antibody; this signal is then amplified with secondary and tertiary complexes generating a distinctive colour based on the chromogen. Using a microscope we can evaluate the location and amount of protein of interest.

2.2.1 Bronchial Biopsy embedding in Glycol methacrylate (GMA) resin

Endobronchial biopsies obtained during the bronchoscopy procedure were fixed in acetone (Sigma, 179973) with 2mM PMSF and 20mM iodoacetamide for 24 hours at -20ºC. After fixation the biopsies were washed in water free acetone for 15 minutes and then placed in methyl benzoate for another 15 minutes. The biopsy was then processed for embedding by incubating them in a solution with JB4 solution A (Polysciences, 0226A) supplemented with 5% methyl benzoate at 4ºC for 6 hours (solution replaced every 2 hours). After

the incubation period, the biopsies were embedded with JB-4 Plus embedding kit (Polysciences, 18570) and set for polymerisation at 4ºC for 48 hours in polythene capsules. Biopsies embedded were subsequently stored at -20ºC, as previously described by (Shikotra *et al*, 2012; Britten, Howarth and Roche, 1993).

2.2.2 Staining of tissue sections using immunohistochemistry

Glycol methacrylate (GMA) sections were cut at 2µm using a microtome (Leica, RM225) and placed in 0.2% ammonium bath for 1 minute. The sections were captured with charged microscope slides (Pyramid Innovation, R530001) and dried at room temperature for 1-4 hours.

Once the slides had dried, the endogenous peroxidase enzyme was inhibited for 30 minutes with a 0.1% sodium azide solution containing 0.3% H_2O_2 v/v. The slides were washed with 1x TBS (appendix 1) and subsequently blocked for 30 minutes (appendix 1)

Primary antibodies were added overnight in a humidified chamber (antibody list shown in table 6). Secondary antibody was added for 2 hours, followed by 2 hours incubation with Vectastain ABC complex kit (vector labs), consequently amplifying antibody binding.

Antibody binding was visualised with the AEC substrate kit (A.Menarini Diagnostics, MP-810-K100) as recommended by manufacturer. Biopsy sections were counterstained with Mayer's haematoxylin (Pioneer Research Chemicals LTD, PRC/R/42). The staining was preserved with a Super mount coat (Launch Diagnostics LTD, HK079-7K) and then cover slipped with DPX

(Sigma). This technique was pioneered by (Britten, Howarth and Roche, 1993) and previously described by (Parmar, 2013; Shikotra et al, 2012). Tryptase an enzyme present in mast cells determined a positive staining run of the experiment.

Antibody	Company	Clonality	Concentration for IHC	Concentration for IF	Concentration for WB
WNT5a LSBio	Lifespan Biosciences, LS-C47384	Polyclonal	10-5µg/ml	10-5µg/ml	2µg/ml
WNT5a RnD	RnD systems, MAB645	Monoclonal	20µg/ml	20-10µg/ml	2µg/ml
WNT5a 6F2	Abcam, ab110073	Monoclonal	5µg/ml	2.5µg/ml	2.5µg/ml
TGF-β1	Santa Cruz, SC-146	Polyclonal	4µg/ml	4µg/ml	4µg/ml

total-SMAD2/3	Cell signalling Technology, 5678s	Polyclonal	-	174ng/ml	-
Vimentin		Monoclonal	-	5µg/ml	
CK-5	Santa Cruz, SC-32721	Monoclonal	400ng/ml	4µg/ml	-
E-Cadherin		Monoclonal	-	5µg/ml	-
α-SMA		Monoclonal	142ng/ml	710ng/ml	-
Collagen 1α1		Polyclonal	-	5µg/ml	-
Mouse IgG2a	DAKO, X0943	Monoclonal	142ng/ml	710ng/ml	-
Mouse IgG2b	RnD systems	Monoclonal	-	5µg/ml	-
Rabbit IgG	DAKO, X0936	Polyclonal	10-4µg/ml	10-4µg/ml	-

Rat IgG2a	RnD systems, MAB006	Monoclonal	20-10µg/ml	20-10µg/ml	-
Mouse IgG1	DAKO, X0927	Monoclonal	5µg/ml	5-2.5µg/ml	-
β-actin	Abcam, ab8229	Monoclonal	-	-	1µg/ml
DAPI	Sigma,	-	-	0.1µg/ml	-

Table 6: Primary antibody list employed for protein detection at relative concentrations.

2.2.3 Immunohistochemistry-GMA limitations

Fixation of tissue in GMA resin allows better preservation of tissue structures over the common fixative 10%nuetral buffered formalin. This is because; the later fixative forms covalent bonds for protein crosslinking. As a result during IHC these covalent bonds need to be broken, this can in turn also denature and destroy delicate structures such as the epithelium. Conversely, GMA resin embedded tissue sections are fixed in acetone that disrupts the hydrophobic bonds of the proteins 3D structure and as the resin is water-miscible, harsh antigen retrieval methods are not required to expose epitopes of interest. Furthermore, the GMA resin can be cut at a thickness of 2µm, allowing co-localization of tissue structures but also preserve the limited resource of bronchial biopsies.

2.3 Immunohistochemistry (IHC) Image Analysis

2.3.1 Bright field IHC image acquisition

Bright field multiple aligned images (MAI) of top and bottom tissue section were taken using an automated Zeiss Microscope. The microscope objective was set at x200 magnification with a light intensity of 3.9V and with 80% exposure of Camera AxioCam HR R3. All images were white balanced. The tiled images were fused using the Zen 2012 software with a minimal overlap of 5% and Max shift of 10%. All area measurements were expressed in per square millimetres (mm^2) and were conducted using the Zen 2012 software on α-smooth muscle actin and haematoxylin stained sections (figure 17). An average area was determined from the top and bottom tissue sections.

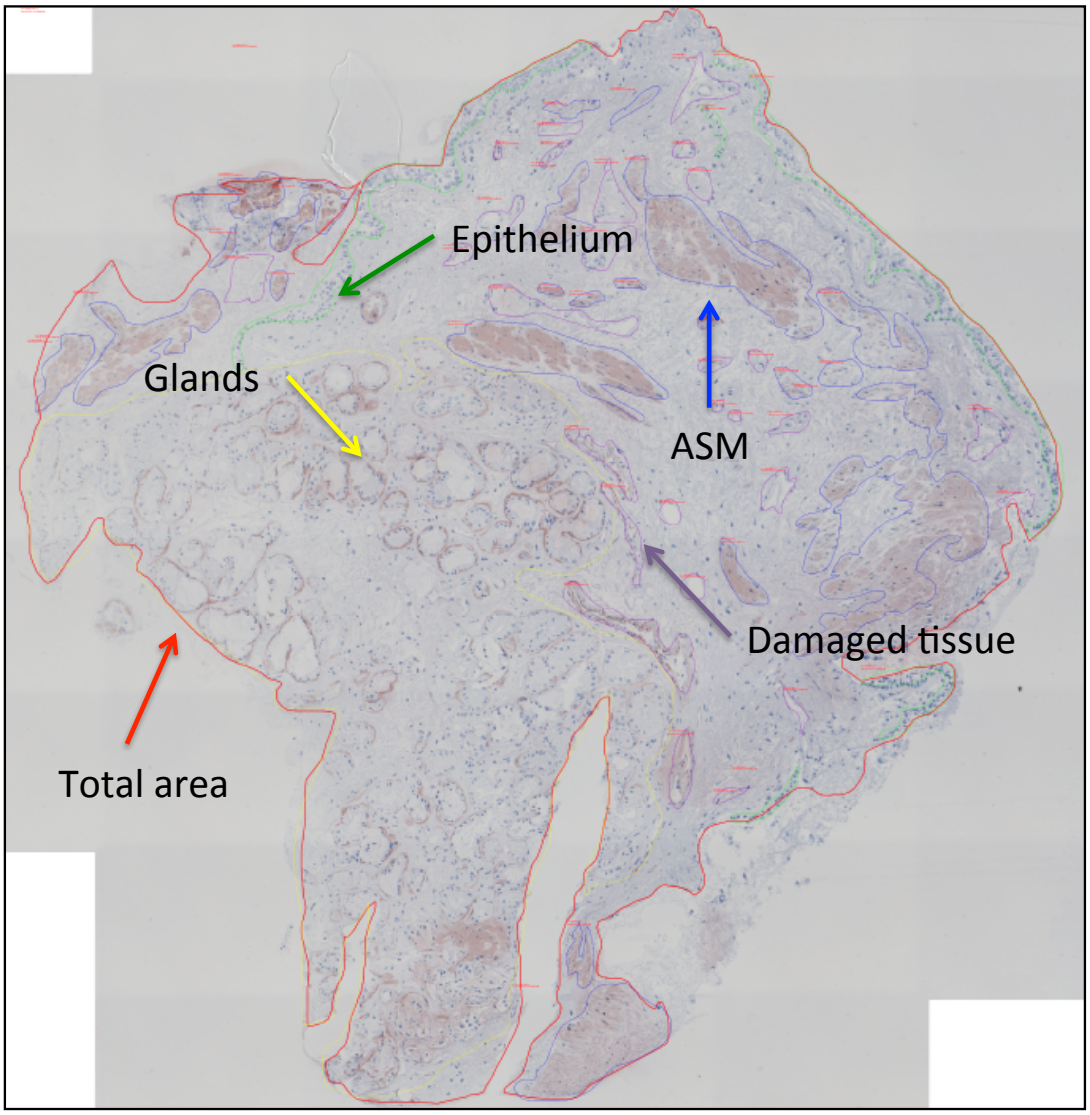


Figure 17: Bronchial biopsy stained with α-SMA and measured for tissue compartments.

2.3.2 Area measurement in IHC tissue sections

The RBM thickness was measured on images taken at x400 objective. Each measurement was 20μm apart, perpendicular to attached epithelial cells and a total of 50 consecutive measurements per section were taken, as described previously by (Sullivan *et al*, 1998).

The total area of the biopsy was determined from 2 non-contiguous tissue sections (top + bottom) from the same biopsy. The area of the lamina propria (submucosa) was determined by subtracting the total area of the biopsy from the epithelial area, ASM area, glandular area and non-structure area (includes damaged tissue and blood vessels). Nucleated positive stained cells were counted in tissue sections at least 20μm apart and expressed in per square millimetre of the lamina propria. Epithelial integrity was measured by determining the length in millimetres of intact, damaged epithelium attached to the RBM and denuded epithelium was determined by RBM length with no epithelial cells attached as previously described by (Ordonez *et al*, 2000) as shown in figure 18. The length of each epithelial measurement was divided by the total RBM length and multiplied by 100 to determine the percentage of intact, damaged and denuded epithelium.

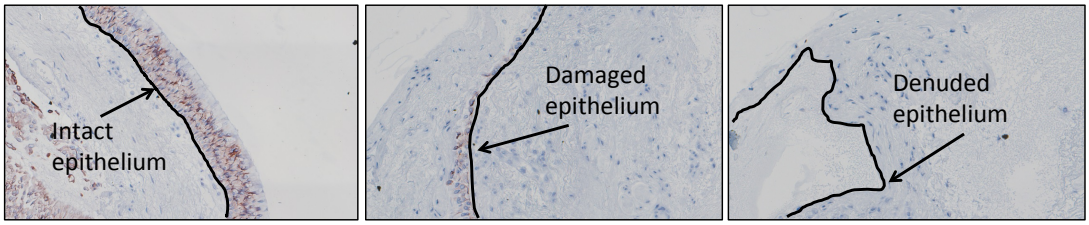


Figure 18: Depicting the selected area measured for analysis for epithelial integrity.

2.3.3 Quantitative assessment of IHC staining

Ubiquitous staining of WNT5a in the epithelium and ASM, TGF-β1 in the epithelium and Collagen III in the lamina propria was assessed qualitatively using a semi-quantitative score (SQS) as previously described by (Siddiqui et al, 2008b). Structure absent was omitted from the score.

Quantitative measurement (percentage staining) was assessed by developing a thresholding technique, based on the H (Hue), Saturation (S) and Intensity (I) index for detecting colour using the Zen 2012 software (Zeiss). The HSI scale ranged from 0-360 for hue and 0-255 for both saturation and intensity. Initially, a mean HSI value was determined manually in 3 donors with an SQS score of grade 3, appropriately highlighting the chromogen staining. The threshold value was then applied to all the donors in the respective tissue compartment including isotype controls. The measurement was deduced as a mean of 2 non-contiguous sections subtracted from the isotype. All counts and threshold measurements were conducted blindly.

2.4 Cell culture

Recombinant TGF-β1 and WNT5a were re-suspended in growth medium before stimulation. BOX-5 inhibitor was added for 30 minutes prior to stimulation.

2.4.1 Primary Human Bronchial Epithelial Cells (HBEC) culture

Bronchial brushings obtained during the bronchoscopy procedure were placed immediately into a chemically defined basal epithelial growth medium (Lonza, Basel, Switzerland) supplemented with 1% Antibiotic-Antimycotic solution (Gibco, Paisley, UK) and 0.3% Fungizone (Gibco). The brushings were placed at 4^{o}C overnight, promoting the growth of epithelial cells only. The brushing was gently agitated to dislodge the cells and sedimented at 600g for 7 minutes. The cell pellet was re-suspended in basal epithelial

growth medium. 12-well plates were coated with 1% pure collagen (appendix 2) for 1 hour and subsequently drained and rinsed in Hank's Balanced Salt Solution (Gibco). The re-suspended cell pellet from each brushing was cultured into 2 wells per brush achieving passage 0.

After confluence, HBEC were enzymatically dissociated with 0.25% trypsin-EDTA (Gibco). The cell pellet was re-suspended in basal epithelial growth medium and subcultivated into a T-75 flask pre-coated with 1% pure collagen, achieving passage 1. HBEC used for experimental purposes were between passage 2-3 and used at 100% confluence (Figure 17).

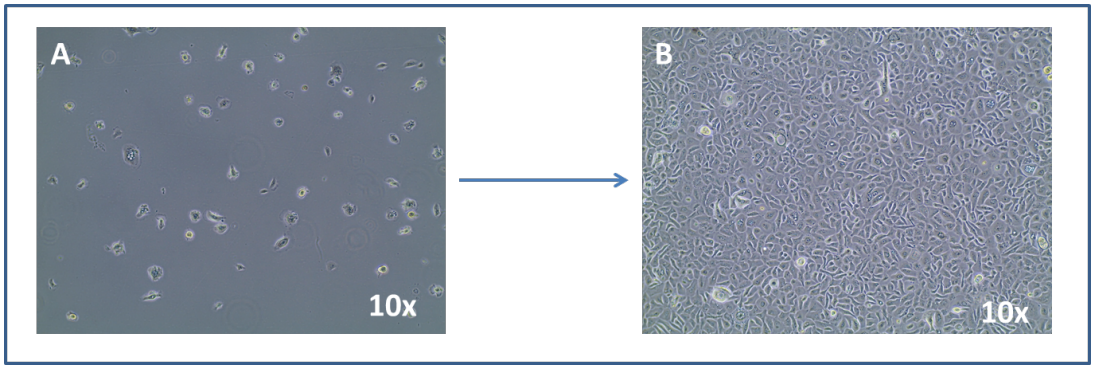


Figure 19: Primary human bronchial epithelial cells in culture.

(A) Primary human bronchial epithelial cells 24 hours after subcultivation. (B) Primary human bronchial epithelial cells at 100% confluence. Both images were taken at Passage 2 with EVOS XL Core-AMG, AMEX-1200.

The submerged culturing of HBEC described above consists of undifferentiated monolayer of basal epithelial cells as extensively characterized previously by (Wan, 2013; Kicic *et al*, 2006; Hackett *et al*, 2011).

Cryopreservation of HBEC was conducted in ProFreeze-CDM NAO 2x (Lonza) with 7.5% DMSO. Cryogenic vials were defrosted in running hot water for 2 minutes and diluted in growth medium, after adherence media was replaced to remove the DMSO.

2.4.1.1 Trouble shooting primary Air-Liquid interface (ALI) cultures

Growth of ALI-cultures was conducted in 12-well transwells inserts (Corning) coated with 1% pure collagen. HBEC were seeded in the apical chamber at $1x10^5$ per well. After confluence basal epithelial growth medium was removed from the apical and basolateral chamber and replaced only in the basolateral chamber with ALI medium, composed of 1:1 of basal epithelial growth medium and Dulbecco's Modified Eagle Medium (Gibco) supplemented with 1% Antibiotic-Antimycotic solution (Gibco, Paisley, UK), 0.3% Fungizone (Gibco) and 100nM of retinoic acid to promote differentiation. Trans epithelial electrical resistance (TEER) was measured using EVOMX with STX2 electrode (World Precision Instruments, USA).

During the course of the project cryogenic stocks of HBEC did not yield any successful ALI-cultures. Furthermore, fresh bronchial brushings also did not yield any epithelial cells. This was replicated with different brushes (3cm and 5cm) during the fibre-optic bronchoscopy procedure; this was confirmed by conducting a cell count on bronchial brushings immediately and 24h after the procedure. Due to the expense of setting a dedicated ALI-culture environment and reagents, we opted to use BEAS-2B cells as an epithelial basal cell model.

2.4.2 BEAS-2B cell culture

BEAS-2B (LGC Standards, UK, CRL-9069) is a cell line model of epithelial cells isolated from normal human bronchial epithelium and infected with an adenovirus 12-SV40 virus hybrid and cloned (figure 20). Beas-2B cells were cultured in a chemically defined basal epithelial growth medium (Lonza, Basel, Switzerland) supplemented with 1% Antibiotic-Antimycotic solution (Gibco, Paisley, UK) and 0.3% Fungizone (Gibco).

After confluence, Beas-2B cells were enzymatically dissociated with 0.25% trypsin-EDTA (Gibco). The cell pellet was sedimented at 600g for 5 minutes and then re-suspended in basal epithelial growth medium. All culture vessels were pre-coated with 1% pure collagen. Beas-2B cells were used at a confluence of 80-90% for culturing and experimental purposes to prevent change in morphology.

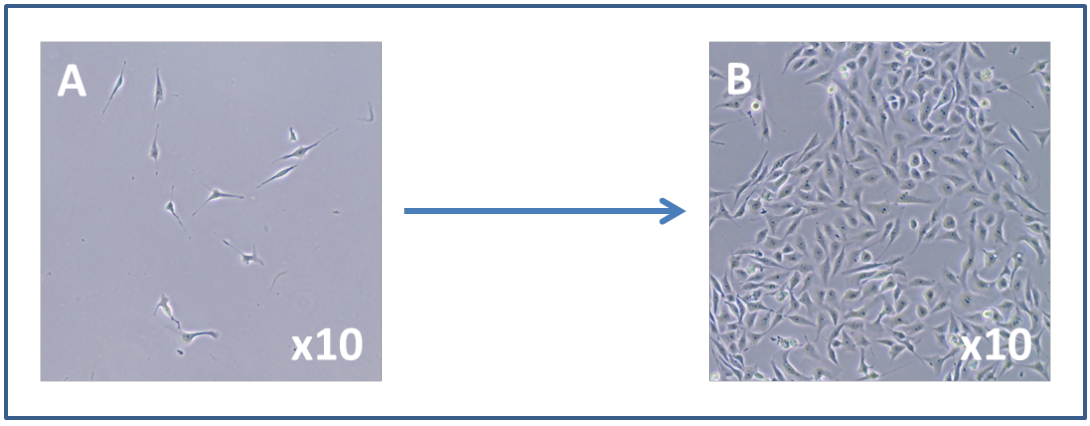


Figure 20: Depicting the morphology of BEAS-2B cell line.

(A) Beas-2B cells 24 hours after subcultivation with a density of 75,000 cells. (B) Beas-2B cells at 90% confluence. Both images were taken with EVOS XL Core-AMG, AMEX-1200.

2.4.3 Primary Human airway smooth muscle (HASM) culture

Endobronchial biopsies obtained during the bronchoscopy procedure were placed in ice cold PBS (Sigma, Missouri, USA). Pure ASM bundles were isolated by dissection, cultured and characterized by Dr Amanda Sutcliffe as previously described by (Sutcliffe *et al*, 2012; Kaur *et al*, 2010; Brightling *et al*, 2005).

Once the cells had been characterized as airway smooth muscle cells with >80% purity determined by α-smooth muscle actin expression, cells were enzymatically dissociated with 0.25% trypsin-EDTA (Gibco). Primary HASM were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), 1% Antibiotic-Antimycotic solution (Gibco), 1% Non-Essential Amino Acids (Gibco) and 1% sodium pyruvate (Sigma, S8636). HASM cells were subcultivated into uncoated T-75 flask.

HASM used for experimental purposes were used between passages 3 at 100% confluence (Figure 21). Furthermore, before each experiment HASM cells were serum deprived and placed in DMEM supplemented with 1% Insulin-Transferrin-Selenium (Gibco), 1% Non-Essential Amino Acids, 1% Sodium Pyruvate, Linoleic acid and oleic acid for 24 hours to synchronize the growth phase as previously done by (Kumawat *et al*, 2013).

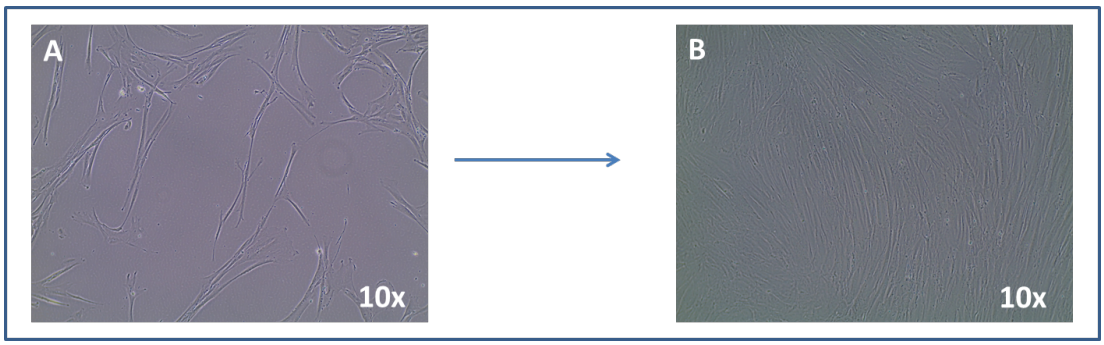


Figure 21: Primary human airway smooth muscle morphology in culture.

(A) Primary human airway smooth muscle cells 24 hours after subcultivation. (B) Primary human airway smooth muscle cells at 100% confluence. Both images were taken at passage 3 with EVOS XL Core-AMG, AMEX-1200.

2.4.4 L-cell WNT5a (mouse fibroblast) cell line culture

Frozen L-cell line (ATCC, Manassas, USA CRL-2814) was kindly provided by Professor Reinoud Gosens (University of Groningen) that overexpressed the WNT5a protein.

Cells were cultured as recommended by ATCC, in DMEM (ATCC) supplemented with 10% FCS (Gibco) and 0.6mg/ml Geneticin G418 (Fisher Scientific, Loughborough, UK). L-cells were enzymatically dissociated with 0.25% trypsin EDTA (Gibco) and subcultivated into a T-75 flask. All L-cells used for experimental purposes were used at passage <10 at 100% confluence (Figure 22)

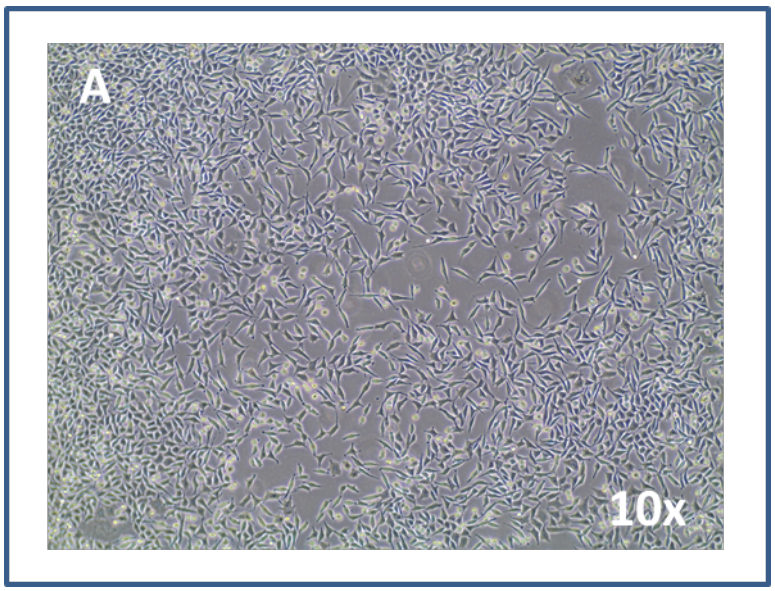


Figure 22: Mouse fibroblast L-cell line morphology over expressing WNT5a.

(A) Mouse fibroblast L-cell line over expressing WNT5a (ATCC, CRL-2814) at 100% confluence. Images were taken at passage 5 with EVOS XL Core-AMG, AMEX-1200.

For immunohistochemistry (IHC) L-cells were dissociated with 0.25% trypsin-EDTA, sedimented 600g for 7 minutes and suspended in HistoGel (Thermo Scientific, Massachusetts, USA) following manufactures instructions. HistoGel with L-cells were cut into pieces and placed in acetone (Sigma) with 2mM PMSF and 20mM iodoacetamide. Cells were fixed and embedded in GMA resin as described above.

2.5 Protein and RNA isolation from cell cultures

2.5.1 Protein lysates

Protein lysates for each cell type were isolated by, placing the culture vessel on ice and washing them with cold PBS. RIPA lysis buffer (Santa Cruz,

Texas, USA) was added as recommended by Santa Cruz Biotechnology and the cells were scraped. Cell lysate was subsequently incubated on ice for 30 minutes followed by centrifugation at 9600g for 15 minutes at 4ºC. The cell pellet was discarded and protein assay (Bio-Rad DC) was carried out on the supernatant. Samples were stored at -80ºC.

2.5.2 RNA lysates

RNA was extracted using the RNeasy mini kit (Qiagen, 74104) as described by the manufacturer. Briefly, cells were washed with ice cold PBS and Lysis buffer with 10μM β-mercaptoethanol was added to the culture vessels and scarped. RNA lysates were placed in spin columns and isolated per kit's instructions. RNA was eluted in RNase-free water and quantified using a Nano drop for concentration and RNA purity determined by the A_{260}/A_{280} ratio, samples were stored at -80°C. Additionally a RNA bio analyser (Agilent) was utilised to check the quality of the RNA (details) with a value of >9.0 used for experimental purposes.

2.6 Western Blotting

Protein lysates were prepared in 4x Laemmli buffer (Appendix 2) and heated for 5 minutes at 99ºC. Protein samples were equally loaded at 40-50μg per lane. SDS-PAGE protein separation and transfer of proteins onto a PVDF membrane was performed using a Bio-Rad system. The SDS-page gels were made at 12% (Appendix 3). Each gel had 15μl of Molecular weight marker (Fisher Scientific) loaded into one lane before electrophoresis was performed in 1X Running Buffer (Appendix 3) at 150V, followed by a semi-dry transfer in

1X Transfer Buffer (see Appendix 10) for 1 hour at 15V using Transblot turbo (Bio-Rad, California, USA).

Subsequent washing of membranes was done with 1x TTBS (Appendix 2) and blocking of non-specific binding of antibodies was done for 1 hour with blocking solution 1x TTBS + 5% Milk w/v (MARVEL, Dried Skimmed Milk).

The primary antibody was applied overnight and incubated at 4ºC with constant agitation, antibody list shown in table 1. The secondary antibody was applied for 1 hour. Antibody binding was visualised with Image Quant LAS4000 with the Pierce ECL substrate kit (Thermo Scientific).

2.6.1 Densitometer (semi quantitative) analysis of protein bands

Protein bands and subsequent protein expression obtained were analysed with the Image J software (Pictures were in 8-bit). Background was subtracted and each band was measured manually by drawing around it, generating an integrated density value. Protein expression was normalised to the loading control.

2.7 Immunoprecipitation

Immunoprecipitation can be used to test which proteins the antibody of interest can bind to. This is achieved by first attaching the antibody to an agarose bead which is then subsequently incubated with protein lysates. After incubation the heavy bead can be separated from the lysate solution capturing the antibody attached to it and any proteins attached to the antibody. The following pellet can be re-suspended and separated by gel electrophoresis. The protein bands can then be visualised with a stain and

cut for analysis. We utilised the liquid chromatography tandem mass spectrometry (LC MS/MS) to analyse the spliced bands.

2.7.1 Sample preparation and quantification

Primary antibody was incubated with protein A/G beads for 4 hours at 4°C. Antibody bead mixture was then washed (to remove unbound antibodies). Antibody bead mixture was applied to the protein lysates (500-1000µg) overnight at 4°C under constant agitation. After incubation, the immunoprecipitate was collected by centrifugation at 2500g for 5 minutes at 4°C. Supernatant was discarded and the pellet was washed in cold PBS.

Protein lysates were re-suspended and prepared in 4x Lamelli buffer (Appendix 3) and heated for 5 minutes at 99ºC. After heating, protein samples were centrifuged (to sediment the beads) and supernatant was equally loaded per lane. SDS-page protein separation and transfer of proteins onto a PVDF membrane was performed using a Bio-Rad system. The SDS-page gels were made at 12% (Appendix 3). Each gel had 15µl of Molecular weight marker (Fisher Scientific) loaded into one lane before electrophoresis was performed in 1X Running Buffer (Appendix 3) at 150V. To prevent the diffusion of proteins, the SDS-page gel was fixed in 7% glacial acetic acid (to prevent shrinkage) and 40% methanol for 1 hour under constant agitation at room temperature. The SDS-page gel was stained with Comassie blue (Sigma) stain for 2 hours under constant agitation at room temperature. SDS-page gel de-stained in 10% acetic acid and 25% methanol for 1 minute. Image of gel obtained and bands cut (by size) and sent to PNACL for mass spectrometry

analysis (University of Leicester, Leicester, UK). The raw data was analysed using Scaffold Q+S (version 3.6.1, Proteome Software).

2.8 Immunofluorescence

Initially, 4% paraformaldehyde was utilised a fixation solution, however due to cross-linking of structures it required antigen retrieval steps which would damage the delicate cell structure. Therefore we used ice-cold methanol, which disrupted hydrophobic interactions of proteins and also permeabilized the cell structure. The diluent for all staining solutions was PBS without calcium and magnesium.

Briefly, culture vessels were placed on ice and washed with PBS, 100% ice-cold methanol (Fisher Scientific) was added for 20 minutes to fix and permeabelize the cells. The methanol was removed and the culture vessels were air dried for 15 minutes. Non-specific binding was blocked with 3% BSA w/v for 1 hour at room temperature. Blocking solution was removed and primary antibody was added for 90 minutes at room temperature. Following incubation, primary antibody was removed and the culture vessels were washed three times with PBS with 0.05% tween 20. Secondary antibody was applied for 90 minutes in dark for 90 minutes at room temperature. After incubation, secondary antibody was removed and the culture vessel were washed three times with PBS 0.05% tween 20. Nuclear DAPI (Sigma) was added [1μg/ml] for 1 minute and then washed six times with PBS without calcium and magnesium. The cells were then coated with Prolong gold anti-fade reagent (Molecular Probes, Paisley, UK) for preserving the staining.

2.8.1 Immunofluorescence cytospins troubleshooting

Cells that could not adhere to glass chamber slides were smeared on glass slides using centrifugal force. In brief, cells were dissociated with 0.25% trypsin-EDTA, and sedimented at 600g for 5 minutes. The pellet was re-suspended at $5x10^5$ cells per ml. The microscope slides were placed in a cytospins device, which was then placed in the Cytospin 4 machine (Shandon, Thermo Scientific). Cell suspension was fed and centrifuged at 475rpm for 6 minutes at room temperature. The smeared cells were fixed in 4% paraformaldehyde. However, the smearing resulted in loss of morphology and damage to the cells. This was overcome by utilising plastic chamber slides that could be coated with collagen for adherence of cells.

2.8.2 Immunofluorescence image acquisition

Fluorescent images were taken using an automated Zeiss Microscope. The objective was set at x200 magnification with the fluorescent bulb at 50% intensity. FITC exposure was set to 1000ms (milliseconds) and DAPI exposure was set to 400ms. Images were captured with AxioCam HR R3.

2.8.3 Quantification of IF staining

The Zen 2012 software (Zeiss) was used to measure the intensity sum of FITC (gray scale value), area was determined in μm^2. Isotype control (background fluorescence) was subtracted prior to FITC measurement; this has been previously described by (Arthur *et al*, 2015) . Cell elongation was measured in a total of 10 cells. The length and width was defined and subsequently a ratio was calculated using the formula

$$Ratio = Average\ Length \div Average\ Width$$

The nuclei visualised by DAPI had variable sizes in different cells, to eliminate the error that more nuclear FITC stain was present because of a larger nucleus. Nuclear FITC staining was measured by first defining the nuclei area with DAPI staining in a total of 20 cells. That same area highlighted was measured for FITC channel only using the imaging software. The Intensity sum of FITC was divided by the DAPI defined area giving an average of FITC staining per nuclei; this value was subtracted from background fluorescence. All counts and threshold measurements were conducted blindly.

2.9 Calcium Flux measurement in BEAS-2B cells

2.9.1 Ratio metric Calcium imaging

To measure the calcium change we initially used a ratio metric assay. This involved incubating the cells with two different calcium dyes, Fluo-3 that increased fluorescent emission upon calcium binding and the other Fura red that decreased fluorescent emission upon calcium binding (Figure 23). This principle allowed measuring calcium flux by measuring the change in geometric mean fluorescence intensity using flow cytometry as previously described by (Kaur *et al*, 2015).

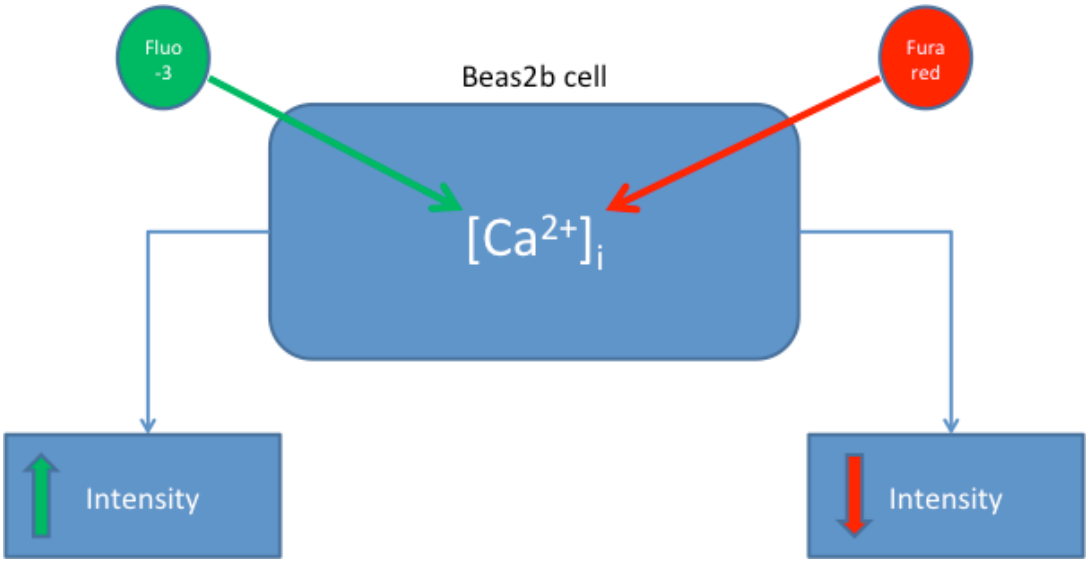


Figure 23: Depicting the fluorescent changes upon calcium binding.

In brief, BEAS-2B cells were cultured to confluence and dissociated with accutase for 5 minutes at 600g. Cells were re-suspended in 500ul PSS solution with 2mM calcium (appendix 4) and labelled with Fura-red [10μg/ml] and Fluo-3 [4μg/ml]. BEAS-2B cells were incubated for 45 minutes at 37°C in incubator covered with foil. After incubation, cells were sedimented and calcium dye was removed and cells were re-suspended in PSS solution with 2mM calcium. BEAS-2B cells were placed in BD FACSCanto II system (BD Bioscience) at $2.5x10^5$, and fluorescent emission was recorded for 1 minute to determine baseline, tube was removed and stimulant was added, the fluorescent was then recorded for an additional 3 minutes. Ionomycin [0.375mg/ml] was used as a positive control.

2.9.1.1 Troubleshooting Calcium Ratio metric assay

Our data consistently showed a deviation of the baseline Δ geometric mean fluorescence intensity as we measured more samples per experiment. This

led to a higher baseline in subsequent conditions being measured on the same day. Our positive control Ionomycin, consistently displayed large increase in Δ geometric mean fluorescence intensity, however upon stimulation with WNT5a, small changes in fluorescence were being masked by the deviation in baseline. This was observed by other members of the lab and was determined to be primarily due to dye leakage.

2.9.2 FLIPR calcium assay

An alternative way of measuring calcium was adopted using the FLIPR calcium 5 assay kit following the manufacturers protocol (Molecular Devices). BEAS-2B cells were seeded in triplicates at $1x10^4$ in black-walled 96-well plates (Thermo Fisher). The proprietary loading buffer that contained the dye was prepared with 2.5mM probenecid and added at 100μl per well, cells were subsequently incubated for 1h at 37°C. Stimulants were prepared with the loading buffer as a diluent in a separate 96-well plate at x5 concentration, this is because 50μl of stimulant was added to the total 200μl cell suspension. Following incubation 96-well plates were placed in the Flexstation 2 machine (Molecular Devices). The excitation wavelength was set at 485nm, the emission wavelength was set at 525nm and the automatic emission cut-off was set at 515nm.

2.10 Wound closure and migration in BEAS-2B cells

2.10.1 ORIS migration assay

This ORIS migration assay (Tebu-bio, 175CMAUFL4) is designed to allow control over the growth area of the cells using cell seeding stoppers as

recommended by the manufacturer. Briefly, 96-well plates were pre-coated with 1% pure-collagen and cell stoppers were inserted manually to prevent growth and adherence of cells in a pre-determined area. Beas-2b cells were plated at a culturing density of 10,000 cells per well in triplicates. After 24 hours, to allow cells to adhere, the cell stoppers were removed exposing a pre-coated but uncultured area. Appropriate stimulant was added to assess migration of the area.

After stimulation cells were fixed in 100% methanol (Fisher Scientific) for 20 minutes on ice and subsequently air-dried for 10 minutes. Nuclear label DAPI (Sigma) was applied for 1 minute followed by washing with PBS. Migration area was visualised using the EVOS FL-AMG, AMF4300 and placing a mask under the 96-well plate highlighting the area covered by the cell stoppers. The images were analysed using Zeiss 2012 software, briefly area of mask was highlighted and total DAPI dots were enumerated.

2.10.1 *in Vitro* Scratch assay

Another viable method for evaluating migration is to manually determine the area of insult and following this, observe the closure of the wound over a period of time with defined reference points. In order to achieve this, BEAS-2B cells were cultured in 6-well plates at a seeding density of $2x10^5$ with a confluence of 80%. Cell monolayer was scratched vertically with a sterile 200μl pipette tip in a predetermined grid pattern drawn under the 6-well plate (Figure 24). Following wounding, cell monolayer was washed with PBS four times to prevent any cell debris from influencing wound closure, as described previously by (Kaur *et al*, 2006).

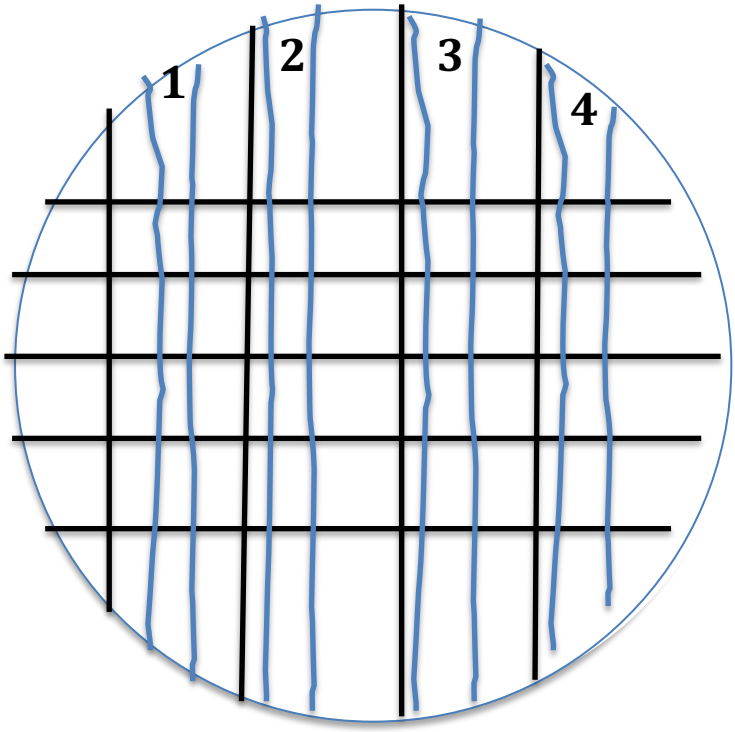


Figure 24: Depicting the grid pattern for wounding BEAS-2B cell monolayer, numbers 1-4 denote the scratch and the black gridlines highlight the reference markers made on the 6-well plate to trace the same wound.

Inhibitor BOX-5 was added 30 minutes prior to stimulation. All stimulants were made in equivalent DMSO concentration. TGF-β1 and WNT5a were added for 24hrs. Images were taken with EVOS XL Core-AMG, AMEX-1200 at baseline (immediately after wounding), 4h, 20h and 24h and wound image references were noted down. A total of four different wound images were taken per condition and per time point. Area of wound was measured using Photoshop. The area of baseline was first measured and traced on to the following stimulated conditions. The percentage of wound closure was determined by, first calculating the percentage of wound to basal and then subtracting that value by 100.

2.11 MTT proliferation assay

MTT is a yellow tetrazolium dye with the chemical formula 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The following dye can be chemically reduced by NADPH enzymes in live cells, forming insoluble formazan crystals and generating a subsequent purple colour. Using a spectrophotometer we can use this colorimetric assay to determine the viability of cells in certain conditions but also the number of cells. However the later can be manipulated by the metabolic activity of the cell as an increase in metabolic activity would increase the NADP enzymes activity and generate more formazan crystals.

MTT was prepared in PBS at stock concentration of 5mg/ml. BEAS-2B cells were seeded in triplicates at $1x10^4$ in 96-well plates. Stimulants were prepared in DMSO and applied for 24hrs. Inhibitor BOX-5 was added 30 minutes prior to stimulation. After 24hrs or 48hrs MTT was added at 20μl per well and incubated at 37^0C for 4hrs. Following incubation, cell suspension was aspirated carefully and formazan crystals were solubilised by adding 100μl of DMSO on a shaker for 15 minutes. The optical density was then measured using a spectrometer at 590nm.

2.12 Qiagen Polymerase Chain Reaction array

To evaluate downstream markers of WNT-TGFβ1 signalling, we selected a total of 16 genes (appendix 5) that can participate in cross talk from previous literature reviews (Miyoshi *et al*, 2012; Katoh and Katoh, 2009a; Kumawat *et al*, 2014; Dao *et al*, 2007; Attisano and Labbe, 2004) and our own microarray

data from epithelial brushing. Qiagen designed the PCR array for use in Stratagene Mx3000p PCR machine in an 8x3 96-well format, allowing 4 samples to be analysed per 96-well plate.

2.12.1 RNA conversion to complementary DNA (cDNA)

Following RNA extraction, we converted RNA to complementary DNA (cDNA) using the RT^2 first strand kit (Qiagen) as per manufacturers instructions. Briefly, 500ng of RNA was incubated for 5 minutes at 42°C in a genomic DNA elimination mixture. The following solution was prepared in a reverse transcription mix, homogenised and incubated at 42°C for 15 minutes, followed by incubation at 95°C for 5 minutes to stop enzymatic reaction. Each reaction solution was topped up with 91μl of RNase-free water and stored at -20°C.

2.12.2 Quantitative Real Time-PCR

Real-time PCR was conducted using RT^2 Profiler PCR arrays kit (Qiagen) as recommended by manufacturer. SYBR green mastermix was prepared with cDNA and topped with RNase-free water. The following solution was carefully homogenised and pipetted at 25μl per well. Each 96-well plate was centrifuged for 1 min at x1000g at room temperature to remove any bubbles. The thermal profile was created with 3 segments for each well:

- Segment 1 (1 cycle) = A total of 10 minutes at 95^0C to activate the hotstart DNA Taq polymerase enzyme.

- Segment 2 (40 cycles) = each cycle to begin at 95^0C for 15 seconds and then drop to 60^0C for 1 minute for annealing of double stranded DNA (dsDNA) and subsequent fluorescence data collection.
- Segment 3 (1 cycle) = each cycle to begin at 95^0C for 1 minute to denature any dsDNA, followed by 55^0C for 30 seconds for annealing of dsDNA and lastly, temperature increased back to 95^0C for 30 seconds to measure the dissociation and subsequent loss of fluorescence data and generate the melting curve.

The internal reference dye ROX was selected for normalization of fluorescent signal on the Stratagene Mx3000p. SYBR green dye has a high affinity for dsDNA therefore, to confirm the presence of specific and non-specific dsDNA formation a melt curve was used with a single peak highlighting a single product, furthermore a hotstart Taq polymerase was used to prevent non-specific dsDNA formation.

2.13 Data analysis

Statistical analysis was conducted using PRISM 6 software. Data sets were analysed by D'Agostino-Pearson omnibus normality test to check if the values come from a Gaussian distribution. The means were compared by a student (unpaired, two-tailed) t-test for parametric data sets and the Mann-Whitney test was used for non-parametric data sets. For multiple comparisons, one-way analysis of variance (ANOVA) (parametric) and Kruskal-Wallis test (non-parametric) were used to compare means in 3 or more groups. Changes were

deemed significant if P value < 0.05. The ‘n’ value denotes the number of biologically independent experiments.

Chapter 3: Clinical Characteristics of Genentech cohort

3.1 Clinical and Demographic data

A total of 9 healthy and 25 asthmatic subjects' bronchial biopsies were selected for analysis. In addition, Th-inflammatory status was determined previously by calculating the mean centre value of each gene (by Dr Matthew Richardson). The genes used to construct Th2-score are POSTN, CLCA1 and SERPINB2. The genes used to construct Th17-score are CXCL1, CXCL2, CXCL3, IL8 and CSF3.

To construct TH2 and TH17 the data set needs to have the log ratios for the genes above as columns, the rows in the data set are patients. The log ratio for each gene is mean centred, for example mean centred POSTN_MC[i]=POSTN[i]-mean(POSTN), i =1 to N, where N is the number of patients (rows). Once the log ratios have been mean centred, the scores are constructed as follows:

TH2[i]= mean(POSTN_MC[i], CLCA1_MC[i], SERPINB2_MC[i])

TH17[i]=mean(CXCL1_MC[i], CXCL2_MC[i], CXCL3_MC[i], IL8_MC[i], CSF3_MC[i])

This was only applied to asthmatic patients, as normal patients did not have differential expression of the selected genes to generate a Th-inflammatory score (Choy *et al*, 2015; Choy *et al*, 2011a). Furthermore, gene expression was expressed as log ratio values based on fluorescence intensity of perfect match (binding of labelled mRNA from cells to the probe on Affymetrix array) and mismatch (binding of non-specific gene and background noise).

The demographics of the patients are shown in table 7. A significant increase in eosinophils in Th2 asthma versus health is confirmed as previously observed by (Woodruff *et al*, 2009). Interestingly asthmatics with a Th-17 phenotype have lower lung function than Th-2 phenotypic patients, however the disease duration is similar, albeit patients are older with Th-17 phenotype and consume more ICS.

Demographic	Control n=9	Th2 Asthma n=7	Th0 Asthma n=10	Th17 Asthma n=8
Age (yrs)	30.00 (23 - 57)	25.00 (22 – 47)	37.00 (27.75 – 53)	35.50 (23.75 – 54.5)
Gender M:F	4:5	4:3	6:4	5:3
Disease Duration (yrs)	-	17.00 (12 – 23)	5.00 (2.75 – 10.5)	16.50 (2.25 – 52.25)
Beclometasone (BDP) μg/day	N/A	250 (0.0 – 875)	1000 (600 – 1600)	800 (575 – 1700)
IgE IU/ml	5.0 (1.0 – 60.0)	300.5 (227.3 – 3910)	154 (56.35 – 416)	111.5 (59.55 – 2424)
FEV1% predicted (Pre)	107.0 (93.5 – 123.5)	94.30 (62 – 107)	93.00 (77 – 98.5)	72.50 (47.5 – 89.00)

Demographic	Control n=9	Th2 Asthma n=7	Th0 Asthma n=10	Th17 Asthma n=8
eosinophils/mm^2	4.59 (2.17 – 12.38)	24.43 (17.53 – 56.62) *‡	2.36 (1.46 – 14.84)	11.21 (3.73 – 19.31)
Neutrophils/mm^2	13.77 (3.15 – 23.8)	3.38 (2.45 – 9.79)	5.46 (2.97 – 13.29)	10.86 (1.22 – 17.14)
mast cells/mm^2	17.62 (12.69 – 20.61)	13.99 (6.78 – 20.72)	6.75 (3.16 – 15.22)	10.66 (4.27 – 11.17)

Table 7: Displaying the clinical characteristics of the Genentech cohort. Data table displaying median and IQR values; p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with control subjects, † p=<0.05 as compared with Th2-asthma, ‡ p=<0.05 as compared with Th0-asthma, ¶ p=<0.05 as compared with Th17-asthma.

3.2 Bronchial biopsy morphology

Biopsy images were obtained, and subsequent tissue compartments were analysed and expressed as a percentage of total biopsy area as shown in table 8.

Demographic	Control n=9	Th2 Asthma n=7	Th0 Asthma n=10	Th17 Asthma n=8
ASM (%)	17.26 (5.51 – 22.41)	19.13 (11.93 – 23.85)	9.850 (3.19 – 31.49)	8.530 (2.03 – 16.44)
Epithelium (%)	8.99 (4.79 – 17.95)	15.87 (12.09 – 28.45)	15.46 (6.46 – 41.05)	13.32 (6.61 – 27.34)
Reticular basement membrane thickness (µm)	4.910 (2.99 – 7.55)	13.15 (9.72 – 14.90) *	7.590 (7.31 – 13.24)	12.84 (8.46 – 14.33)
Lamina propria (%)	66.43 (59.72 – 68.61)	65.58 (42.05 – 68.64)	55.89 (35.22 – 64.38)	49.34 (39.85 – 69.54)
Damaged tissue (%)	8.81 (5.57 – 13.60)	6.36 (3.11 – 14.4)	6.21 (3.69 – 7.79)	8.43 (5.88 – 11.64)
Glands (%)	0.0 (0.0 – 0.55)	0.0 (0.0 – 1.92)	0.0 (0.0 – 0.65)	0.11 (0.0 – 4.10)

Table 8: Displaying immunopathological characterisation of the Genentech cohort bronchial biopsies. Data table displaying median and IQR values; p

value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with control subjects.

Interestingly, tissue compartments did not vary significantly, however the reticular basement membrane was significantly thicker in Th-2 asthma phenotype compared to healthy. This trend of RBMT was also apparent in Th-17 asthma phenotypes.

3.2.1 Epithelial damage in asthma

Epithelial functionality in asthma has been shown to be impaired, including but not limited to, increasing susceptibility to apoptosis (Bucchieri *et al*, 2002), secretion of cytokines and growth factors (Takahashi *et al*, 2011) and mucus goblet cell hyperplasia (Aikawa *et al*, 1992; Crosby and Waters, 2010). However, in bronchial biopsies the epithelium appears with physical injury. To determine if this is an artefact of biopsy collection we measured the length of intact epithelium attached to the BM, damaged epithelium that was attached to the BM but not fully pseudo-stratified and denudated epithelium where the BM had no epithelial cells attached.

The results expressed as a percentage of total BM length, show no significant difference between asthma and healthy in figure 25A.

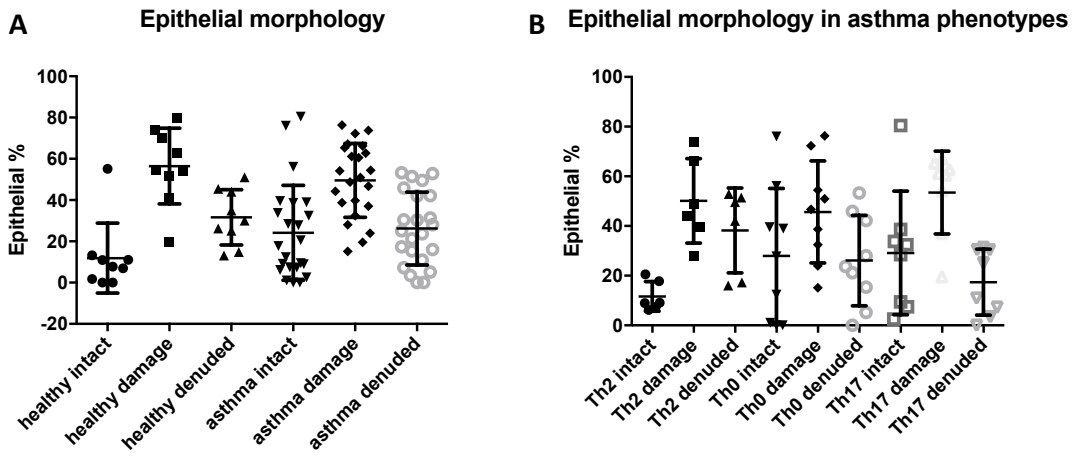


Figure 25: Epithelial morphology expressed as a percentage of total RBM length in (A) healthy versus asthma and in (B) asthma phenotypes.

Additionally, no difference was observed in epithelial morphology in asthma phenotypes figure 25B.

3.3 Gene expression in Genentech cohort

As part of the Genentech cohort, bronchial biopsies homogenates were processed for microarray analysis. We initially selected WNT5a and TGF-β1 related signalling molecules and compared against healthy and asthma phenotypes as shown in table 9 (data extracted by Dr Matthew Richardson).

Gene	Healthy n=7	Th2 Asthma n=7	Th0 Asthma n=10	Th17 Asthma n=8
WNT5A	-0.57 (-0.73 - -0.47)	-0.41 (-0.60 - - 0.25)	-0.61 (-0.90 - - 0.34)	-0.64 (-0.76 - - 0.49)
TGF-β	-0.80 (-0.88 - -0.55)	-0.82 (-0.93 - - 0.59)	-0.78 (-0.79 - - 0.69)	-0.86 (-0.93 - - 0.64)

ROR2	0.06 (-0.11 – 0.22)	0.08 (-0.06 – 0.32)	0.10 (0.06 – 0.17)	0.15 (0.04 – 0.21)
FZD5	-0.43 (-0.69 - -0.35)	-0.03 (-0.26 – 0.003) *‡	-0.43 (-0.49 - - 0.32)	-0.23 (-0.40 - - 0.17)
FZD4	0.45 (0.20 – 0.58)	0.23 (0.11 – 0.34)	0.29 (0.21 – 0.39)	0.18 (0.00 – 0.35)
ROR1	-0.59 (-0.82 - -0.29)	-0.56 (-0.65 - - 0.35)	-0.53 (-0.84 - - 0.32)	-0.76 (-1.0 - - 0.64)
DVL2	-0.26 (-0.32 - -0.20)	-0.24 (-0.35 - - 0.16)	-0.27 (-0.37 - - 0.16)	-0.27 (-0.33 - - 0.15)
JNK	0.31 (0.19 – 0.32)	0.22 (0.11 – 0.33)	0.22 (0.10 – 0.28)	0.28 (0.23 – 0.33)
NFATc1	-0.05 (-0.16 - -0.04)	0.01 (-0.01 – 0.15)	0.13 (-0.08 – 0.28)	0.10 (-0.01 – 0.26)
TAK-1/MAP3K7	0.04 (-0.06 – 0.12)	0.19 (0.13 – 0.22) *	0.08 (-0.04 – 0.15)	0.08 (0.04 – 0.09)
SMAD2	0.23 (0.13 – 0.27)	0.25 (0.21 – 0.39)	0.28 (0.22 – 0.30)	0.25 (0.19 – 0.27)
SMAD3	0.57 (0.53 – 0.62)	0.59 (0.57 – 0.68)	0.64 (0.41 – 0.77)	0.70 (0.57 – 0.78)
SMAD4	-0.30 (-0.43 - -0.24)	-0.17 (-0.24 - - 0.11)	-0.31 (-0.40 - - 0.19)	-0.21 (-0.34 - - 0.15)

β-catenin	-0.33 (-0.51 - -0.31)	-0.36 (-0.53 - -0.23)	-0.43 (-0.52 - -0.40)	-0.47 (-0.55 - -0.38)
β-arrestin 2	0.09 (0.048 – 0.33)	0.01 (-0.15 – 0.05)	0.08 (0.01 – 0.19)	-0.05 (-0.09 – 0.11)

Table 9: Gene expression (expressed in log ratio values) of the Genentech cohort. Data table displaying median and IQR values; p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with control subjects, † p=<0.05 as compared with Th2-asthma, ‡ p=<0.05 as compared with Th0-asthma, ¶ p=<0.05 as compared with Th17-asthma.

A significant difference was found in Th2 asthma FZD5 mRNA expression in comparison to healthy and Th0 asthma. Interestingly, TAK-1/MAP3K7 a downstream target of TGF-β1-smad independent pathways was significantly elevated in Th2 asthma in comparison to healthy.

3.3.1 WNT signalling gene expression in Genentech cohort

We next looked at the expression of WNT related signalling molecule in the total Genentech cohort whom had microarray analysis in biopsy homogenates and some in epithelial brushings shown in figure 26. We found no significant difference in WNT ligands and receptor expression in health vs asthma (adjusted p-values (q-values) determined shown in Appendix 6 calculated by Dr Matthew Richardson). Interestingly the trend of WNT related signalling molecules expression was consistent in epithelial brushing figure 26B. This analysis was extended to different asthma phenotypes in biopsy

homogenates, however no significant difference was observed (data shown in appendix 6).

Lastly, we compared WNT5a and TGF-β1 gene expression in biopsy homogenates and epithelial brushings based on GINA classification as shown in figure 27. In both, biopsy and epithelial brushings WNT5a expression was not significantly elevated figure 27A-B. In the case of TGF-β1 gene expression, the epithelium had a significant increase in asthmatics classified as GINA 5, conversely, TGF-β1 gene expression was decreased in biopsy homogenates of asthmatics with GINA 5 classification.

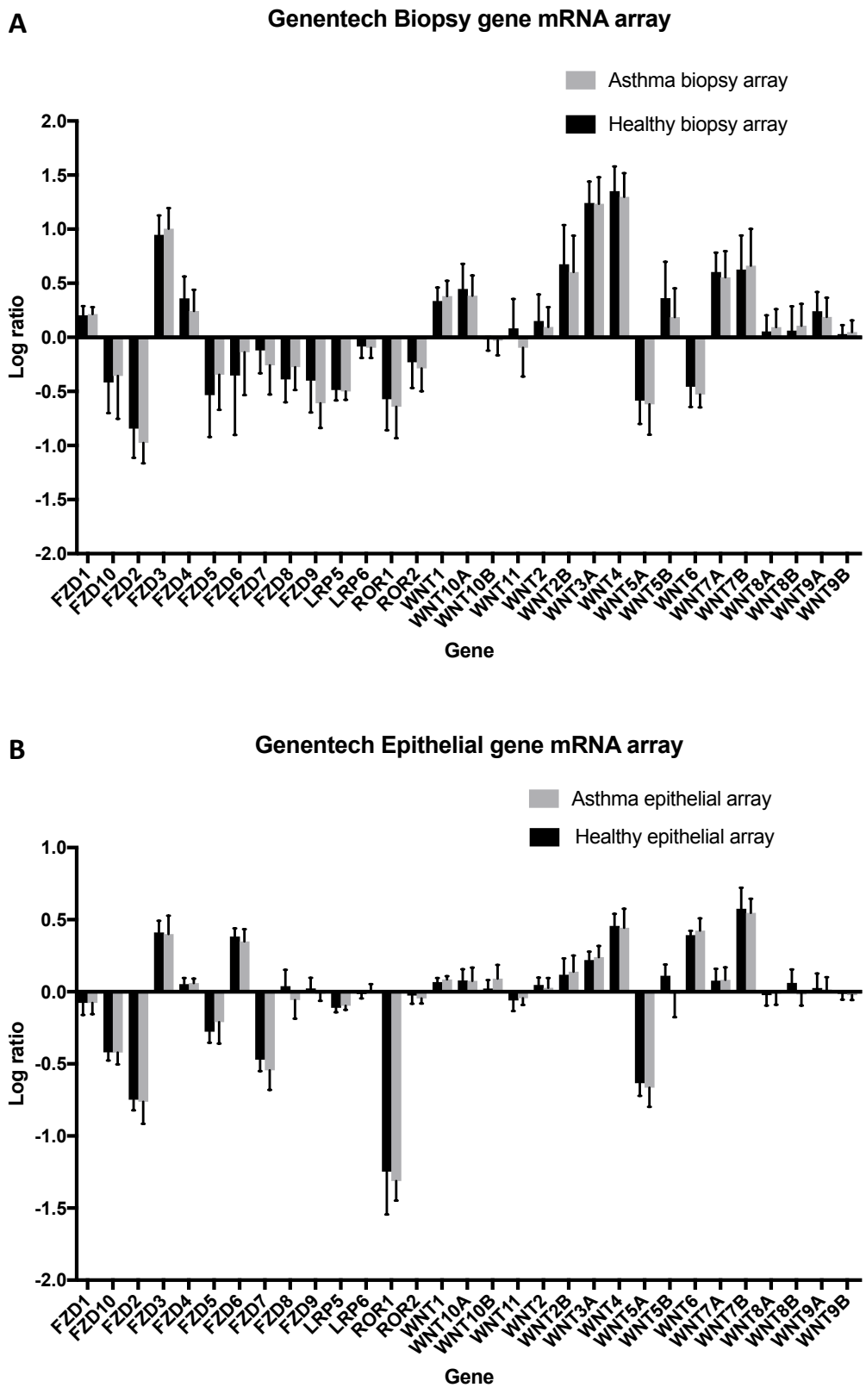


Figure 26: Gene expression of multiple WNT components in (A) biopsy homogenates and (B) epithelial brushings. p value determined from Wilcoxon

rank sum test, the adjusted p values were obtained using the FDR method (benjamini and Hochberg).

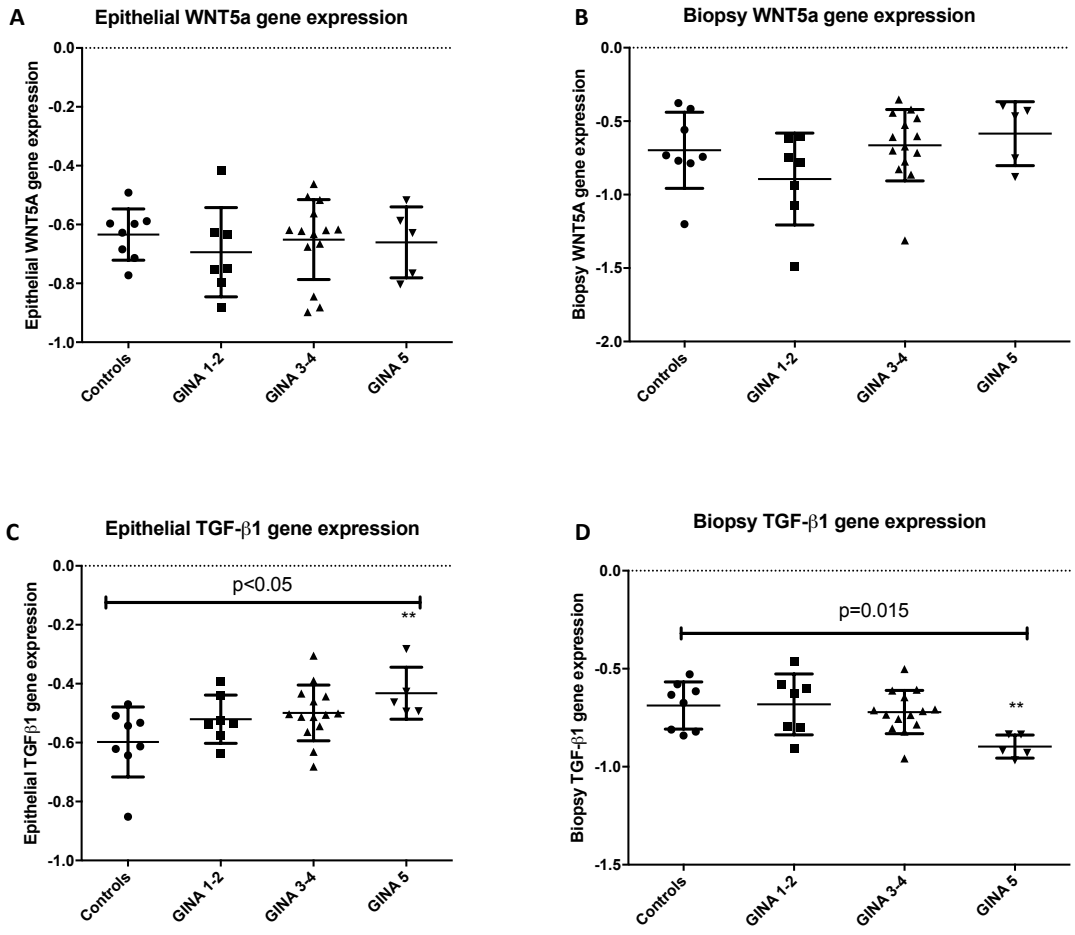


Figure 27: WNT5a gene expression (A-B) and TGF-β1 gene expression (C-D) in healthy and asthma classified by GINA.

3.4 Discussion

We selected an array of patients randomly from the Genentech cohort previously characterised (Choy *et al*, 2015). The mean age and gender diversity was consistent across the groups. Interestingly, the duration of disease was higher in Th2 and Th17 asthma phenotypes, but only Th17

asthma phenotype had a more pronounced reduction in lung function. Consistent with the literature Th2 asthmatic patients had an increase in eosinophils in the submucosa (Woodruff *et al*, 2009) and higher serum IgE, with neutrophils highest in Th17 asthma phenotypes (Newcomb and Peebles, 2013; Cosmi *et al*, 2011). The reduction of neutrophils in Th2 asthma can occur as IL-13 a typical type 2 cytokine can attenuate IL-17a production and promotion of Th-17 cells (Newcomb *et al*, 2011), this in turn can inhibit neutrophil recruitment. Surprisingly, mast cell number was not significantly elevated in asthma, suggesting that mast cell localization rather than mast cell number is a more important determinant of asthma pathophysiology (Brightling *et al*, 2005; Siddiqui *et al*, 2008).

Quantitation and measurement of tissue compartments of bronchial biopsies showed typical asthmatic features of RBM thickening (Sobonya, 1984; Benayoun *et al*, 2003) but no difference in ASM area or epithelial area. With regards to the latter physical damage was devoid of any associations with disease status. Supporting this observation (Loxham, Davies and Blume, 2014) show that epithelial shedding or denudation is primarily attributed to bronchial biopsy collection and as a consequence an artefact. Furthermore, damaged epithelial cells similar to our data represent the large pool of epithelium coverage on the basement membrane.

Microarray analysis of the Genentech cohort displays no significant difference in WNT signalling components. Interestingly WNT ligands and receptors are differentially regulated in asthma and health. Previously, (Choy *et al*, 2011b) have shown WNT ligands particularly WNT5a is elevated at an mRNA level

in asthma with a Th2-high phenotype and are not treated with steroids. This was not apparent in our cohort and may be attributed to the fact our patients were on corticosteroid treatments. Furthermore, the gene expression signal may be diluted in bronchial biopsy homogenates, which contain many structural and inflammatory cells. This is apparent in TGF-β1 gene expression where it is increased at an mRNA level in severe asthmatics in the epithelium. However in biopsy homogenates TGF-β1 gene expression is significantly suppressed in severe asthma as shown in figure 27D and no difference is observed between asthma phenotypes as shown in table 9. Conversely, protein expression of TGF-β1 in asthma has been shown to be elevated in the submucosa and attributed to the eosinophils (Flood-Page *et al*, 2003), ASM also have the capacity to induce TGF-β1 protein expression in the presence of mast cells (Woodman *et al*, 2008) a feature of asthma. Therefore, this may indicate that WNT5a protein expression may differ from gene expression and requires investigation.

Chapter 4:

Immunostaining of bronchial biopsies

4.1 Validation of WNT5a antibody

4.1.1 WNT5a antibody staining in IHC-GMA

To observe the distribution of WNT5a protein expression, bronchial biopsies sections embedded in GMA were immunostained with multiple WNT5a antibodies. Initially, to observe the distribution of WNT5a protein expression, bronchial biopsies sections were immuno-stained with WNT5a LSbio antibody (Figure 28). The results displayed excessive staining for the rabbit IgG isotype control (Figure 28B), suggesting that it is non-specific. Reducing the concentration of the WNT5a LSbio antibody from 10µg/ml to 5µg/ml (Figure 28B-C) resulted in an equal reduction of staining intensity for the isotype control and WNT5a staining (Figure 28D-E). Further reduction of the antibody at 4µg/ml (data not shown) resulted in no isotype control or antibody staining. This concludes that WNT5a LSbio antibody is non-specific.

Utilising a different antibody raised against human WNT5a protein from RnD systems, no staining was observed in GMA embedded bronchial biopsy sections (Figure 29). This was repeated in a different biopsy; however no staining was observed again (data not shown). Suggesting this antibody is probably incompatible with IHC conducted in GMA sections.

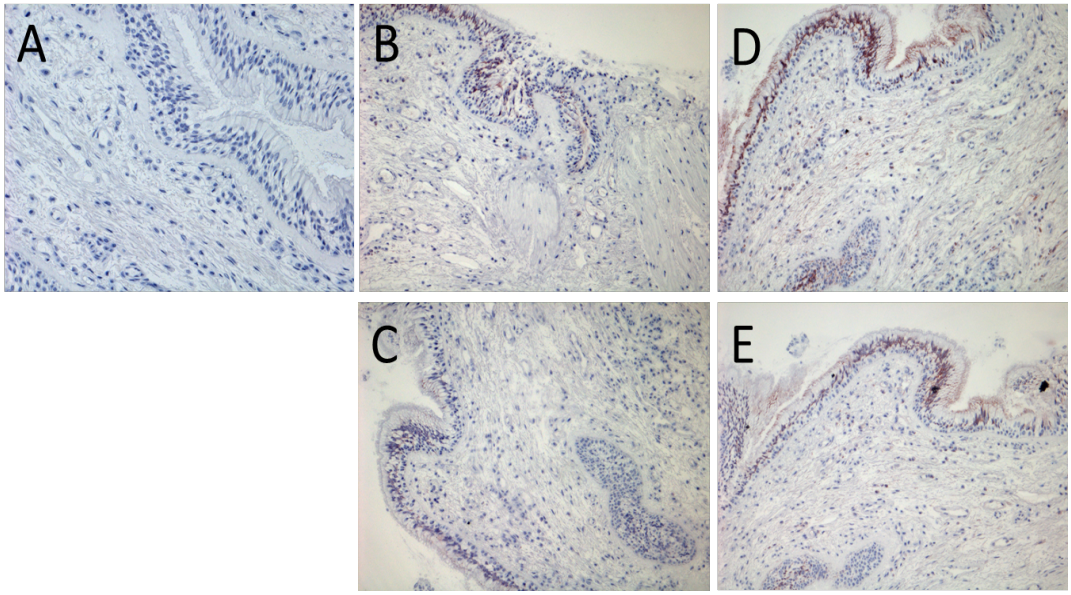


Figure 28: Photomicrograph of a bronchial biopsy section (x100 magnification) stained with WNT5a LSbio antibody. (A) No antibody, (B) rabbit IgG at 10µg/ml, (C) rabbit IgG at 5µg/ml, (D) WNT5a LSbio antibody at 10µg/ml and (E) WNT5a LSbio antibody at 5µg/ml.

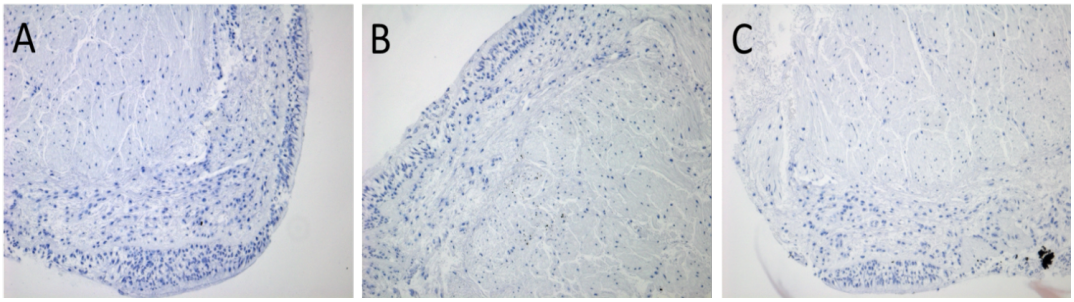


Figure 29: Photomicrograph of bronchial biopsy section (x100) magnification stained with WNT5a RnD antibody. (A) No antibody), (B) Rat IgG2a (isotype control) at a concentration of 20µg/ml and (C) WNT5a RnD antibody at a concentration of 20µg/ml.

To address this issue, a positive control was established by embedding L-cells (that over express WNT5a) in GMA blocks. Staining L-cells sections in GMA with WNT5a LSbio and WNT5a RnD antibody displayed no staining (data in appendix 7). However, staining these L-cells with a new antibody WNT5a 6F2, clear cytosolic staining is visible (Figure 30E) in all the cells. Additionally, multiple structures are stained in the bronchial biopsies (figure 30F) and no staining is observed in the isotype control mIgG1 (Figure 30C-D)

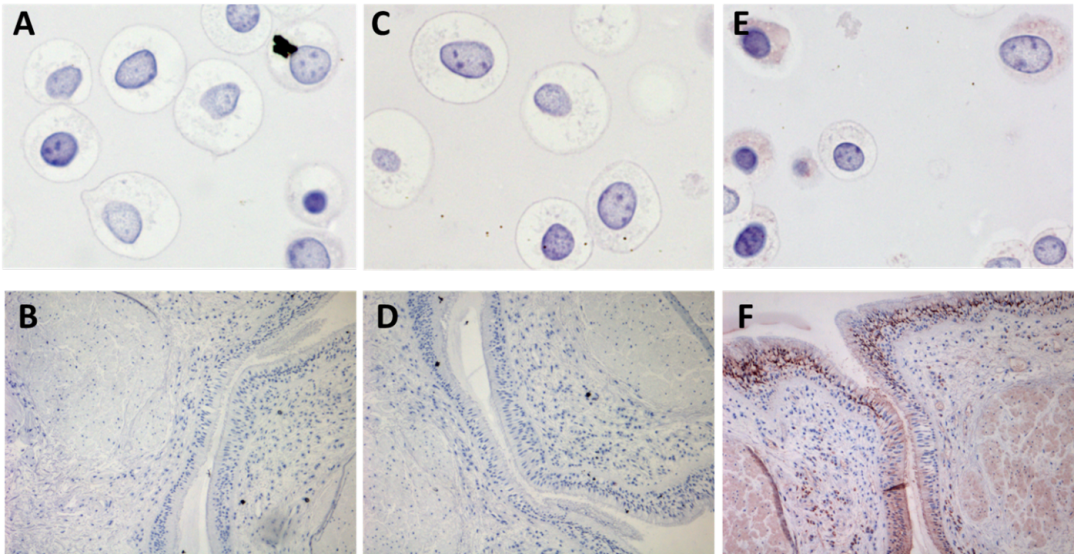


Figure 30: Photomicrograph of a GMA-embedded section stained in L-cells at x400 magnification (A, C and E) and bronchial biopsy at x200 magnification (B, D and E) with WNT5a 6F2 antibody. (A-B) no antibody (C-D) mouse IgG1 at 5µg/ml, (E-F) WNT5a 6F2 antibody at 5µg/ml.

4.1.2 WNT5a antibody validation in *in vitro*

Initially WNT5a protein expression was assessed in lung structural cells (HBEC and HASM) using WNT5a LSbio antibody. The antibody recognised

the recombinant protein, but also displayed multiple bands most prominently at 80kDa and 39kDa. The 39kDa band has previously been shown by (Ghosh et al, 2013). All samples were evenly loaded as depicted by β-actin (data shown in appendix 8).

In comparison, the same samples were immunolabelled with WNT5a RnD antibody. The results showed a clear band for HASM and human lung fibroblasts at ~40kDa and the recombinant protein weakly (data shown in appendix 8). In both cases the recombinant protein was observed between 60-80kDa standard markers as it had a proprietary tag. Furthermore, no protein expression was observed for HBEC with WNT5a RnD antibody.

As a preliminary experiment, we conducted western blot in HASM only with WNT5a 6F2 antibody and L-cells that overexpress WNT5a. There was a consistent band in both cell types at ~30kDa (much smaller than the size of WNT5a), but interestingly bands in asthmatic HASM seem more intense vs. healthy as illustrated by Figure 31.

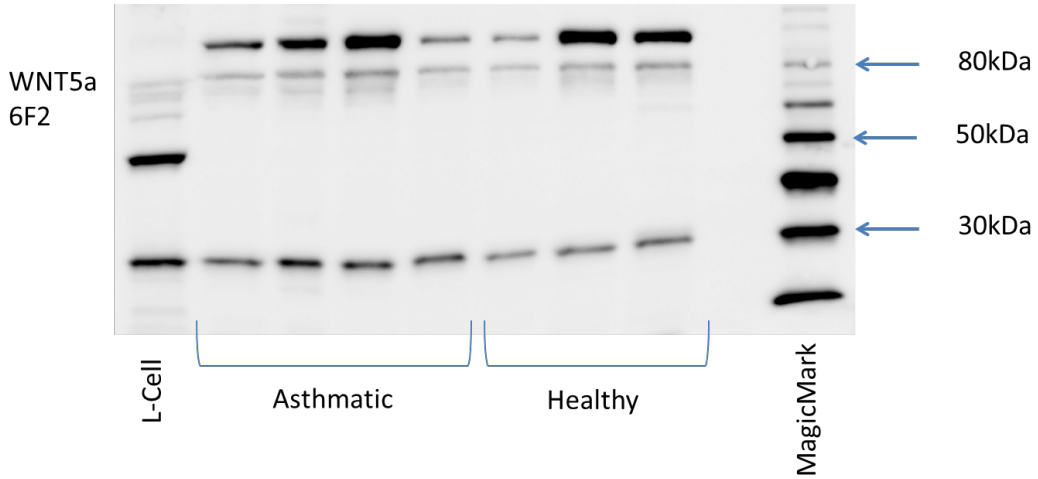


Figure 31: WNT5a protein expression in asthmatic vs. healthy in HASM. Immunolabelled with WNT5a 6F2 antibody, PVDF membrane exposed for 1 minute.

To further evaluate, we ordered a custom peptide to which the antibody was raised against. Containing a proprietary sequence, it contained WNT5a amino acids at position 75-380 and is a recombinant protein expressed in E.coli (Novus Biologicals, NBP1-28873PEP, Gene ID: 7474). We initially conducted blocking peptide experiments by sequestering the primary antibody with its raised Immunogen to no avail (data not shown).

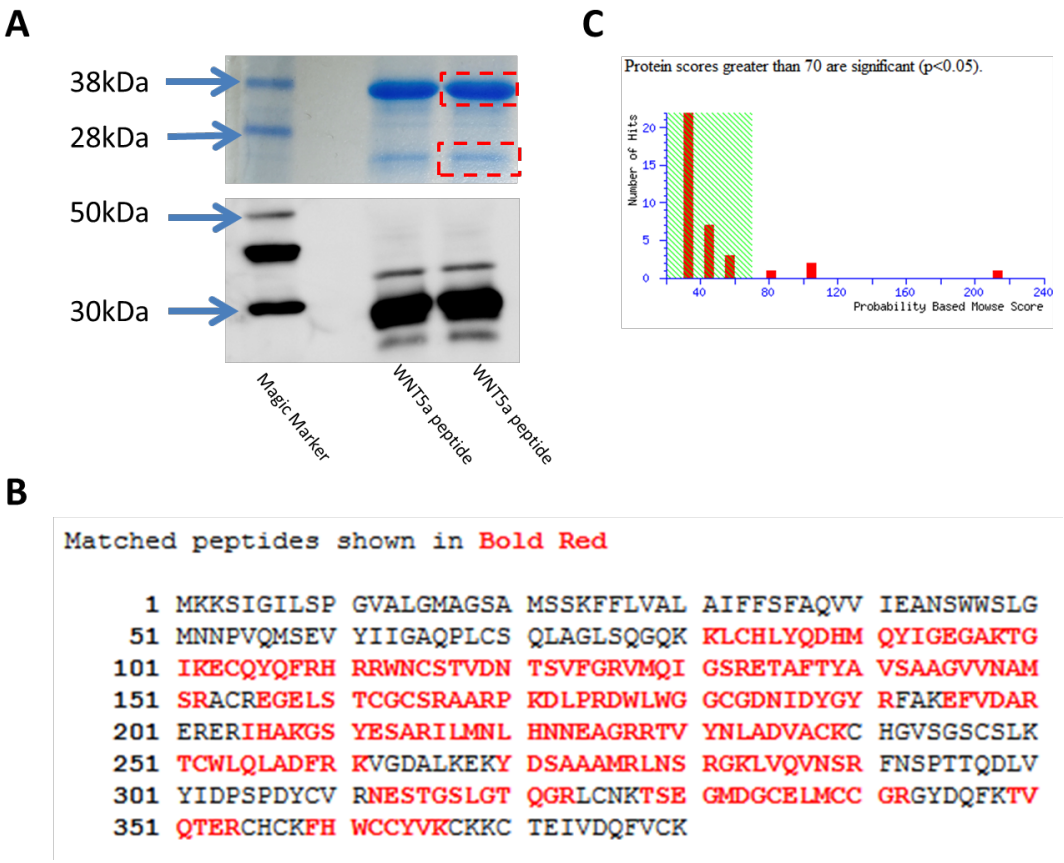


Figure 32: Proteomic analysis of WNT5a (A) SDS-page gel stained with Comassie blue and PVDF-membrane immunoblotted with WNT5a 6f2 antibody. (B) Matched sequences of WNT5a peptide (cleaved by trypsin) as confirmed by PNACL (University of Leicester). (C) Probability based mowse scores of WNT5a peptide sequences cleaved by Trypsin.

To understand if this protein peptide was indeed a WNT5a peptide, we conducted a mass spectrometry analysis. After loading the peptide on a SDS-page gel and conducting electrophoresis and Comassie staining, it revealed a 38kDa and 28kDa band (Figure 32A) of which both were cut and sent to PNACL for analysis. The data showed that the peptide indeed resembled WNT5a P41221 with a relative molecular weight of 43.68kDa. The peptide

had sequence coverage of 58%, this is because the first 75 amino acids were already truncated during the purchase of the peptide and furthermore, the peptide was placed in trypsin digestion prior to mass spectrometry analysis (Figure 32B). All protein scores (probability based mowse score) greater than 70 (i.e. $p<0.05$) matched the protein WNT5a (Figure 32C). Lastly, transferring the peptide to a PVDF membrane allowed the detection of the protein at ~30kDa with the WNT5a 6F2 antibody (Figure 32A). Confirming that WNT5a 6F2 antibody indeed has the capacity to detect WNT5a protein.

To compare WNT5a protein expression in asthmatic vs. healthy in lung structural cells a second technique was developed. Fixing L-cells over expressing WNT5a in 4% paraformaldehyde (and permeabilized with triton X-100) or 100% methanol displayed no positive staining for WNT5a LSbio and WNT5a RnD antibody. The same was observed in HASM and HBEC. On the other hand, staining L-cells with WNT5a 6F2 antibody displayed clear cytosolic protein expression in L-cells (Figure 33).

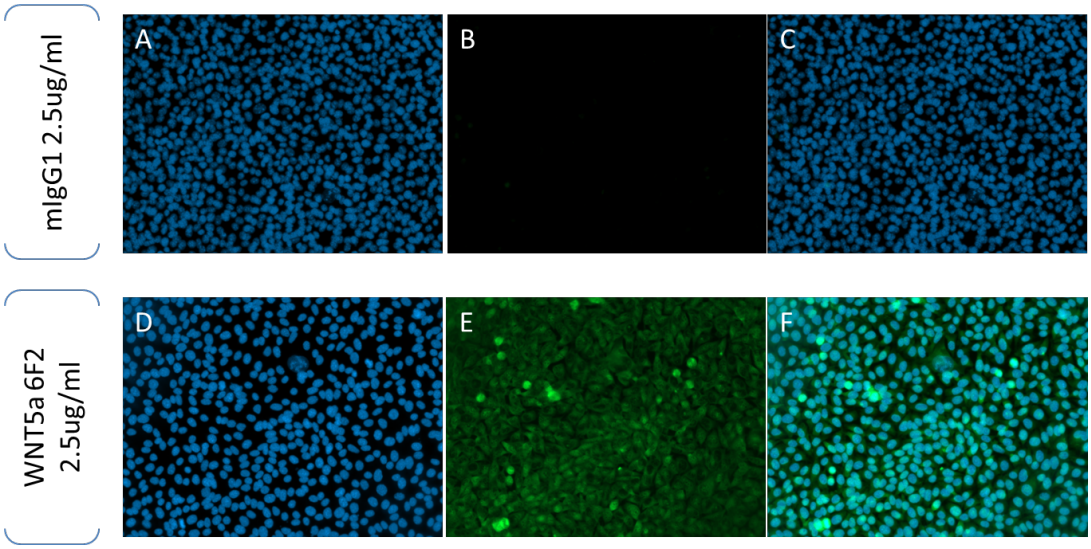


Figure 33: Showing WNT5a protein expression in L-cells (x100 magnification) stained with WNT5a 6F2 antibody. (A) DAPI staining with isotype control at 2.5ug/ml, (B) FITC staining with isotype control at 2.5ugm/ml, (C) FITC + DAPI images merged for isotype control, (D) DAPI staining with WNT5a 6F2 antibody at 2.5ug/ml, (E) FITC staining with WNT5a 6F2 antibody at 2.5ugm/ml, (F) FITC + DAPI images merged for WNT5a 6F2 antibody.

4.2 WNT5a protein expression in asthma

WNT5a immunostaining was observed in multiple airway structures, including the epithelium, the airway smooth muscle, submucosa and endothelial cells with resident leukocytes in blood vessels (Figure 34C).

4.2.1 WNT5a protein expression in the lamina propria

To quantify the amount of WNT5a protein expression we counted positively stained cells in the lamina propria (the tissue interface below the epithelium) in healthy controls and asthmatics.

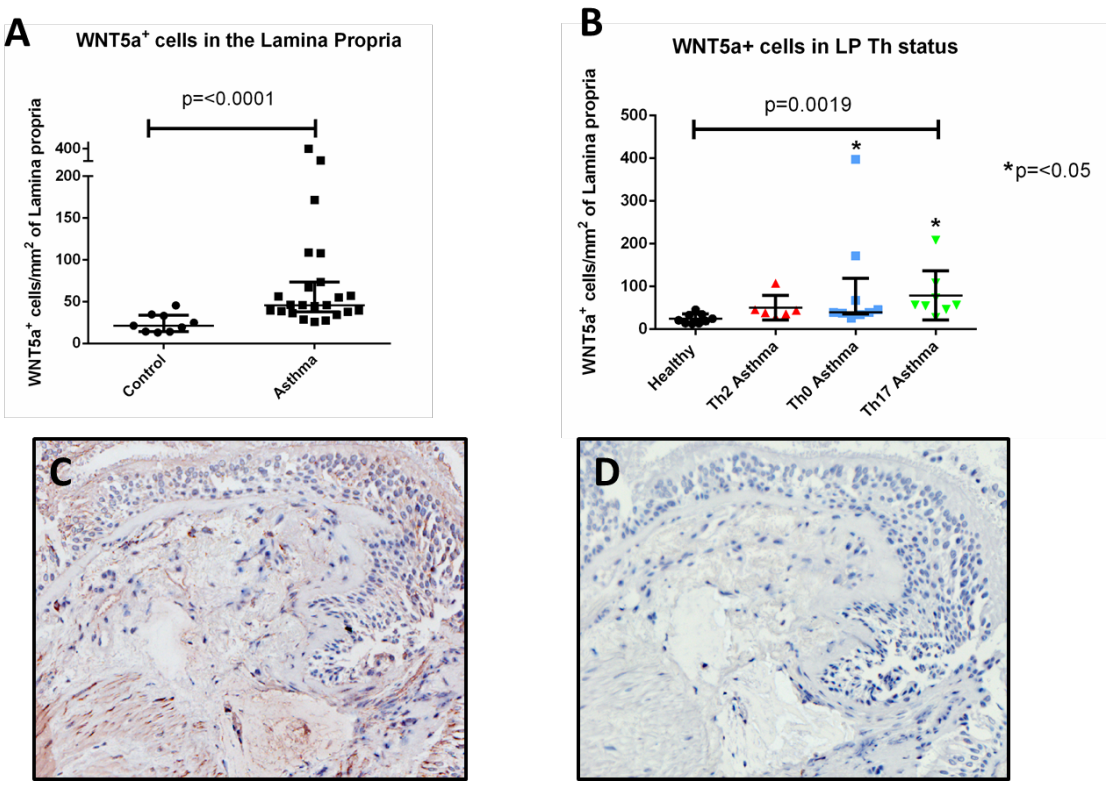


Figure 34: WNT5a protein expression in bronchial biopsies. (A) WNT5a positively immunostained cells in healthy controls and asthmatics. (B) WNT5a positively immunostained cells compared in healthy controls and different asthma inflammatory status based on Th-scores (C) Asthmatic bronchial biopsy stained for WNT5a at x200 magnification. (D) Isotype control mouse IgG1 staining at x200 magnification. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with control subjects.

We found a significant increase in the positively WNT5a stained cells (p=<0.0001) in asthmatics (Figure 34A). Comparing the counts based on Th-status (previously determined using their gene signature) we found a significant increase in Th0 and Th17 patients (Figure 34B).

4.2.2 WNT5a protein expression in the epithelium

To assess the ubiquitous staining of WNT5a in the epithelium we developed a semi-quantitative score (SQS) as depicted in figure 35. Comparing the SQS between healthy and asthmatics, it revealed a significant increase in WNT5a protein expression (p=0.0005) in the epithelium (Figure 36A).

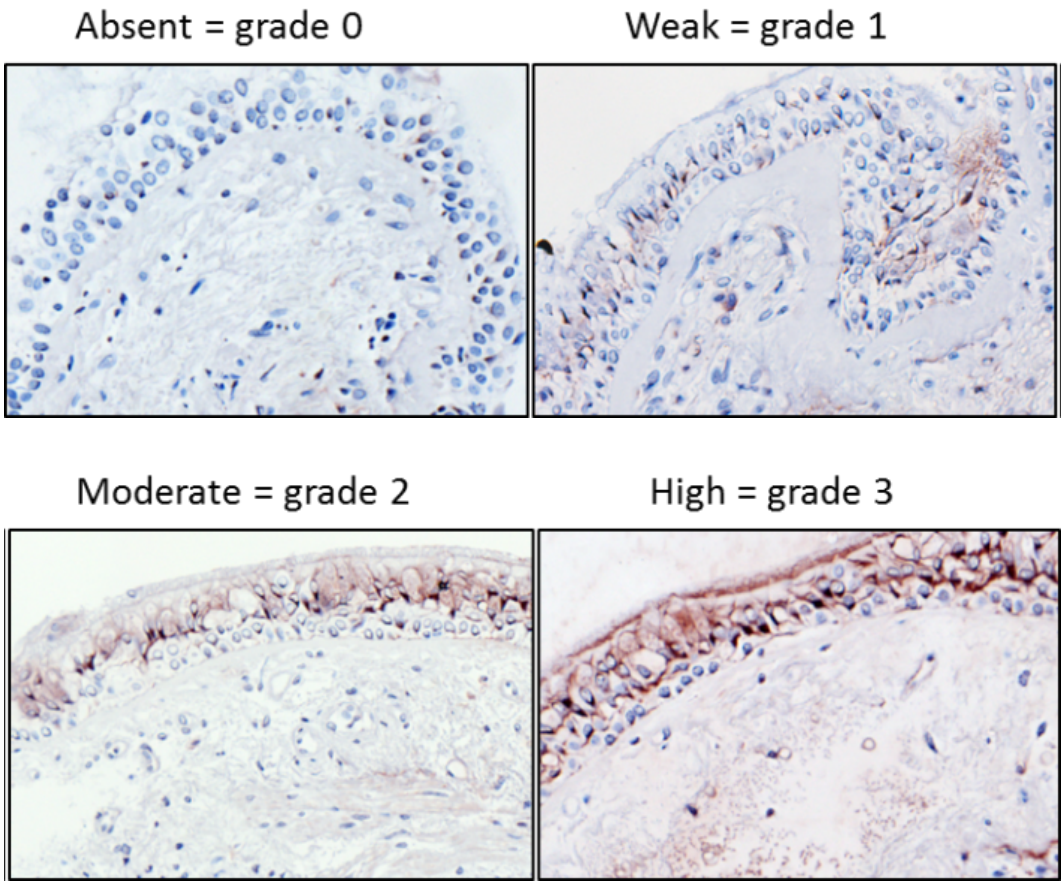


Figure 35: Bronchial biopsy sections stained with WNT5a (red) representing the Semi-quantitative score (SQS) in the epithelium at X200 maginification.

Evaluating this further, we developed a quantitative thresholding measurement using the Zen 2012 software (Zeiss), which essentially highlighted the chromogen staining (Figure 37). Utilising the threshold

measurement, we sketched the area of the epithelium and applied the threshold, generating the percentage staining of the total epithelium in the bronchial biopsy. We tested the thresholding measurements with the epithelial SQS scores and found excellent correlation (r^2=0.6334; p=<0.0001) show in Figure 36B. This validates our observation of the SQS that as the grade increases so does the percentage stained in the epithelium.

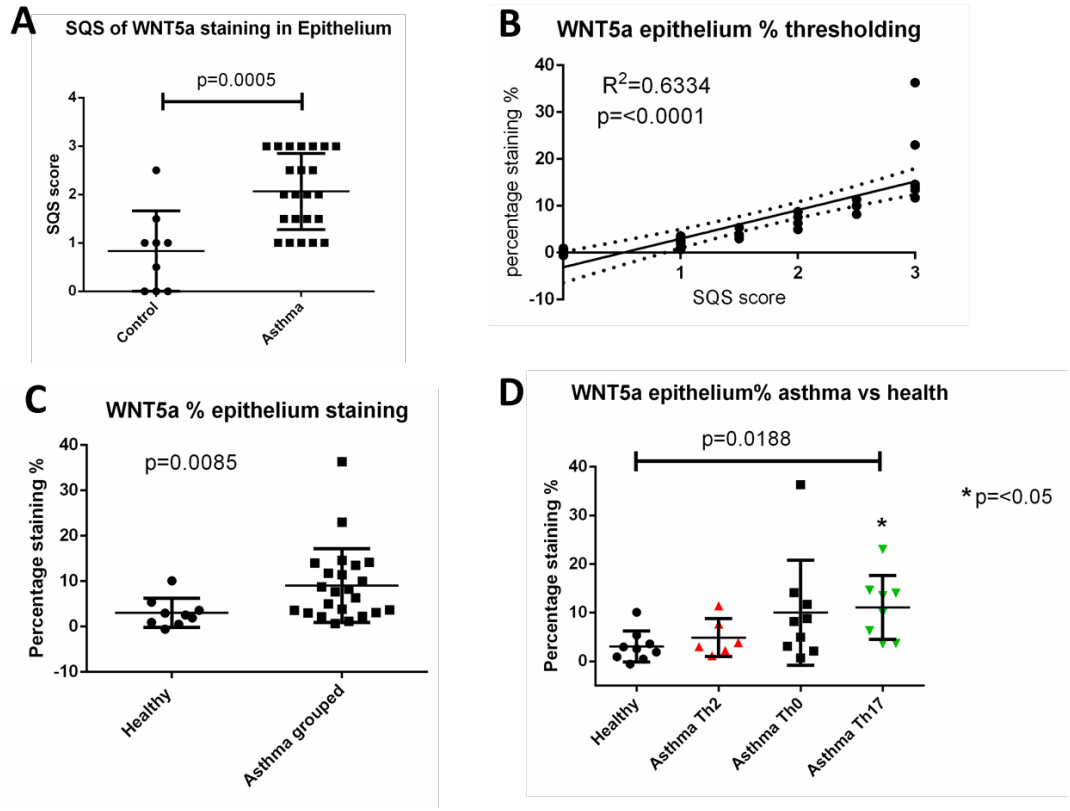


Figure 36: WNT5a protein expression analysis in epithelium in bronchial biopsies.(A) SQS of WNT5a staining in the epithelium. (B) Correlation between SQS score and threshold WNT5a staining in the epithelium. (C) Percentage of WNT5a epithelial staining compared in healthy controls and asthmatics. (D) Percentage of epithelial staining in healthy controls and different asthma inflammatory status based on Th-scores.

We then compared the percentage of staining between healthy and asthmatics and found a significant difference ($p=0.0085$), interestingly this was constrained to patients with a Th17 phenotype ($p=0.0188$) in asthmatic biopsies (Figure 36C-D).

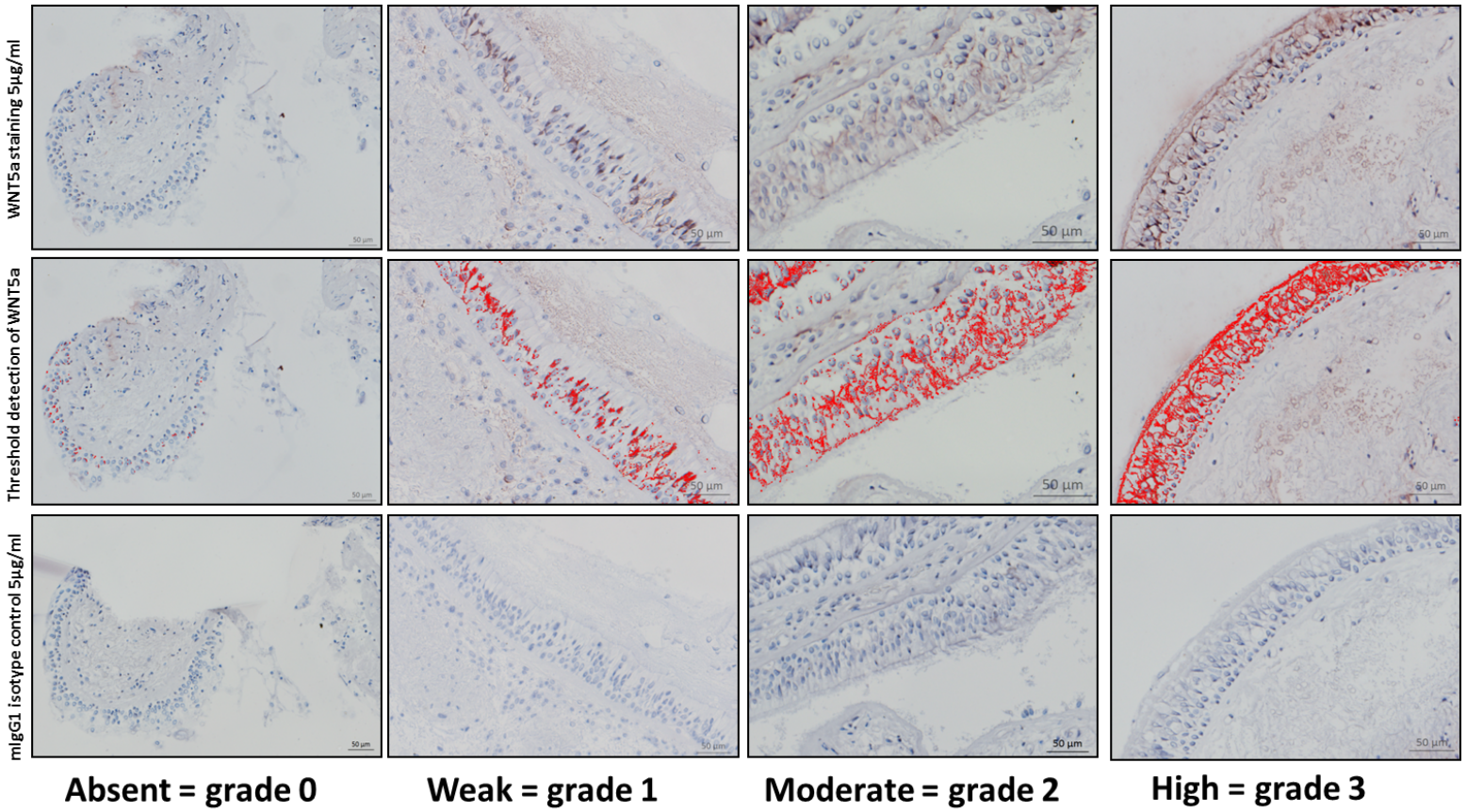


Figure 37: Depicting representative bronchial biopsies stained with WNT5a (Red) with different SQS. Threshold detection of the chromogen shows high accuracy with no detection in isotypes.

4.2.3 WNT5a expression in the airway smooth muscle

The protein expression in ASM for WNT5a was also ubiquitous and as a result we employed the same quantitative thresholding analysis. After establishing the HSI value we tested the thresholding measurements with the ASM SQS scores showed in figure 39 and found excellent correlation (r^2=0.8410; p=<0.0001) show in Figure 38A.

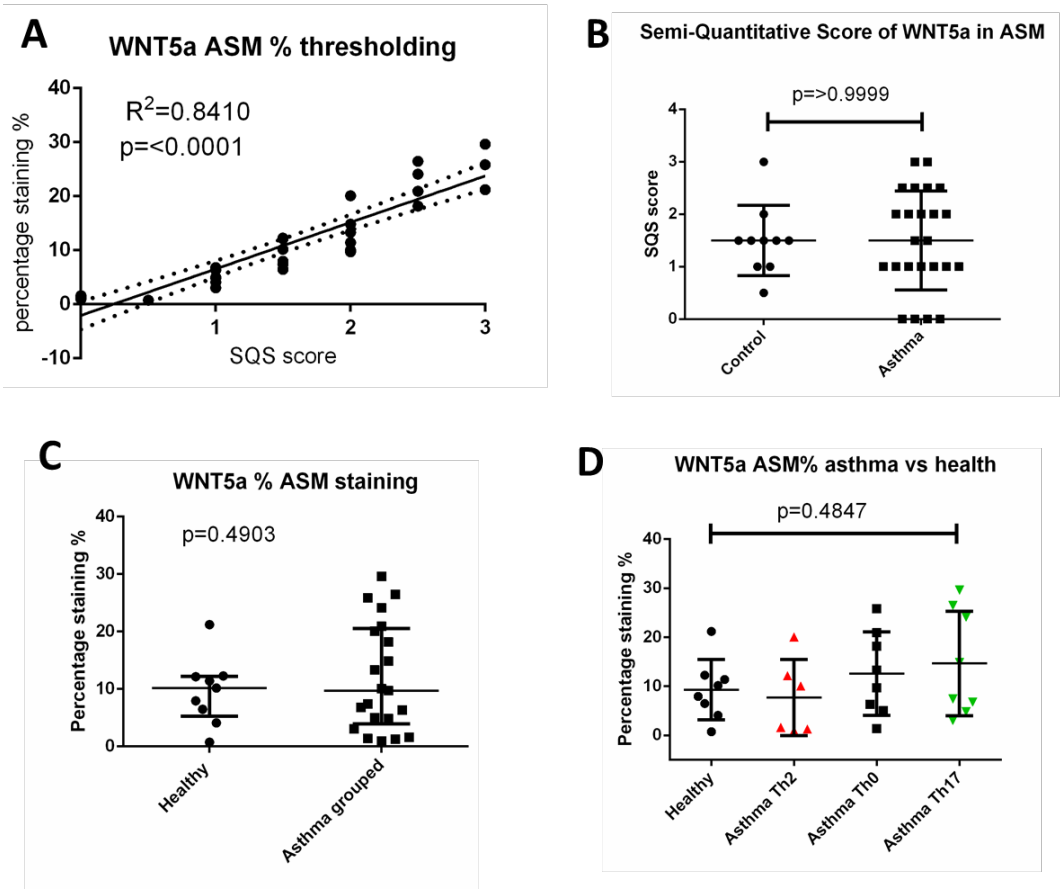


Figure 38: WNT5a protein expression in airway smooth muscle in bronchial biopsies. (A) Correlation between SQS score and threshold WNT5a staining in the epithelium. (B) SQS of WNT5a staining in the ASM. (C) Percentage of WNT5a ASM staining compared in healthy controls and asthmatics. (D)

Percentage of ASM staining in healthy controls and different asthma inflammatory status based on Th-scores.

However, no significant difference was observed when comparing the SQS of WNT5a stain in Asthma (Figure 38B). Extending this to the percentage of WNT5a staining (i.e. the amount of chromogen covering the ASM) we also found no significant difference between healthy controls and asthma (Figure 38C-D).

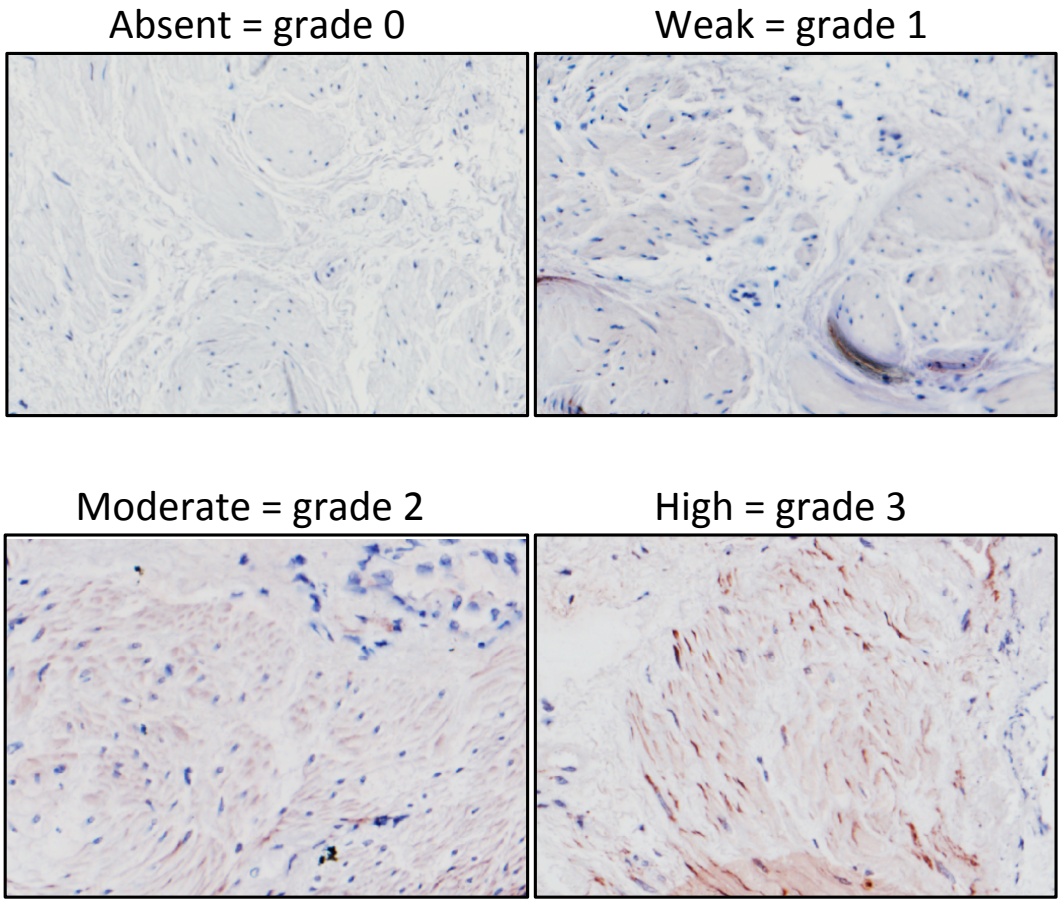


Figure 39: Bronchial biopsy sections stained with WNT5a (red) representing the Semi-quantitative score (SQS) in the ASM at x400 magnification.

4.3 TGF-β1 protein expression in Asthma

TGF-β1 immunostaining was observed in airway structures, including the epithelium and the submucosa (Figure 40C). However, no expression was found in the ASM, making the staining very comparable to (Redington et al, 1998). We therefore subjected TGF-β1 staining to the same analysis as WNT5a immunostaining described previously.

4.3.1 TGF-β1 protein expression in the lamina propria

To quantify the amount of TGF-β1 protein expression we counted the positively stained cells in the lamina propria and found a significant increase in asthmatics ($p=0.003$) compared to healthy controls (Figure 40A).

Discriminating the TGF-β1 positively stained cells by the asthmatic inflammatory Th-status we reveal an increase in the Th2 and Th0 asthma phenotypes. This is in agreement with the literature where Th2 inflammation is associated with airway eosinophilia (Woodruff et al, 2009) and the latter seem to be a major source of TGF-β1 (Flood-Page et al, 2003).

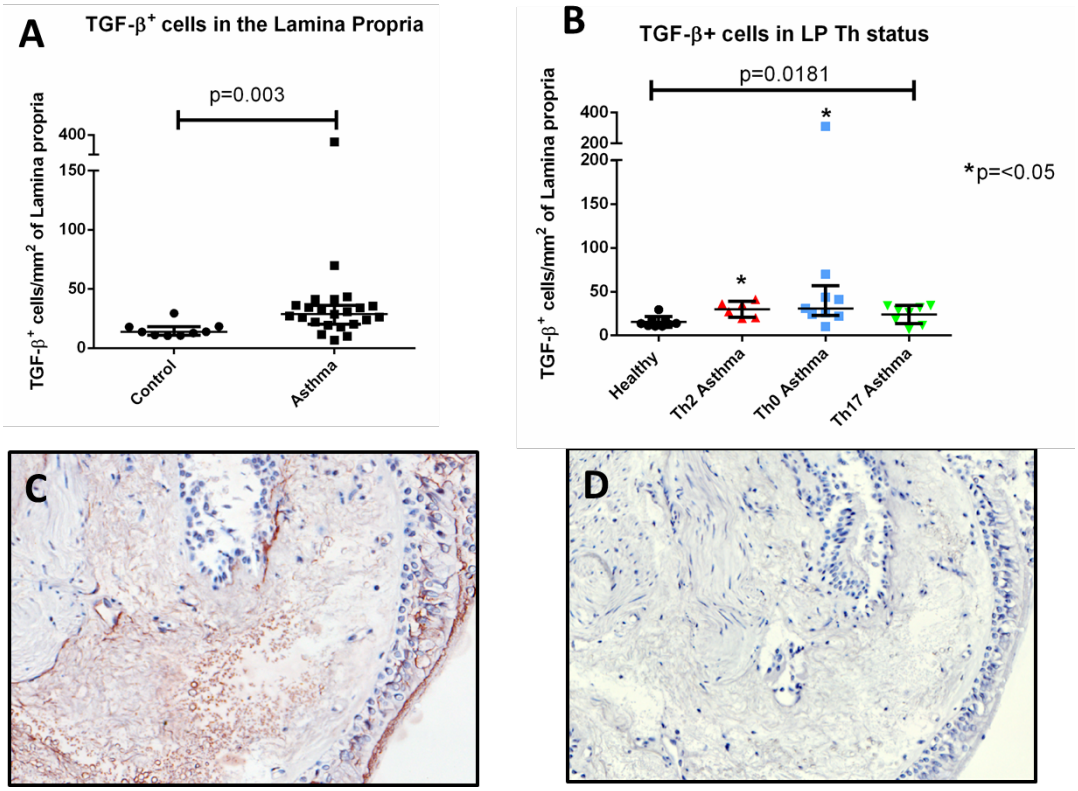


Figure 40: TGF-β1 protein expression in bronchial biopsies. (A) TGF-β1 positively immunostained cells in healthy controls and asthmatics. (B) TGF-β1 positively immunostained cells compared in healthy controls and different asthma inflammatory status based on Th-scores (C) Asthmatic bronchial biopsy stained for TGF-β1 at x200 magnification. (D) Isotype control rabbit IgG staining at x200 magnification. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with control subjects

4.3.2 TGF-β1 protein expression in the epithelium

To assess the ubiquitous staining of TGF-β1 in the epithelium we utilised the same HSI value obtained for WNT5a epithelium staining as the chromogen was the same and only the chromogen distribution/intensity was affected

based on the protein expression. The SQS between healthy and asthmatics revealed a significant increase in TGF-β1 protein expression (p=0.0266) in the epithelium (Figure 41A). Testing the SQS versus the percentage staining displayed a linear correlation (r^2=0.8742; p=<0.0001) (Figure 41B). Nevertheless, unlike the SQS comparing the percentage staining yielded no significance (Figure 41C-D) between health and asthma phenotypes.

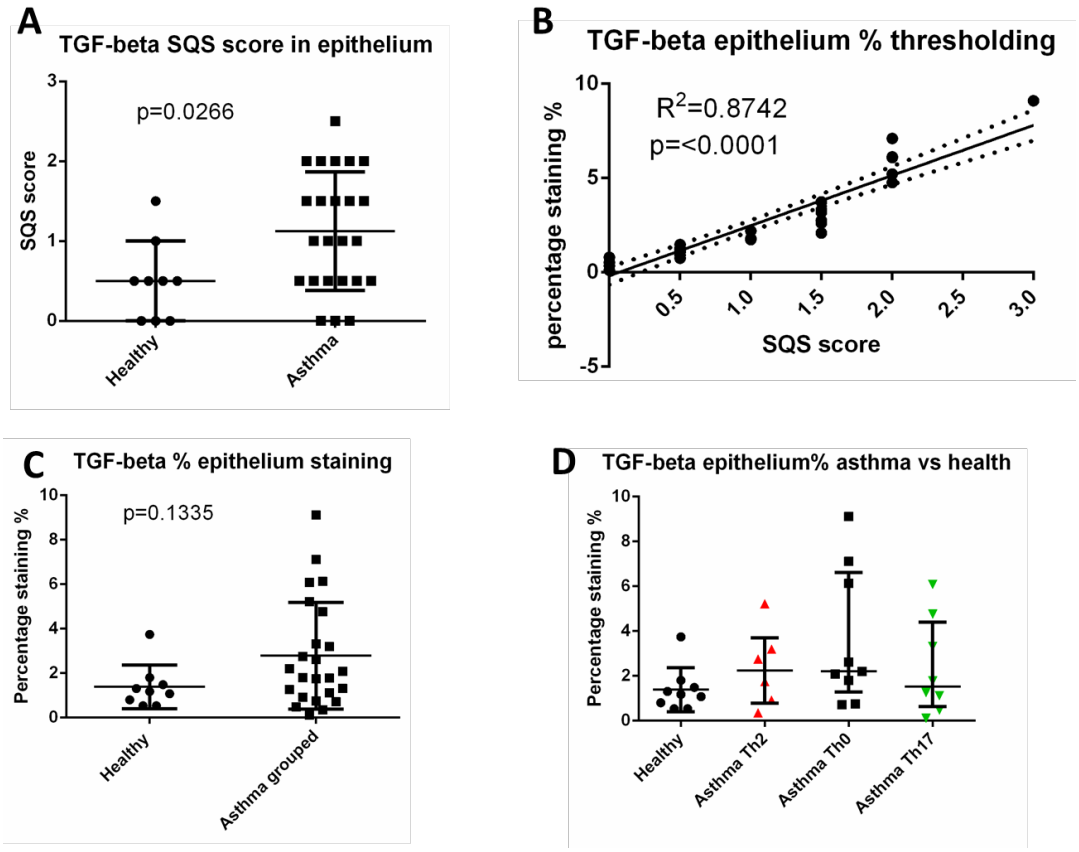


Figure 41: TGF-β1 protein expression analysis in epithelium in bronchial biopsies. (A) SQS of TGF-β1 staining in the epithelium. (B) Correlation between SQS score and threshold TGF-β1 staining in the epithelium. (C) Percentage of TGF-β1 epithelial staining compared in healthy controls and asthmatics. (D) Percentage of TGF-β1 epithelial staining in healthy controls and different asthma inflammatory status based on Th-scores.

4.4 Collagen III protein expression in asthma

Protein expression of ECM in asthma has been previously investigated particularly the expression of collagen III (Benayoun *et al*, 2003; Chu *et al*, 1998) as shown previously in table 3. We wanted to add to this analysing it similar to (Siddiqui *et al*, 2008) to see if aberrant ECM deposition was increased in our cohort similar to RBM thickening. After generating an SQS shown in figure 42 we positively highlighted the submucosa to perform the thresholding on the chromogen.

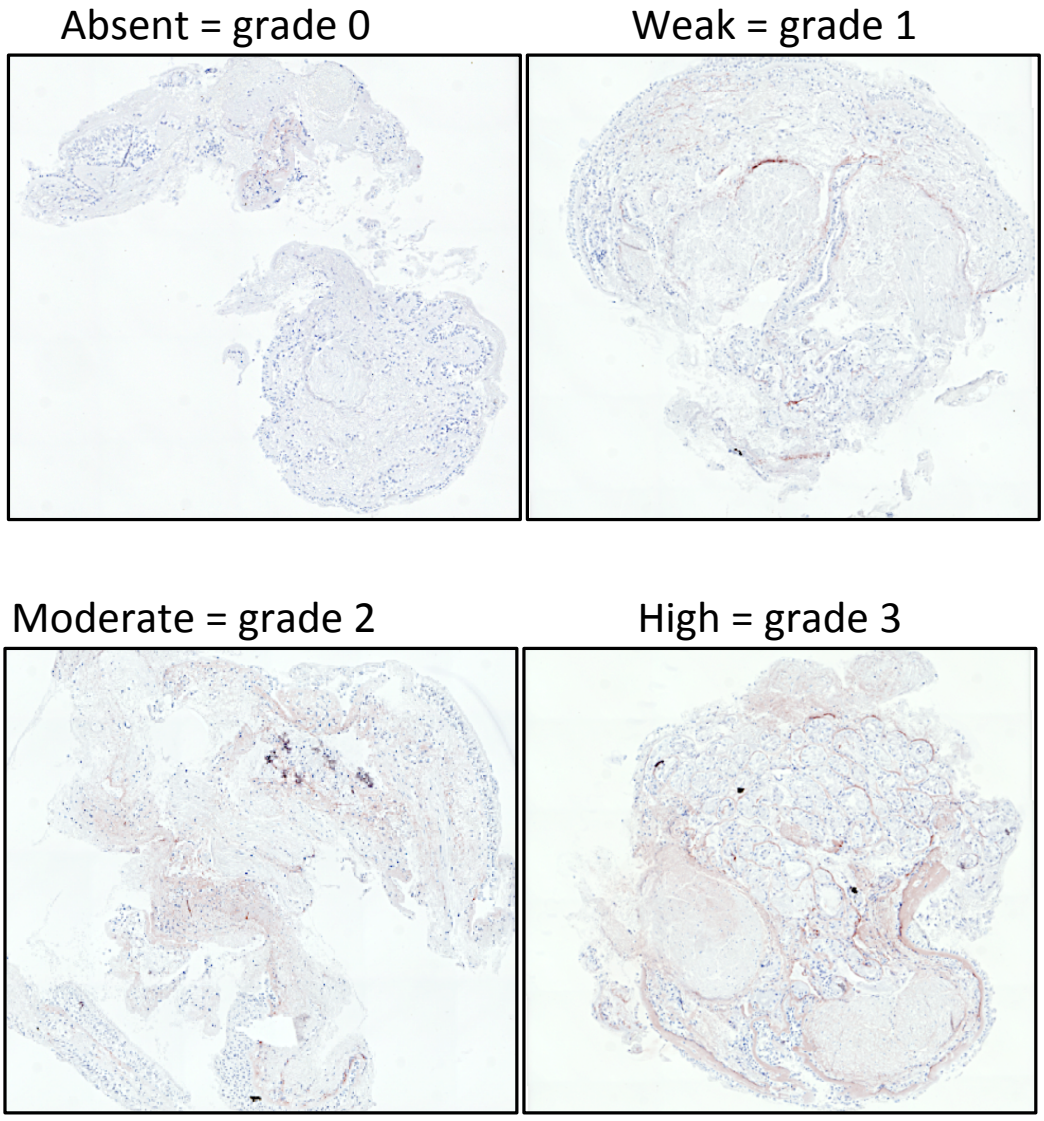


Figure 42: Bronchial biopsy sections stained with Collagen III (red) representing the Semi-quantitative score (SQS) in the submucosa at X20 maginification.

Similar to our previous data we found a significant positive linear correlation with our SQS and percentage collagen III staining in the submucosa (figure 43A). Comparing healthy and asthmatics we found no significant difference in collagen III staining based on the SQS or percentage staining (figure 43B-C). Consistently, we also found no difference between health and asthma

phenotypes (figure 43D). This is the first evidence suggesting that asthmatic inflammatory phenotype does not discriminate between collagen III deposition, albeit asthma individuals were under steroidal treatment.

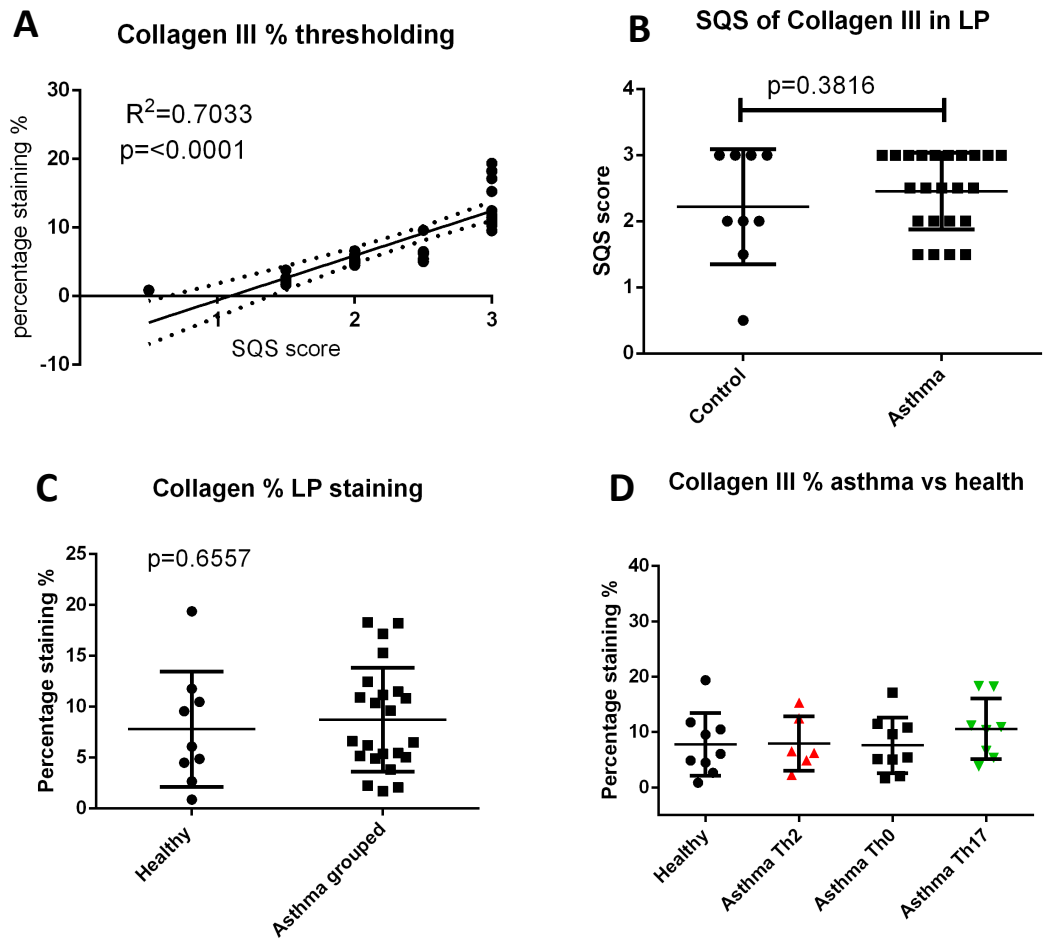


Figure 43: Collagen III protein expression analysis in submucosa of bronchial biopsies. (A) Correlation between SQS score and threshold collagen III staining in the submucosa. (B) SQS of collagen III staining in the submucosa. (C) Percentage of collagen III sub mucosal staining compared in healthy controls and asthmatics. (D) Percentage of collagen III sub mucosal staining in healthy controls and different asthma inflammatory status based on Th-scores.

4.5 WNT5a-TGF-β1 protein expression correlate in biopsies

TGF-β1 expression inducing WNT5a protein expression is well established in the literature (Katoh and Katoh, 2009; Kumawat et al, 2014). In order to investigate this cross talk signalling in vivo we compared WNT5a+ cells versus TGF-β1+ cells and found a positive linear correlation (r^2=0.0.5171; p=<0.0001) (Figure 44A). This was also apparent in the epithelium (r^2=0.6304; p=<0.0001) (Figure 44B).

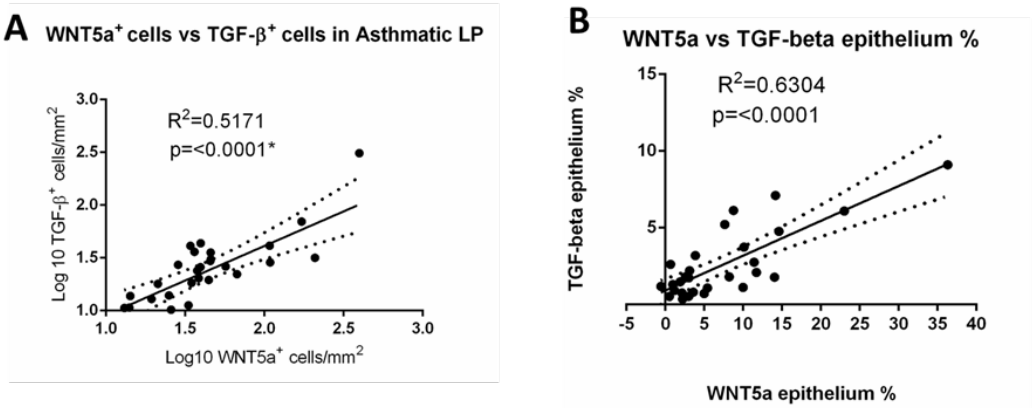
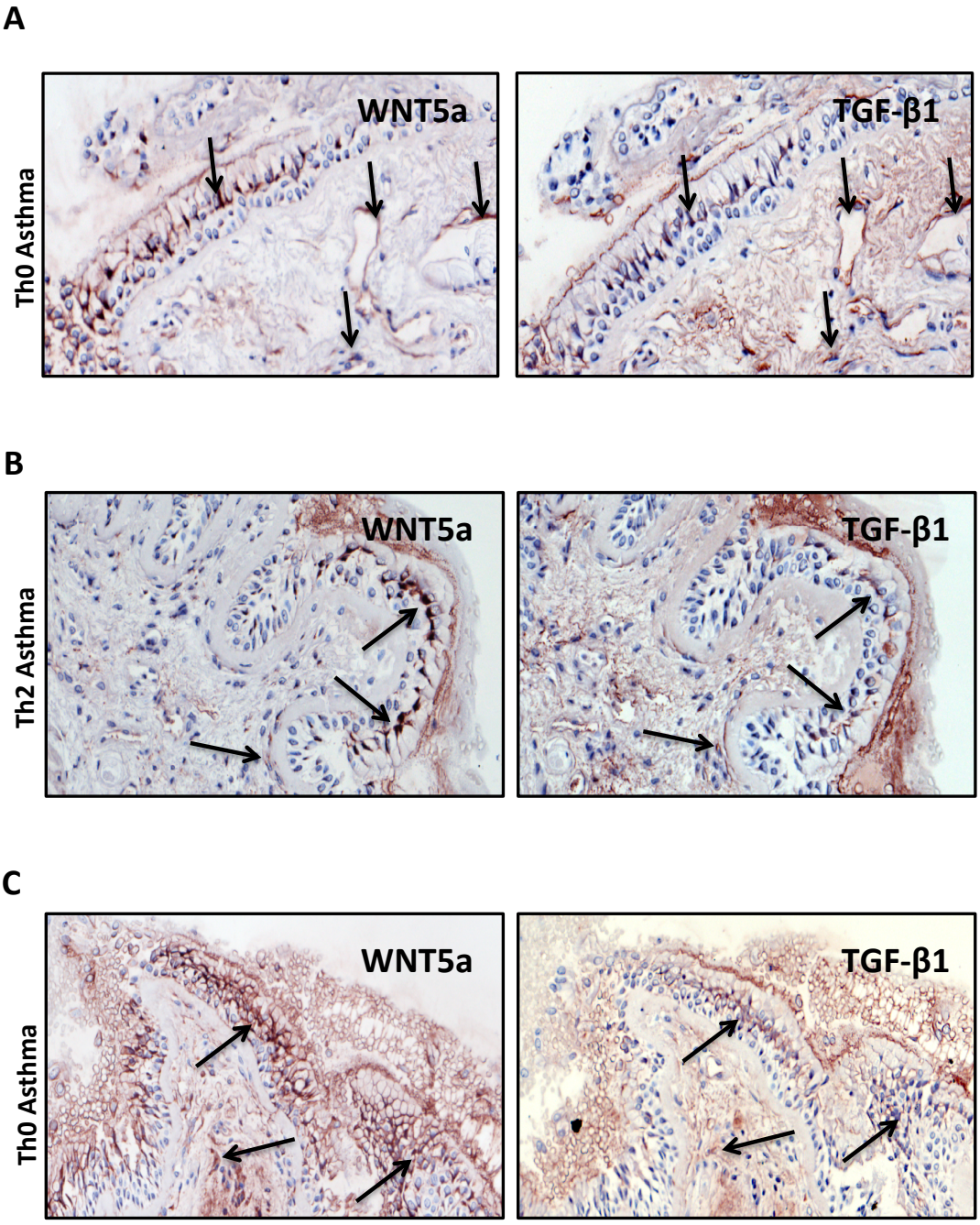


Figure 44: Protein expression of WNT5a and TGF-β1 correlated in bronchial biopsies. (A) Correlation between WNT5a+ cells versus TGF-β1+ cells staining in the lamina propria. (B) Correlation between Percentage of WNT5a epithelial staining versus Percentage of TGF-β1 epithelial staining. p-value and R^2 value determined with a linear regression.

Additionally, Initial observations of the sequential tissue section also revealed multiple co-localization of the WNT5a-TGF-β1 signal. In multiple asthma phenotypes the apical-basal interface is stained for WNT5a and TGF-β1 as shown in figure 45A-D indicated by the arrows. Additionally, we also see

individual cells in the submucosa dual stained for WNT5a and TGF-β1 (figure 45E).



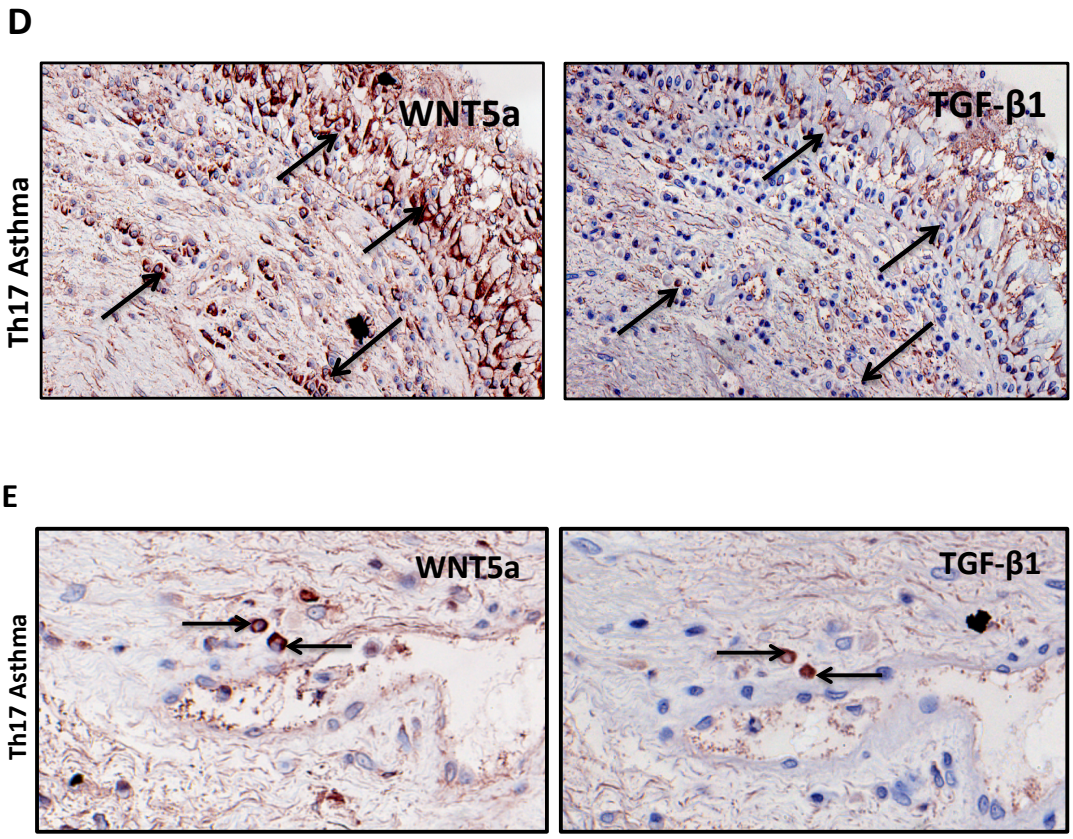


Figure 45: WNT5a and TGF-β1 co-localization in asthma phenotypes indicated by black arrows.

In healthy we find the staining of WNT5a and TGF-β1 is suppressed with limited dual staining in the epithelium (figure 46A-B), however dual staining of WNT5a and TGF-β1 is observed in the submucosa (figure 46B).

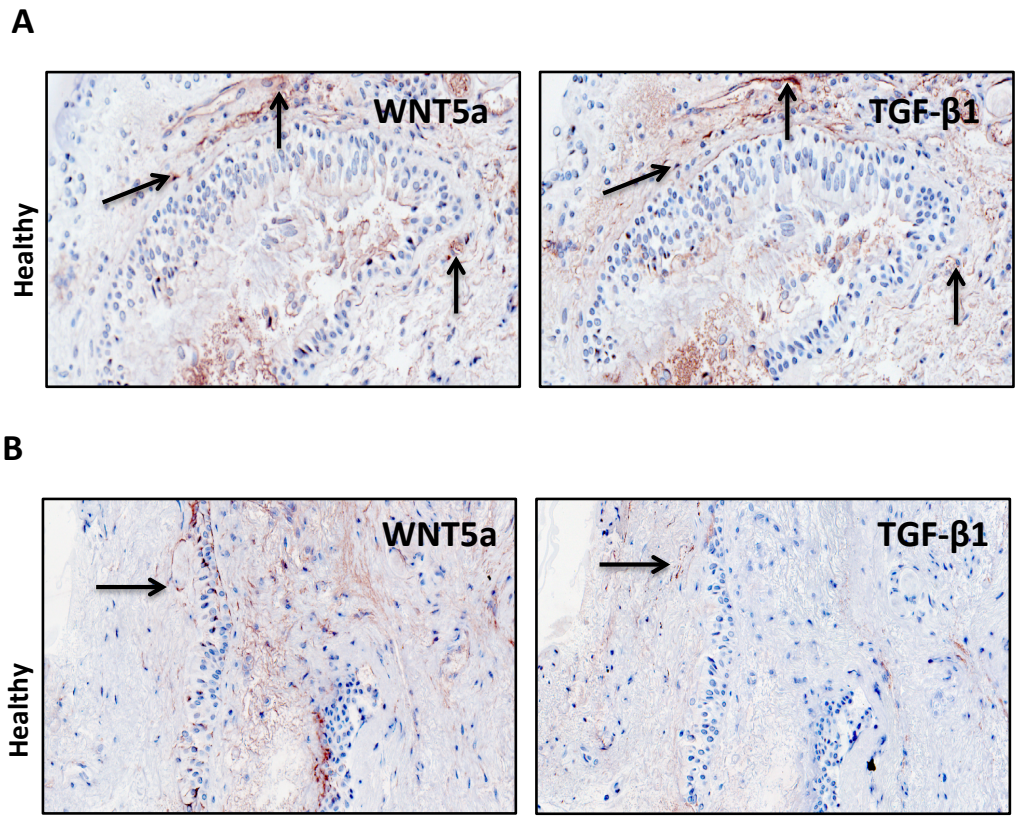


Figure 46: WNT5a and TGF-β1 co-localization in healthy indicated by black arrows.

4.6 The effect of WNT5a on airway remodelling in asthma

Asthma is an inflammatory disorder that can lead to airway wall remodelling encompassing various changes as described previously. As a consequences lung function is deteriorated in asthmatics. We compared asthma lung function against the amount of WNT5a protein expression in the submucosa (figure 47A) and in the epithelium (figure 47B) and interestingly there was a trend towards negative correlation.

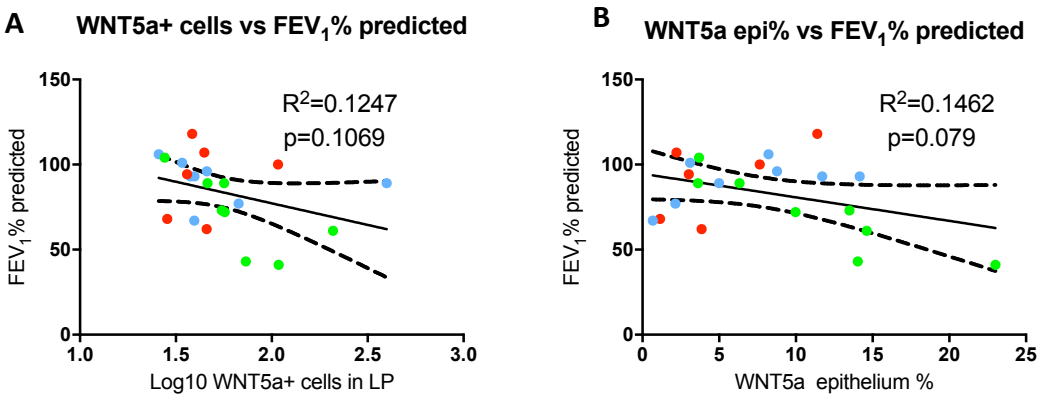


Figure 47: WNT5a protein expression in asthmatics correlated against lung function in (A) submucosa and (B) epithelium. p-value and R^2 value determined with a linear regression.

Nevertheless, comparing WNT5a protein expression against typical ECM remodelling parameters such as RBM thickness or collagen III protein expression we found no significant difference (figure 48). Patients whose biopsies did not meet the criteria for RBM measurement were omitted from analysis in RBMT. Lastly, inflammation is consistently present in asthmatics, particularly the increase in eosinophils and neutrophils.

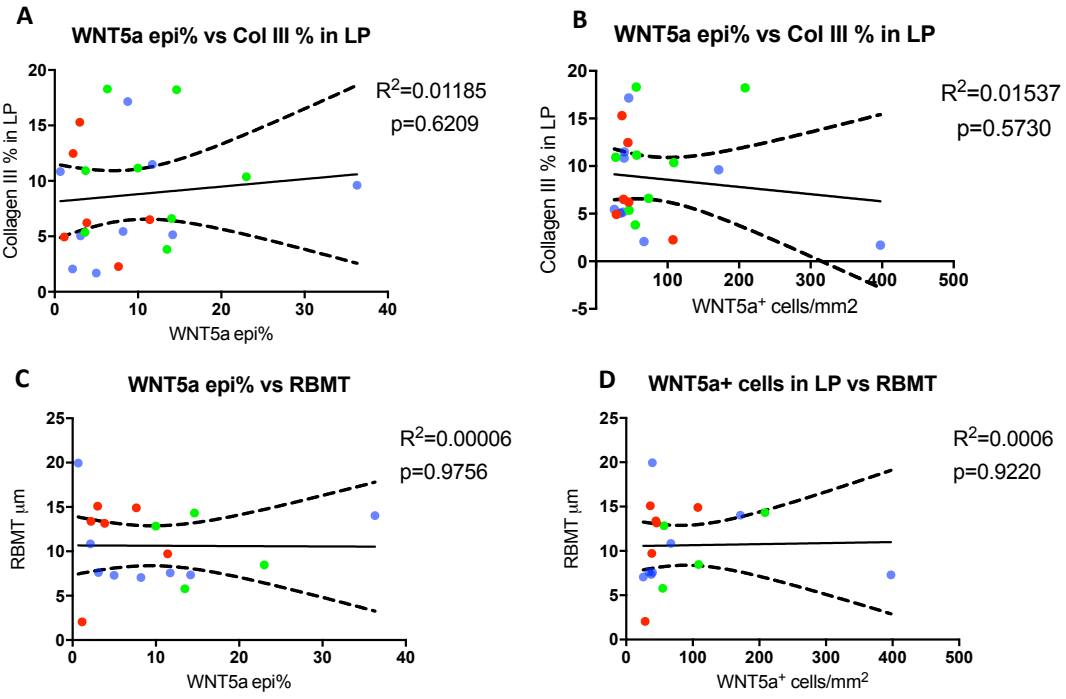


Figure 48: Depicting the expression of WNT5a protein against ECM remodelling features of collagen III protein expression and RBM thickness. p-value and R^2 value determined with a linear regression.

Having access to biopsy data that previously enumerated inflammatory cells in our cohort we compared WNT5a positive stained cells in the lamina propria against eosinophils, which positively stained for major basic protein (MBP) and neutrophils, which positively stained for neutrophil elastase (NE).

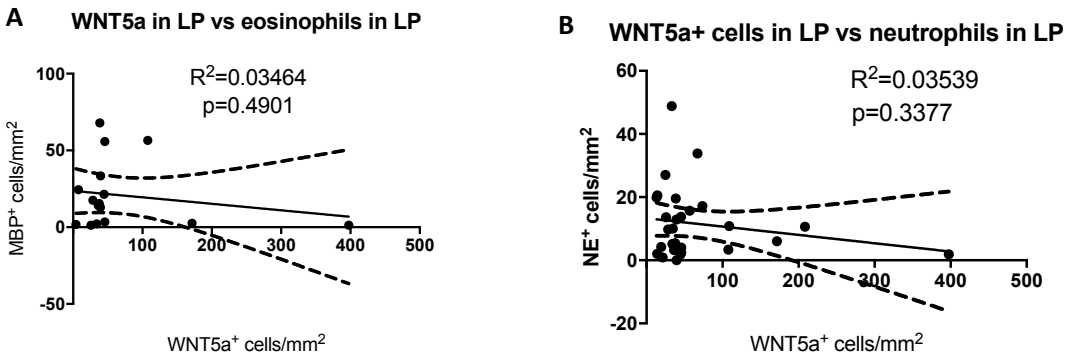


Figure 49: Comparison of WNT5a positive stained cells against eosinophils and neutrophils in bronchial biopsies. p-value and R^2 value determined with a linear regression.

In both instances no significant correlation was observed (figure 49), this might in part be attributed to measurements from different tissue sections cut, making it difficult to co-localise the signal. However, enumeration of positive stained cells in different tissue sections of the same biopsy have confirmed consistent values. Furthermore, we found multiple cells stained in the lamina propria for WNT5a and TGF-β1 and also inside blood vessels (figure 45E), which could indicate WNT5a is expressed by inflammatory cells as shown in peripheral blood by (Syed *et al*, 2007) and promote transmigration into the lung submucosa (Jung *et al*, 2013a).

4.7 Discussion

Initially, we wanted to confirm if our antibody was optimised for the detection of WNT5a. Staining with WNT5a LSbio antibody displayed a high background staining as noted by the isotype control. Reducing the concentration equally reduced antibody staining and as a result we can conclude that the staining

signal is non-specific. Utilizing a different antibody, WNT5a RnD showed that this antibody couldn't stain bronchus tissue. To deduce whether this was the case we established a positive control by embedding L-cells that over express WNT5a in GMA. The processing procedure was exactly the same as how the tissue was processed and we found; in the case of WNT5a LSbio and WNT5a RnD both antibodies could not stain the L-cells. However, WNT5a 6F2 antibody did stain the L-cells suggesting that it has the potential to detect WNT5a protein expression in GMA. We extended this optimisation in *in vitro* by first confirming that WNT5a 6F2 antibody could detect its Immunogen that was a truncated WNT5a peptide. Interestingly, using this peptide as a blocking agent did not sequester the antibody and block the signal in western blotting. This may occur as the peptide is a large protein and its topological conformation could be hiding the epitope for antigen recognition. Denaturing the protein and then subsequently using it as a blocking agent for the antibody could overcome this. Since our western blotting data showed multiple bands, to confirm if our cell lysates contained WNT5a and as a consequence was detected by immunoblotting, we conducted immunoprecipitation of the cell lysates. In all cases, incubation of WNT5a 6F2 antibody with cell lysates and subsequent precipitation with agrose beads revealed no bands during immunoblotting, furthermore protein bands identified during SDS-page electrophoresis at 30kDa and 50kDa were analysed by mass spectrometry only to reveal light and heavy mIgG1 molecules, suggesting that our samples either did not have enough WNT5a or also required thermal shock to expose WNT5a epitope. In conclusion, WNT5a 6F2 antibody has the capacity to

function in GMA unlike other antibodies and *in vitro* data helps confirm that this antibody detects WNT5a.

Building on this, we set out to study the role of two important repair molecules WNT5a and TGF-β in asthmatic tissue characterised by gene expression scoring. These observations are important because tissue repair in asthma may be dysregulated and has not been studied in the context of Th2, Th17, and Th0 inflammation characterised by biopsy gene expression scores. Our results demonstrated evidence of WNT5a protein expression in different compartments of the airway tissue sections. Epithelial cells have previously been shown to increase WNT5a gene expression (Kicic *et al*, 2010) and in this report we show significant WNT5a protein expression in the epithelium and the lamina propria.

In contrast to other reports (Kumawat *et al*, 2013) we did not observe significant change in the ASM derived WNT5a protein expression. We found WNT5a was increased in asthma in the lamina propria with a Th17 and Th0 biopsy gene expression. However, this was not observed in the epithelium for the latter Th0 phenotype possibly because the sample size was underpowered, a power calculation could be performed to deduce the samples size required for significance within each of the Th strata. Additionally, the source of WNT5a in lamina propria in the Th17 phenotype could be neutrophils. Previous reports have shown WNT5a to be a potent stimulant for human neutrophils migration and chemokine production e.g. CXCL8 and CCL2 (Jung *et al*, 2013a) and the Th17 phenotype is associated with increased neutrophils (Cosmi *et al*, 2011). This could be explored with

further co-localization experiments by immunostaining neutrophils and WNT5a in sequential sections and examining the expression of WNT5a in circulating immunomagnetically purified peripheral blood neutrophils.

Our protein expression levels of TGF-β1 have been consistent with previous reports in the epithelium (Magnan *et al*, 1997; Magnan *et al*, 1994) with no expression in the ASM (Redington et al, 1998) and when elevated in the Th2 phenotype (Flood-Page et al, 2003). Lastly, we found that protein expression of WNT5a positively correlated with TGF-β1 protein expression in the epithelium and in the lamina propria.

Remodelling of airways is an irreversible process that occurs in asthma, similar to other reports we found an increase RBMT in asthmatics. However, protein expression of collagen III was not elevated. Previous reports have shown diverse ECM related protein expression as shown in table 3 and since our patients were on steroids this may affect ECM deposition. Furthermore, expression of WNT5a protein in epithelium or lamina propria did not correlate with collagen III or RBMT, suggesting that WNT5a may act on differing ECM remodelling pathways by promoting migration and apical/basal cell polarity (Yamamoto *et al*, 2015; Witze *et al*, 2008; Gon *et al*, 2013).

In conclusion, we are first to extensively evaluate and find evidence of WNT5a protein expression in asthma bronchial biopsies elevated in epithelium and lamina propria stratified by inflammatory phenotypes. The role of WNT5a in ASM has been described previously to elicit an ECM response and promote chemotaxis in PBMC (Kumawat *et al*, 2013; Syed *et al*, 2007; Jung *et al*,

2013b). On the other hand, the role of WNT5a in asthmatic epithelium has not been evaluated and may influence differentiation and repair.

Chapter 5: WNT5a and TGF-β1 signalling in Beas-2B cell line

5.1 Toxicity assay in *in vitro*

Previous data have revealed WNT5a can be expressed in various lung structural cells and in *in vivo* WNT5a protein expression was elevated in the epithelium. To identify and test a suitable model we cultured human bronchial epithelial cells (HBEC), however they could not be sustained due to limited stock and costs. This was overcome by utilising BEAS-2B cell line. As a consequence, we tested the efficacy of a TGF-β1-smad dependent inhibitor known as SIS3 and BOX5 a competitive antagonist for WNT5a. The efficacy of SIS3 inhibitor was determined at 10μM as previously described by (Jinnin, Ihn and Tamaki, 2006), however stimulation of BEAS-2B cells with 10μM of SIS3 resulted in apoptosis as determined by MTT assay shown in figure 50.

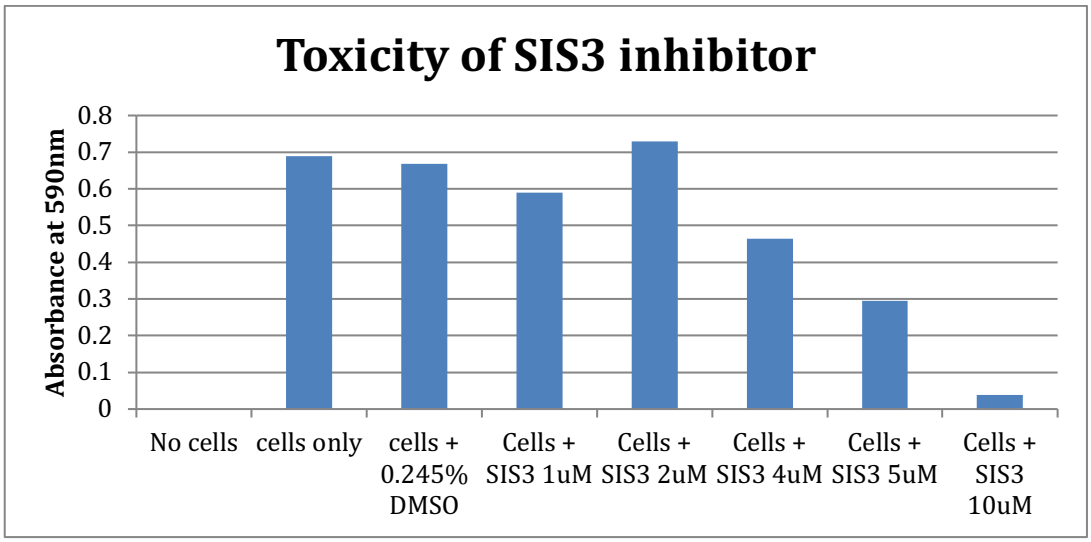


Figure 50: The toxicity of SIS3 inhibitor in cultured BEAS-2B cells.

The inhibitor BOX5 can competitively antagonise the effects of WNT5a at 500μM in the presence of 1μg/ml of WNT5a. We tested the efficacy of BOX5

and found no significant loss of viable cells as determined by MTT assay (figure 51) or in DMSO.

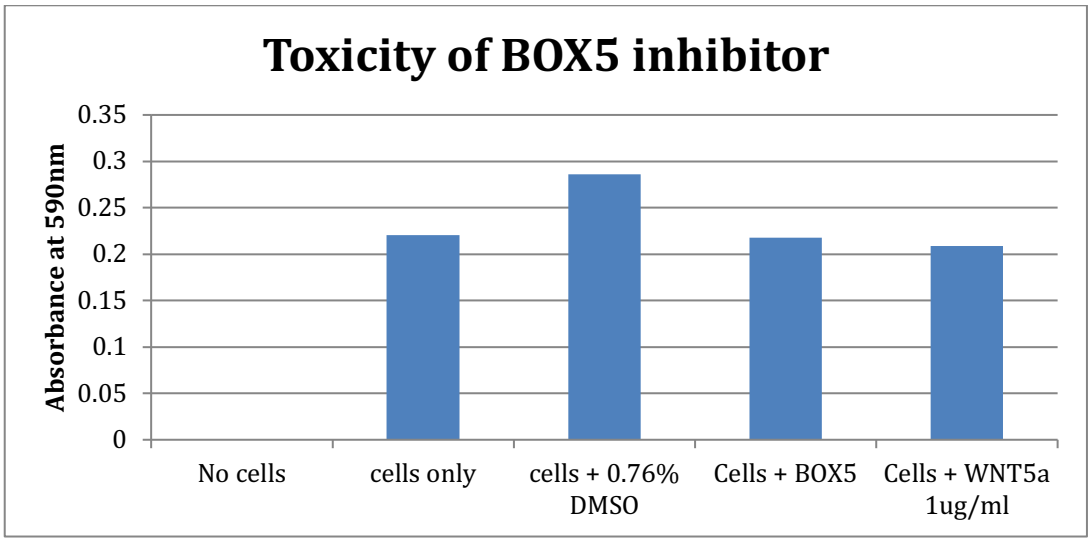


Figure 51: The toxicity of BOX5 inhibitor cultured in BEAS-2B cells.

The following data demonstrate that BOX5 a WNT5a competitive antagonist is safe to use in BEAS-2B cells.

5.2 Morphological changes in BEAS-2B cells

Expression of WNT5a was increased in asthmatic epithelium and therefore we began by testing the sensitivity of BEAS-2B cells to TGF-β1 and found that stimulation with 10ng/ml of TGF-β1 for 1 hour was sufficient to invoke t-SMAD2/3 nuclear translocation and induce morphological changes (Figure 52).

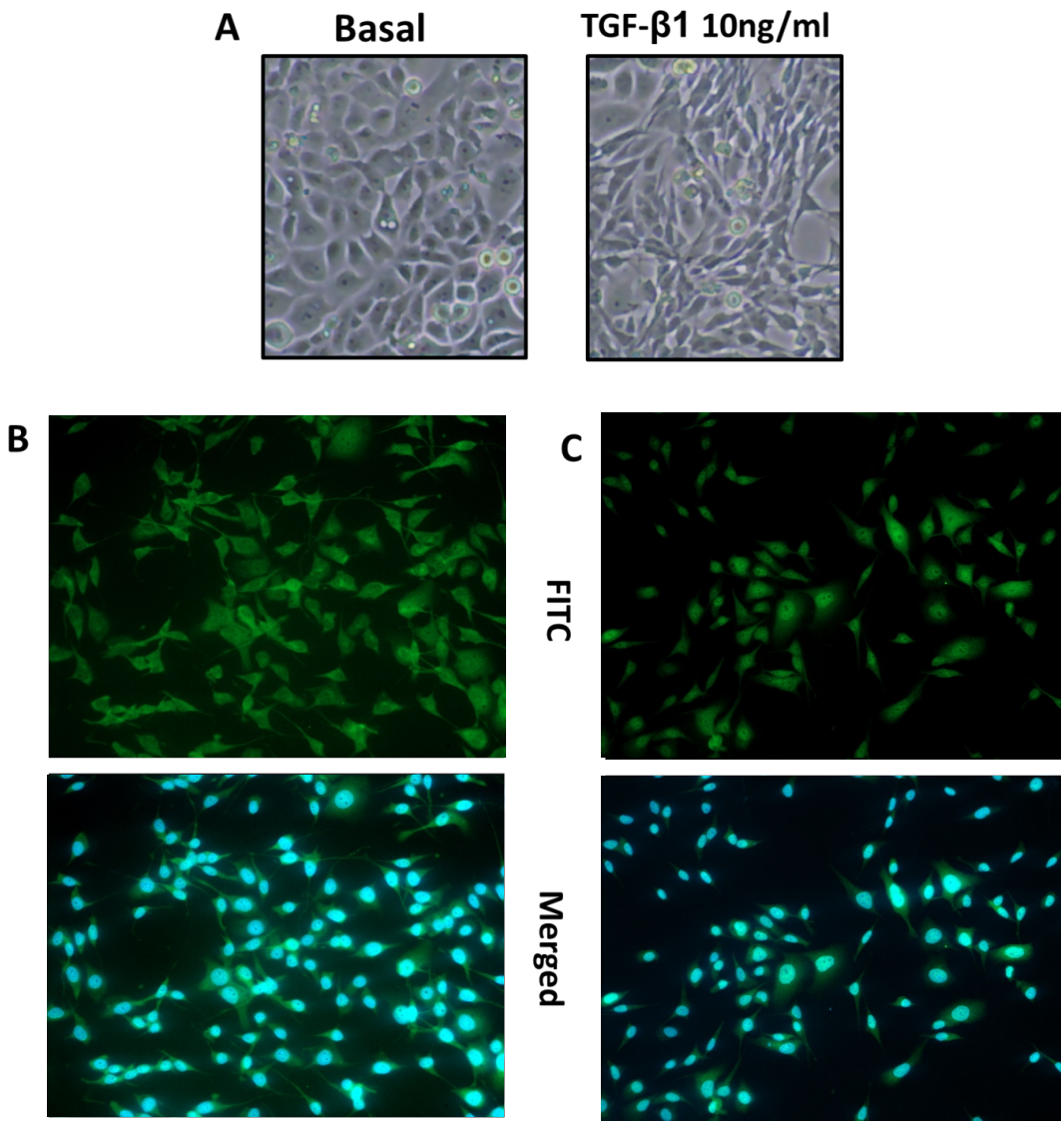


Figure 52: Morphological changes induced by TGF-β1 and SMAD2/3 nuclear translocation. (A) Morphological changes of Beas-2B cell line observed under bright field microscope x200 magnification. (B) Beas-2B cells with no stimulation stained for total-SMAD2/3 at x100 magnification. (C) Beas-2B cells treated with TGF-β1 at 10ng/ml for 1hour stained for total-SMAD2/3 at x100 magnification.

5.2.1 WNT5a mimics TGF-β1 and induces EMT

After an initial insult, the epithelium responds by secreting various cytokines and growth factors (Chung and Barnes, 1999; Hamid et al, 1991; Robinson et al, 1992; Wills-Karp et al, 1998). We later investigated whether if TGF-β1 could induce EMT features as observed by (Doerner and Zuraw, 2009; Hackett et al, 2009) and if WNT5a could participate in these early events during EMT. Accordingly, we stimulated Beas-2B cells with TGF-β1 at a concentration of 10ng/ml or WNT5a at a concentration of 1μg/ml for 48 hours. In contrast to previous reports we found that BEAS-2B cells constitutively expressed Collagen 1αI and Vimentin at a basal state. Additionally, stimulation with TGF-β1 for 72 hours at a concentration of 10ng/ml had no effect on α-SMA expression. Nevertheless, we add to this by showing that WNT5a can also in part enhance EMT changes in BEAS-2B cells.

During EMT, epithelial cells change their morphology from a cobble to a more fibrotic and elongated phenotype. To visualise this, we stained BEAS-2B cells with vimentin in the presence of TGF-β1 and or WNT5a for 48hr. We found that the cells significantly exhibited a more elongated phenotype ($p=0.05$) when stimulated with WNT5a (figure 53).

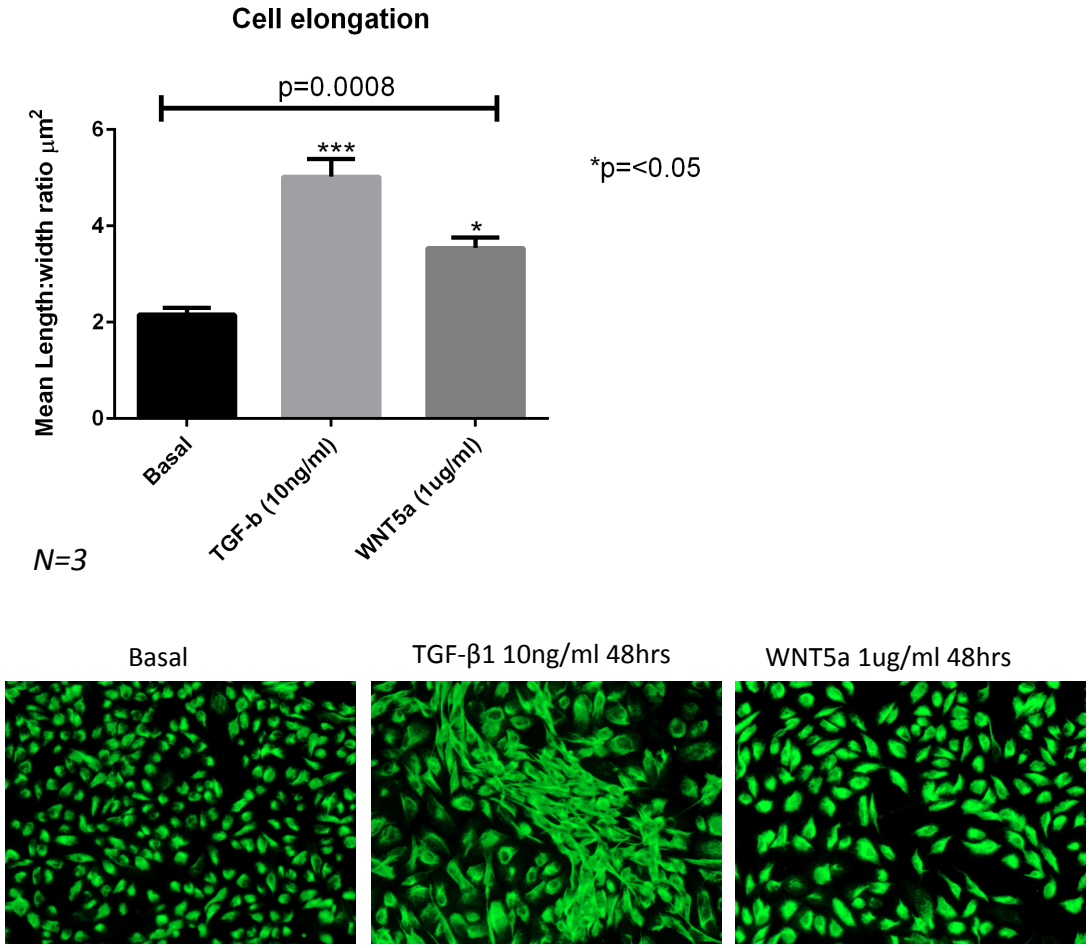


Figure 53: Cell elongation determined in vimentin stained BEAS-2B cells after 48hrs, *p=<0.05. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with basal.

Basal cells are very well characterised based on their unique expression of cytokeratin's and tight junctions (Hackett *et al*, 2011). When a basal cell transitions from an epithelial to a mesenchymal phenotype, one of the processes involves the loss of epithelial type markers that include cytokeratin's and loss of tight junctions. To evaluate this, we stained BEAS-2B cells with cytokeratin-5 (CK-5) and E-cadherin. Previous data has shown

that in the presence of TGF-β1 protein expression of CK-5 and E-cadherin is reduced (Borthwick *et al*, 2009; Doerner and Zuraw, 2009). Here we show that in the presence of WNT5a for 48hr, CK-5 (figure 54) and E-cadherin (figure 55) expression is reduced markedly (p=0.05).

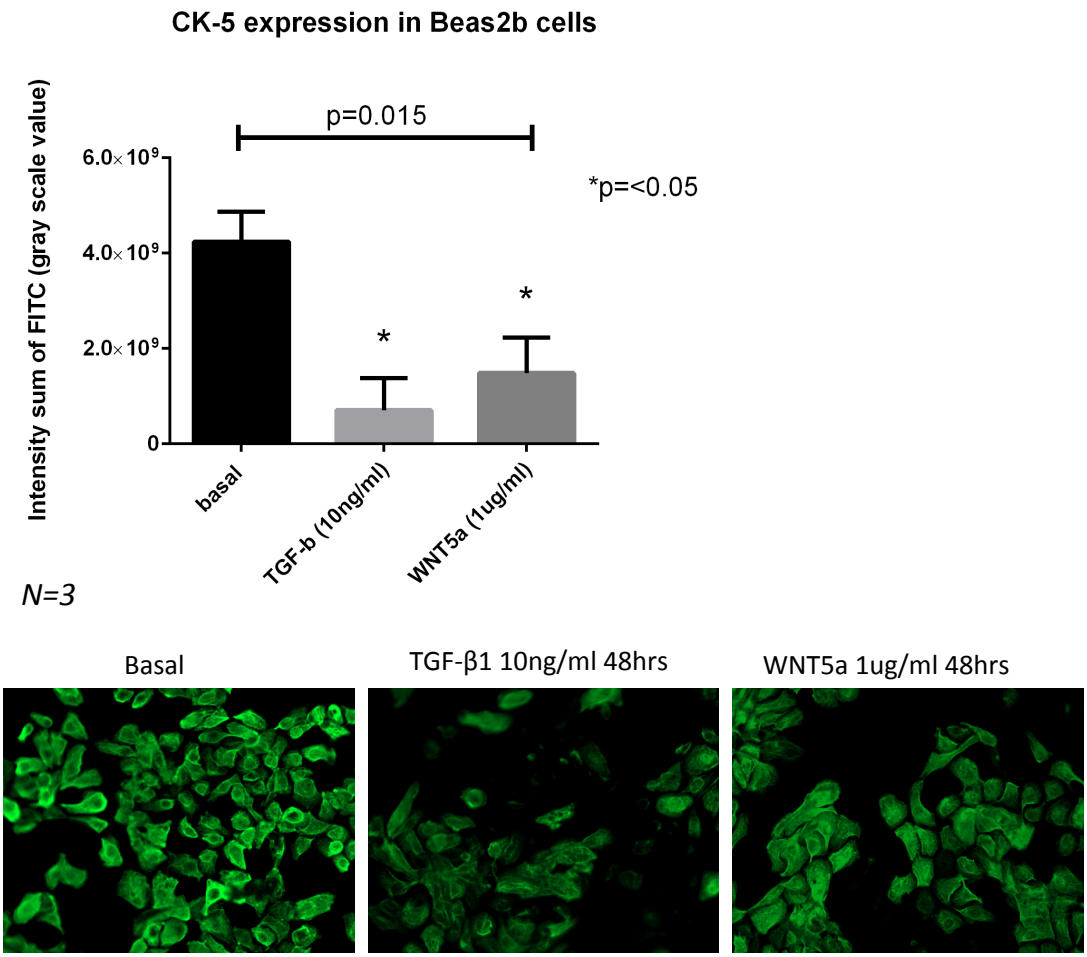


Figure 54: Cytokeratin-5 expression in BEAS-2B cells after 48hrs, *p=<0.05. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with basal.

Interestingly we did not find any expression of α-smooth muscle actin in BEAS-2B even after 72hr in the presence of TGF-β1 (data not shown).

Furthermore, we did not see an increase in Collagen-Iα1 protein expression in BEAS-2B cells (figure 56).

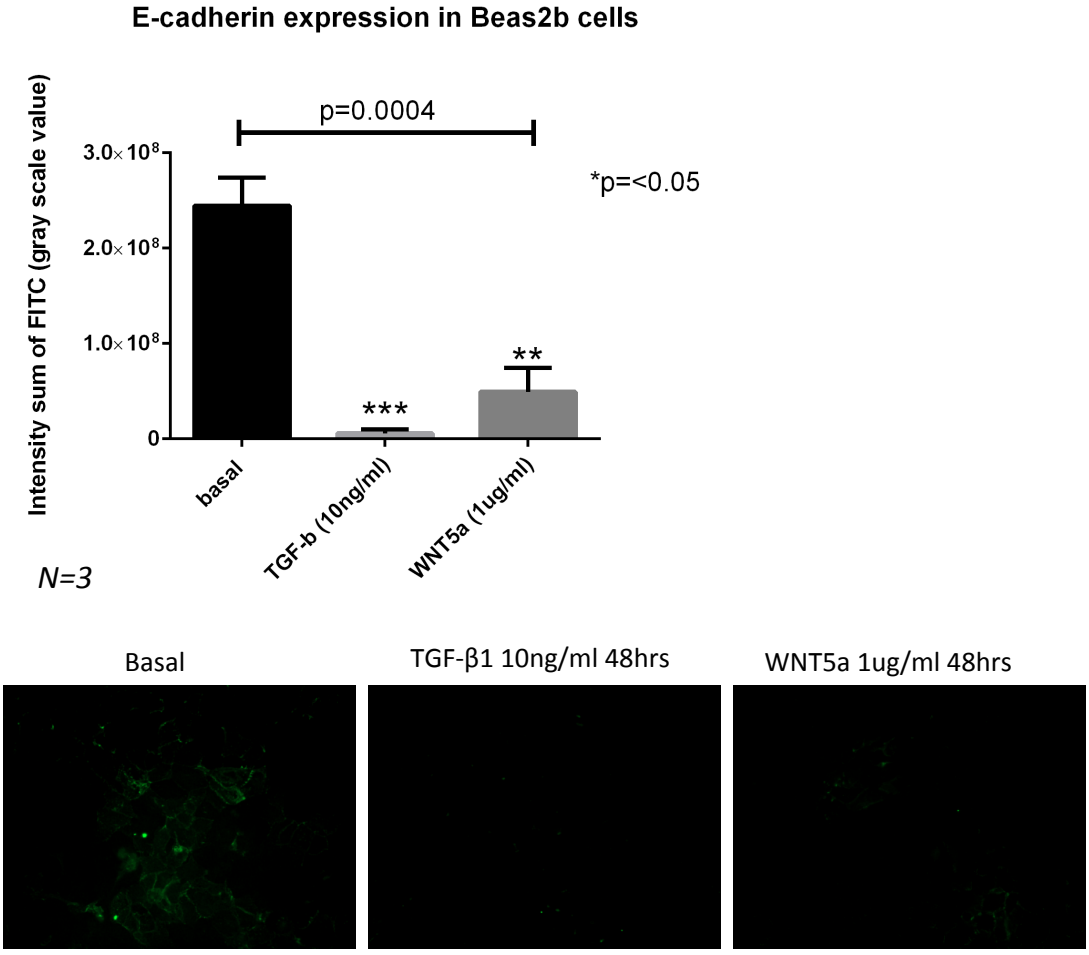


Figure 55: E-cadherin expression in BEAS-2B cells after 48hrs, **p=<0.01. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with basal.

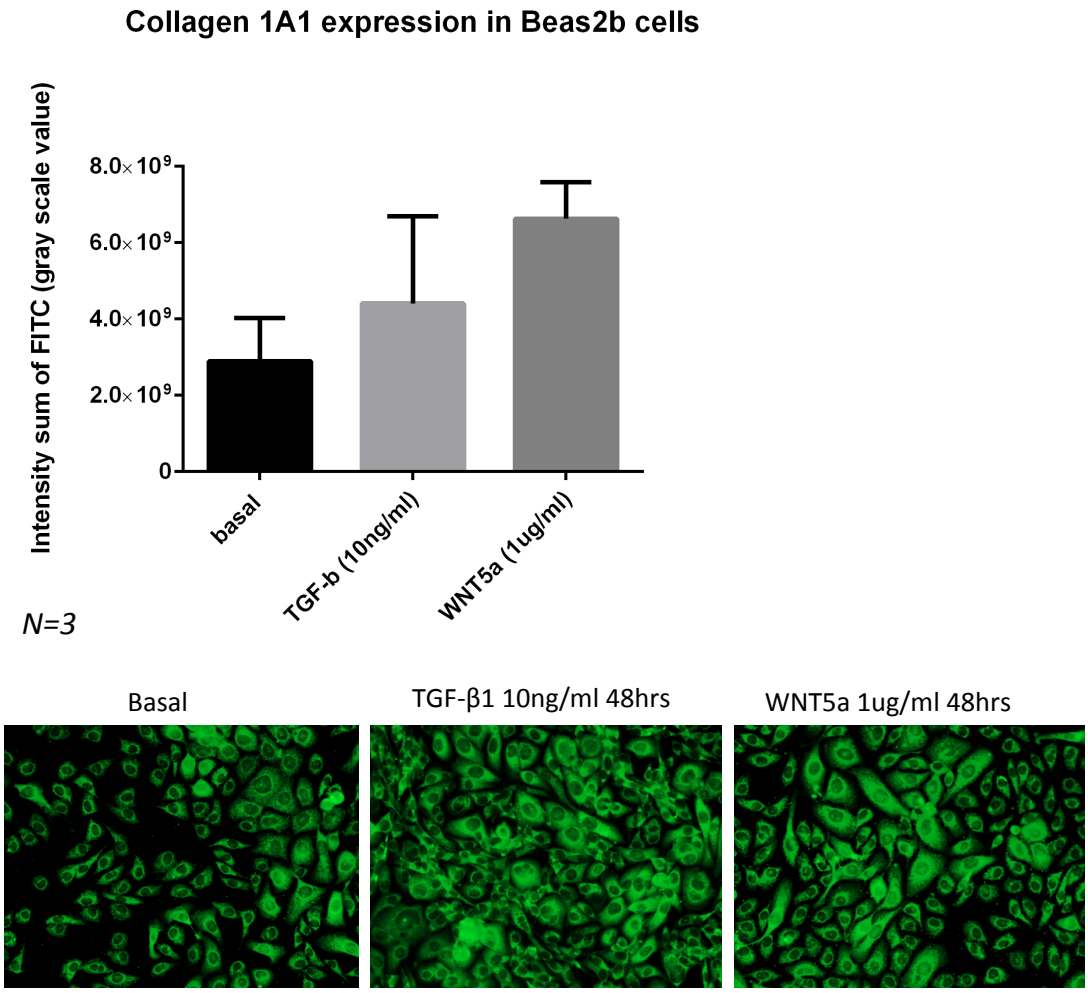
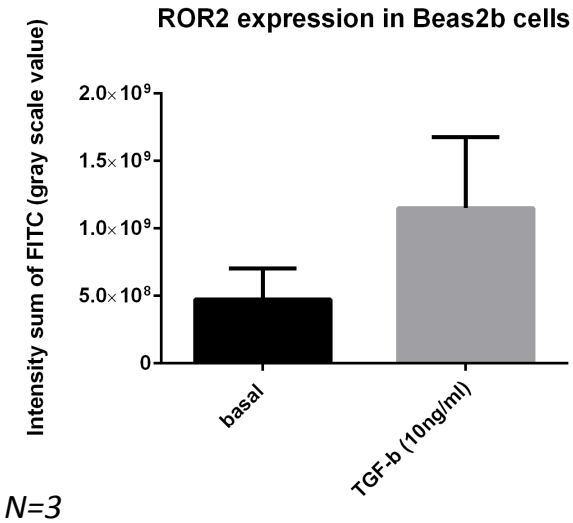


Figure 56: Collagen 1α1 expression in BEAS-2B cells after 48hrs. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with basal.

5.2.2 Effects of TGF-β1 on WNT5a and receptor expression

After observing the effects of WNT5a on EMT we wanted to explore if TGF-β1 could enhance WNT5a related signalling pathway. This is because it has been previously suggested in the literature that TGF-β1 can differentially regulate WNT ligands and receptors (Kumawat *et al*, 2013). Therefore, we stimulated BEAS-2B cells for 48hr in the presence of TGF-β1. Interestingly,

we did not find an increase in protein expression for WNT5a or its receptor ROR2 (figure 57-58). Furthermore, staining for receptors FZD5 and FZD4 showed no protein expression (data not shown).



N=3

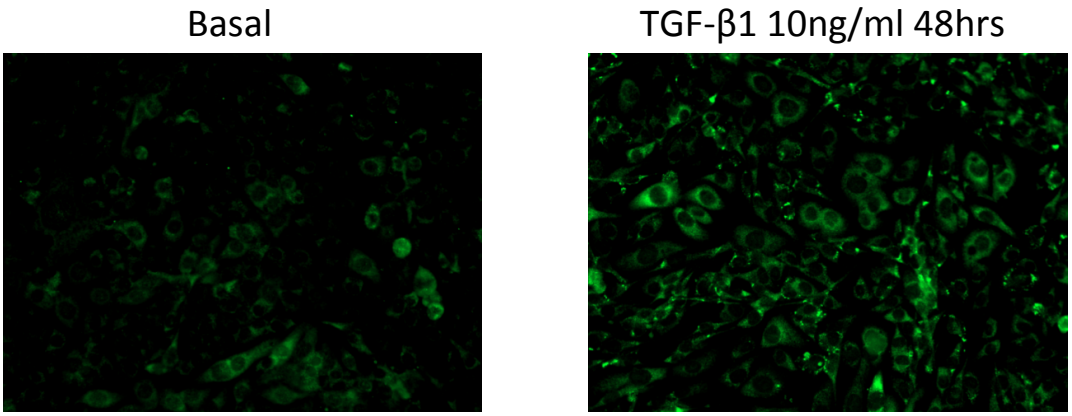


Figure 57: ROR2 protein expression in BEAS-2B cells after 48hrs, n=3. p value determined from unpaired t-test two-tailed. * p=<0.05 as compared with basal.

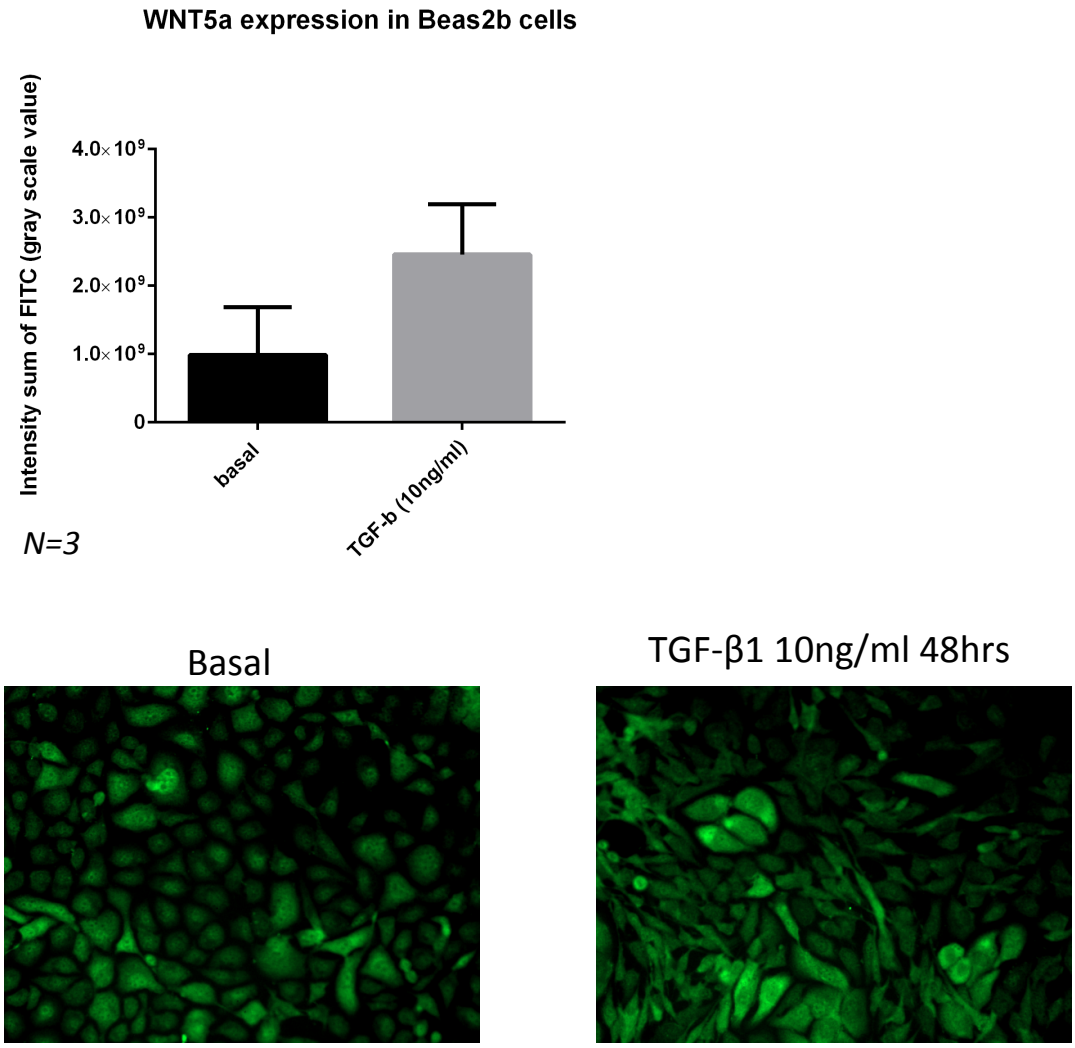


Figure 58: WNT5a protein expression in BEAS-2B cells after 48hrs, n=3. p value determined from unpaired t-test two-tailed.

5.3 WNT5a engages the non-canonical Ca^{2+} pathway in BEAS-2B cells

WNT5a can activate the non-canonical pathway of WNT signalling. Part of this comprises of activating intracellular calcium, which is thought to be Gq coupled. Here wanted to explore if BEAS-2B cells can respond to calcium as

it can promote migration. Initially, we conducted a ratiometric measurement using flow cytometry, however the data was inconsistent primarily due to the shift in the baseline/background fluorescence (Figure 59)

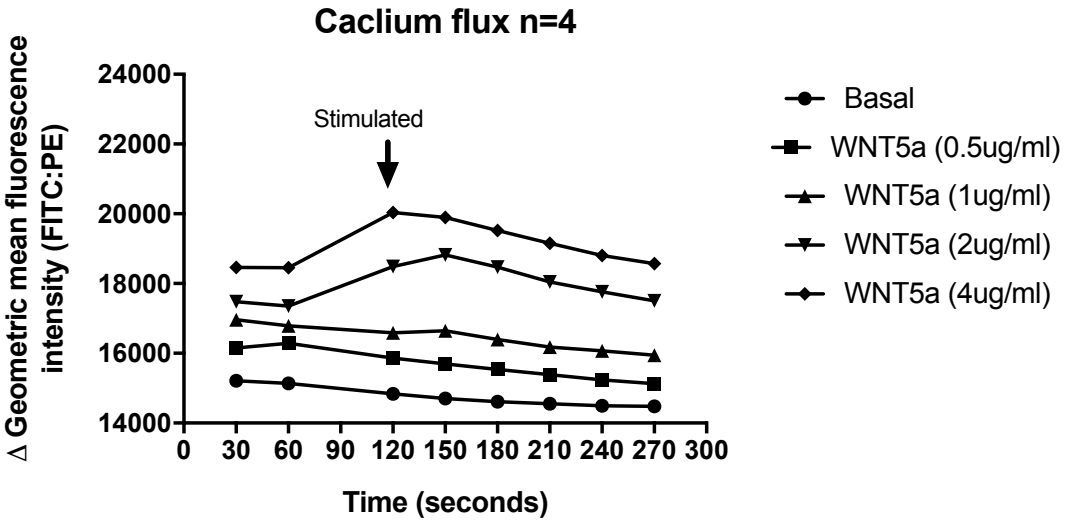


Figure 59: Calcium measurement in BEAS-2B cells before and after addition of WNT5a immediately.

Change in calcium measured by obtaining the mean geometric ratio of FITC:PE, as FITC increases with calcium binding, while PE decreases upon calcium binding, n=4.

To elucidate, this problem we suspected after 1hr incubation with the calcium dyes, there was a form of dye leakage while we were measuring each condition. This could be eliminated with the use of probenecid. Furthermore, the cells were trypsinised prior to flow cytometric analysis; this could contribute to enzymatic cleavage of surface receptors and subsequently attenuate their function. It is worth noting that the extracellular calcium concentration was 2mM in all conditions.

To overcome the limitations of ratiometric calcium assay, we used FLIPR 5 calcium kit. The following technique had a proprietary dye that eliminated background fluorescence from dye leaking; additionally, cells could be incubated with the dye and would not have to be dissociated with trypsin for analysis. We stimulated BEAS-2B cells with WNT5a at various concentrations from 0.5μg - 4μg/ml. Interestingly we found no increase in calcium except at 4μg/ml (figure 60).

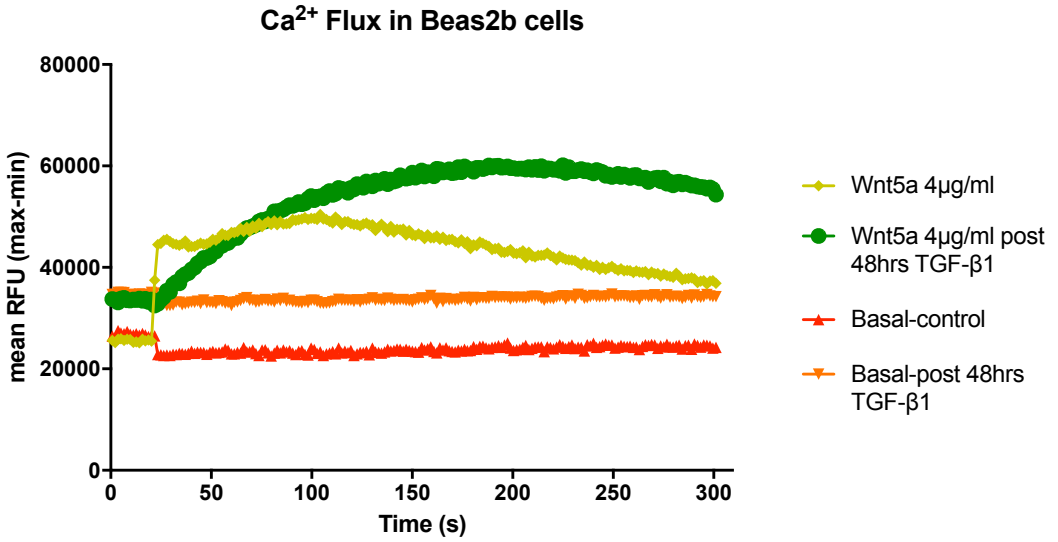


Figure 60: Calcium measurement in BEAS-2B cells before and after addition of WNT5a, pre and post TGF-β1-transformed.

Calcium measurement in TGF-β1-transformed BEAS-2B cells before and after addition of WNT5a, n=3. WNT5a added at 20 seconds by Flexstation.

We found that at 4μg/ml BEAS-2B responded by a mobilisation of intracellular calcium, similar to the flow cytometry data. Interestingly, previous reports have shown that TGF-β1 stimulation can enhance WNT-related molecules; as a result, we hypothesised that this could result in an increase in the efficacy

and sensitivity of WNT5a-induced calcium release, as the machinery would be up regulated.

To test this hypothesis, we incubated BEAS-2B cells with TGF-β1 at 10ng/ml for 48hrs, we then observed the response to WNT5a at various concentrations. However, we found again that calcium mobilisation only occurred at 4μg/ml, this might be because our previous data (figure 60) showed no significant change in WNT5a-related signalling protein expression.

5.4 WNT5a and TGF-β1 promote wound closure in BEAS-2B cells

Previously we investigated functional migration using an ORIS assay, however the data was inconsistent primarily due to the addition of well stoppers manually. The protocol involved coating the 96-well plates with collagen followed by the addition of a well insert that covered a known area preventing cell growth (this allowed an accurate wound area to be created).

After culturing, the cells were stimulated accordingly, and the well insert was removed. The area of wound covered/cells infiltrated was measured by placing a mask at the bottom of the well that mirrored the area covered by the well insert. However, by observing under the microscope this was not the case as the well insert was susceptible to microscopic deviations. This led a to a large variance in actual cells infiltrated the wound area highlighted by the mask (figure 61). Other members of the lab also replicated this problem.

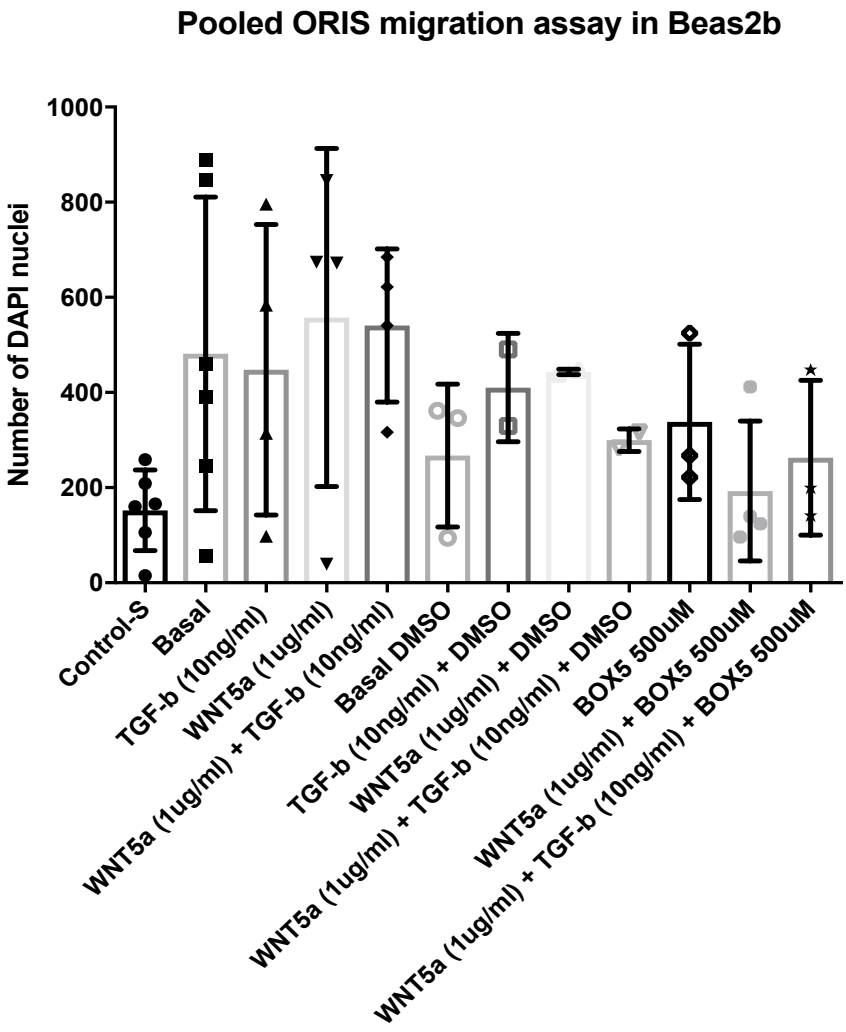


Figure 61: Displaying the number of DAPI stained cells infiltrated in the masked area. Data table displaying mean and IQR values; p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test.

To overcome this problem, we used an *in vitro* scratch assay. Here we manually scratched 4 wounds per condition on a 6-well plate and took a total of 4 images from 4 different reference points per condition to reduce the variance occurred by the scratch. The results showed that concomitant

stimulations of WNT5a and TGF-β1 for 24h enhanced wound closure by 57% in comparison to basal, which was 23% (figure 62)

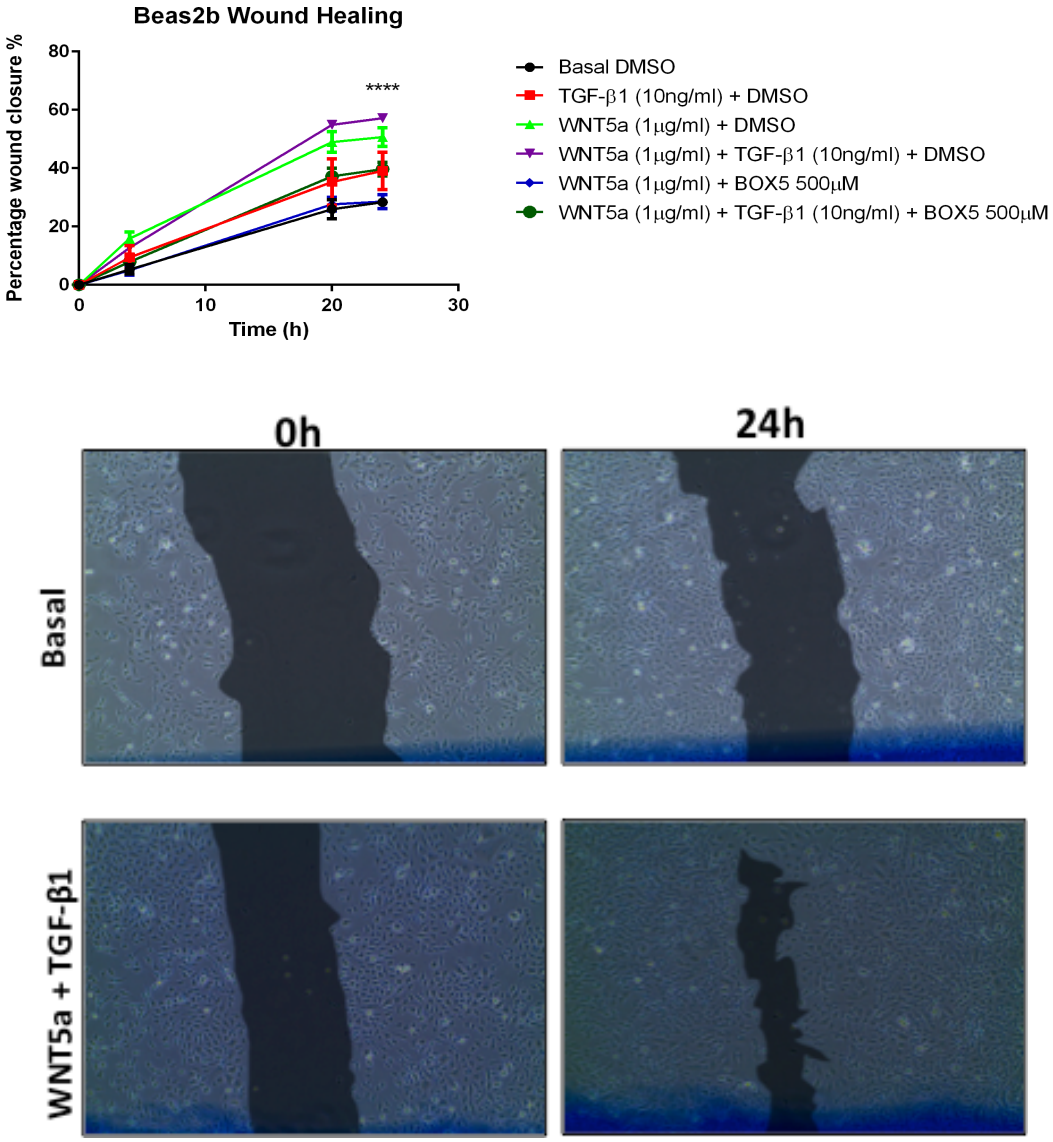


Figure 62: Percentage wound closure in BEAS-2B cells. p=<0.01. ****Basal vs. WNT5a [1μg/ml] + TGF-β1 [10ng/ml], n=3. Representative photomicrograph of BEAS-2B cells at 0h and 24h in the presence or absence of WNT5a + TGF-β1 at x100 magnification. p value determined from two way ANOVA.

Interestingly, when BOX-5 a WNT5a specific antagonist was added, WNT5a-induced wound closure was reduced at basal level. Likewise, BOX-5 in the presence of WNT5a and TGF-β1 returned wound closure to TGF-β1-induced wound closure. This suggests that BOX-5 can only inhibit the WNT5a-induced migration and not TGF-β1.

5.4.1 The effect of TGF-β1 and WNT5a on BEAS-2B cell proliferation

The data for wound closure was obtained over a time period of 24h in BEAS-2B cells. To see if the wound closure effect was primarily due to proliferation of BEAS-2B cells we conducted a colorimetric MTT proliferation assay looking at the effect over 24h and 48h in the stimulated conditions. We initially conducted an assay to generate a linear regression standard to determine the change in cell numbers. From this we could see that as we increased the cell density the optical density increased linearly with $r^2 = 0.9586$ (figure 63)

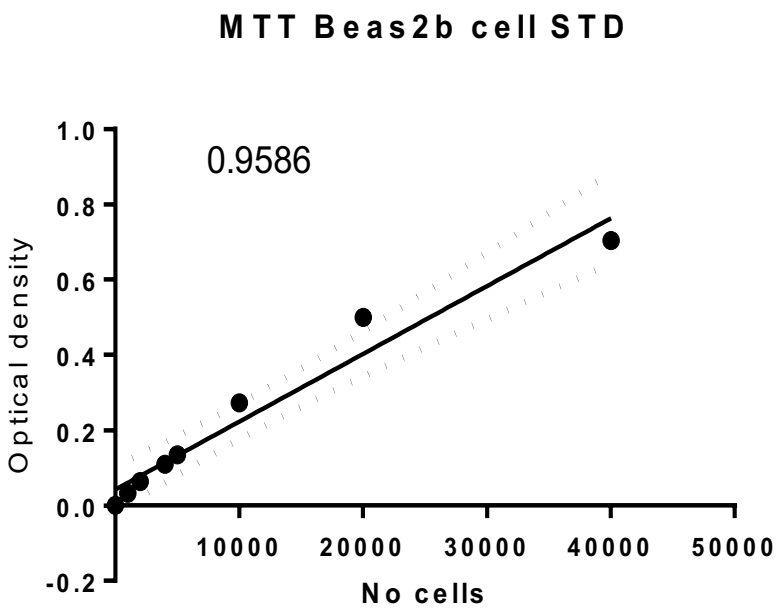


Figure 63: Linear regression of cell standard for MTT proliferation assay. Y = 1.801e-005*X + 0.04235.

After culturing BEAS-2B cells in 96-well plates and stimulated accordingly for 24h (figure 64) or 48h (figure 65) we found no significant increase in proliferation.

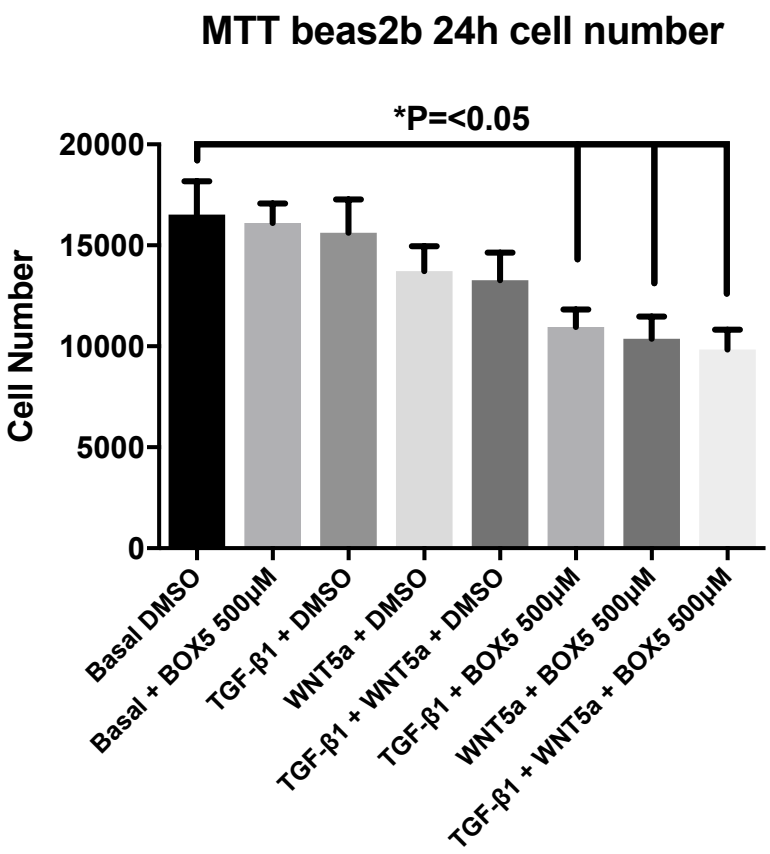


Figure 64: MTT proliferation assay for BEAS-2B cells. Cells stimulated for 24h, n=4. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with basal.

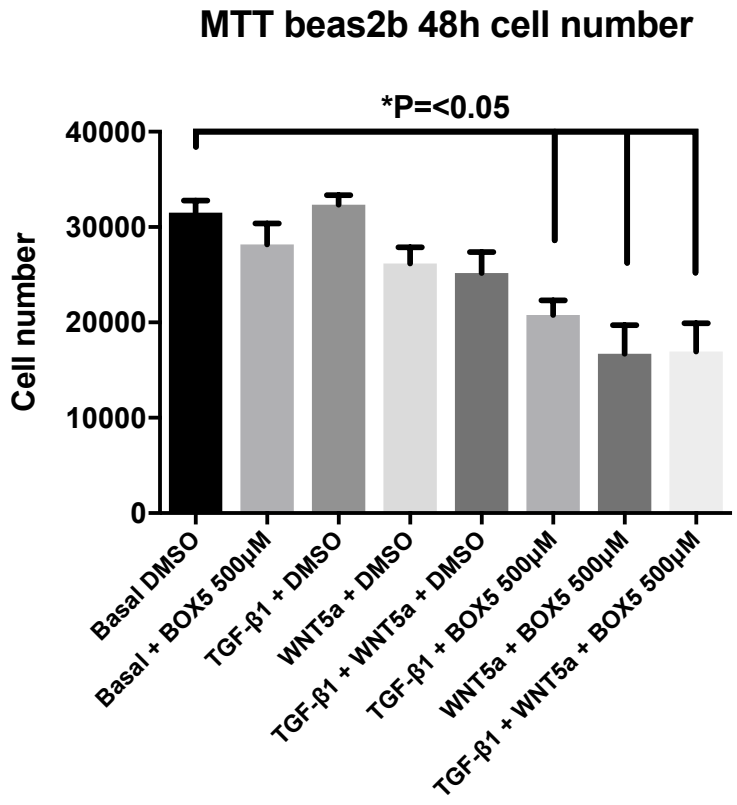


Figure 65: MTT proliferation assay for BEAS-2B cells. Cells stimulated for 48h, n=4. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with basal.

From this we can conclude that proliferation at 24h in the *in vitro* scratch assay had minimal effect. In fact the presence of WNT5a or TGF-β1 reduced proliferation compared to basal. Interestingly, when comparing the cell numbers between 24h and 48h we see that in general in all conditions the cell numbers double, however we observed the wound closure phenomenon at 24h time point.

5.5 WNT5a cross-talk with TGF-β1-SMAD dependent pathway

Exploring the idea of TGF-β1-WNT5a cross talk, it is well known that TGF-β1 mediates its effect through the SMAD2/3 heterogeneous complex. In addition, (Miyoshi *et al*, 2012) have shown in colonic organoids, WNT5a can potentiate SMAD3 nuclear translocation and WNT5a signalling can be influenced by TGF- β1 (Roarty and Serra, 2007; Katoh and Katoh, 2009).

Supporting this, we chelated data from epithelial gene arrays from Genentech and identified 59 biologically important genes in the WNT-TGF-β1 pathway and Th-inflammatory pathway (list displayed in appendix 9). Conducting a bivariate correlation and subsequently ranking them by the correlation coefficient we found a strong positive correlation between WNT5a and SMAD2 (r^2=0.48, p=0.03) and PPP3CA a calcinuerin subunit activated by intracellular calcium release. Interestingly, similar to our *in* vivo data we found a negative correlation between WNT5a and SERPINB2 a gene part of the Th2-inflammatory gene signature (figure 66).

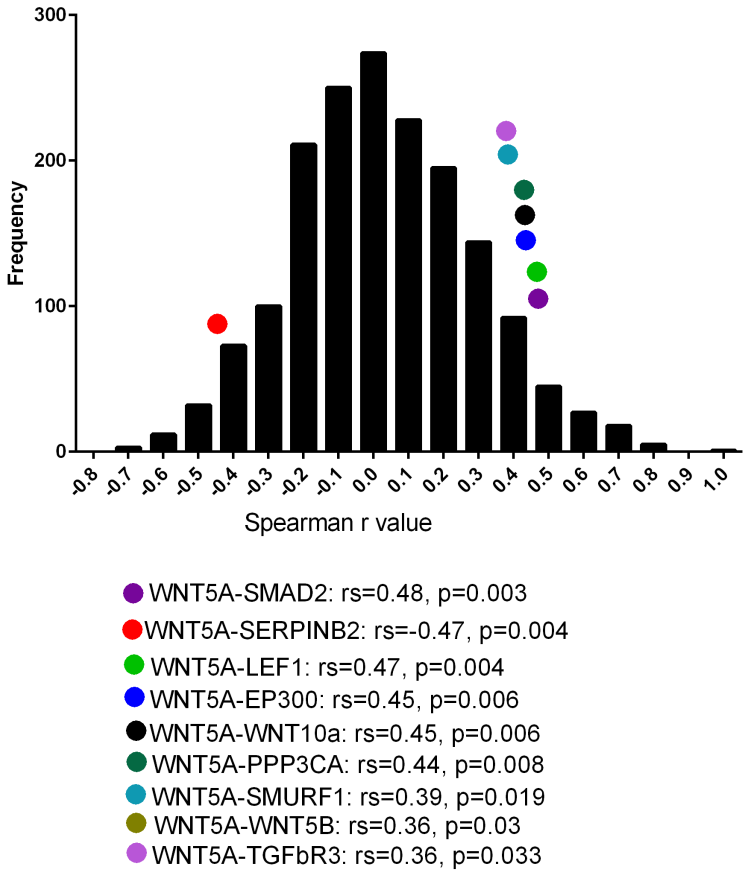


Figure 66: The frequency of gene-gene correlation for given spearman R-value determined from bivariate correlation.

As a result, we wanted to evaluate if WNT5a can affect SMAD2/3 nuclear translocation and the latter act as an intermediary for TGF-β1-WNT5a cross talk. After initial optimisations, we stimulated BEAS-2B cells for 2h in the presence of WNT5a [1μg/ml] or TGF-β1 [10ng/ml]. As expected we observed SMAD2/3 nuclear translocation in the presence of TGF-β1 and was not affected by BOX-5. Interestingly, when stimulated with WNT5a we saw an increase in SMAD2/3 nuclear translocation, which was attenuated in the presence of BOX-5 a WNT5a specific antagonist (figure 67).

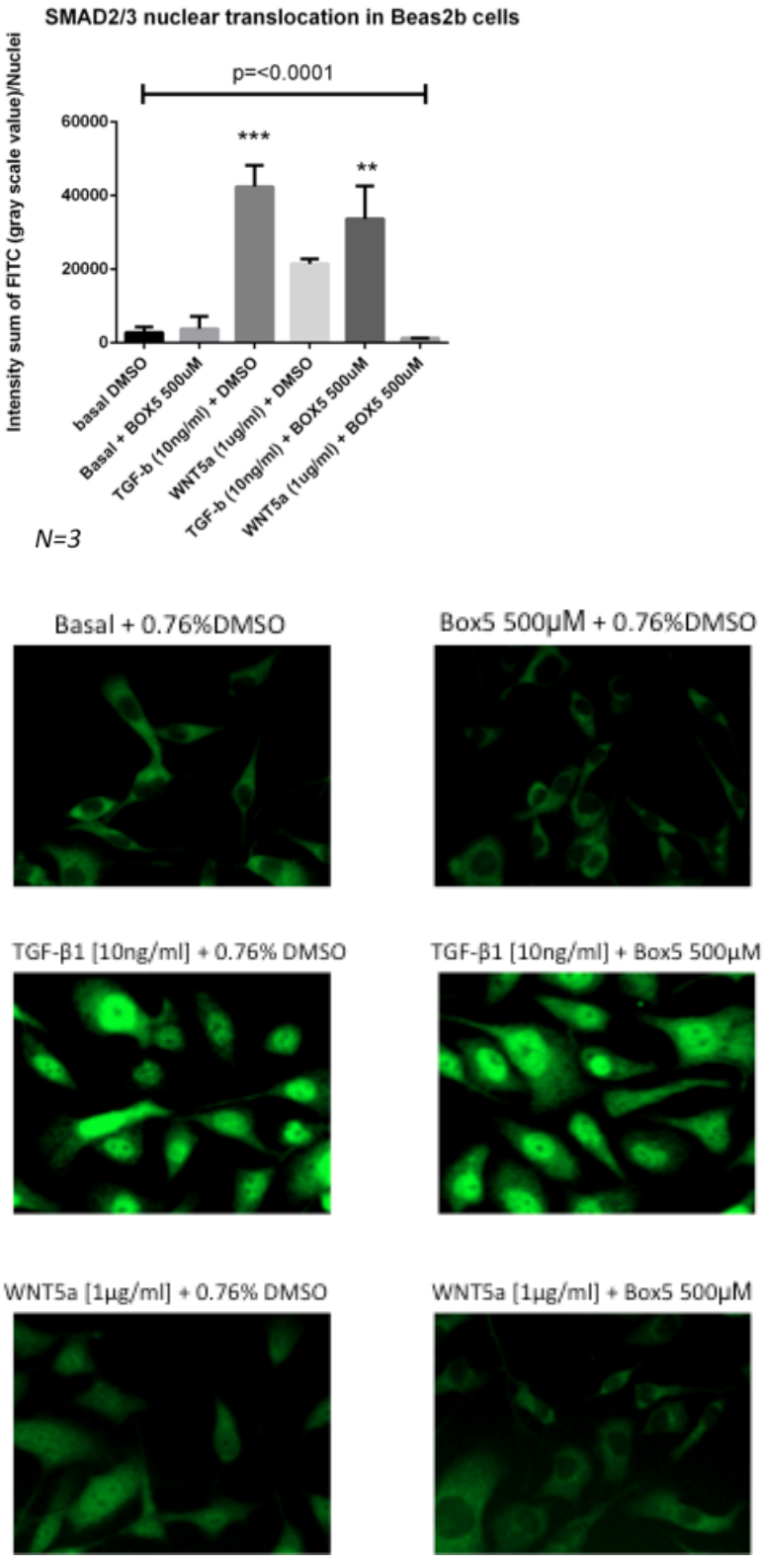


Figure 67: Total SMAD2/3 per nuclei in BEAS-2B cells stimulated with WNT5a, TGF-β1 and or BOX5.***p=<0.001. ***Basal vs TGF-β1 [10ng/ml],

**p=<0.01. **Basal vs WNT5a [1μg/ml], n=3. Representative photomicrograph of total SMAD2/3 in BEAS-2B cells after 2h at x200 magnification. p value determined from one way ANOVA with Kruskal-Wallis test and Dunn's multiple comparisons test. * p=<0.05 as compared with basal.

After establishing the involvement of the SMAD-complex we initially wanted to investigate the changes in WNT5a-TGF-β1 cross-talk related molecules selected from the literature using a custom PCR array. Unfortunately, after extraction of RNA and subsequent cDNA synthesis and amplification, it was evident that the PCR machine was not functioning correctly and as a consequence data was not analysed. Analysis of RNA quality displayed a A_{260}/A_{280} ratio of ≥2.0, additionally RNA integrity was assessed using a bio analyser (Agilent RNA 6000 series II) with no evidence of degradation.

5.6 Discussion

Our previous data showed evidence of WNT5a protein expression in different compartments of the airway tissue sections. However, the role of WNT5a remained unexplored. These observations are important because tissue repair in asthma may be dysregulated and has not been studied in the context of Th2, Th17, and Th0 inflammation characterised by biopsy gene expression.

Here we set out to explore further the i*n vivo* effects of WNT5a and TGF-β1 protein expression signal in *in vitro*. Our data demonstrated that similar to TGF-β1, WNT5a could also mimic EMT changes in BEAS-2B cells, by reducing the expression of epithelial markers and altering the morphology.

Additionally, WNT5a can induce intracellular calcium in BEAS-2B cells with an immediate response similar to (Jenei *et al*, 2009), in addition (Prgomet *et al*, 2015) show WNT5a can invoke a calcium response in oral squamous tongue carcinoma cell lines, however the response was sustained for 4 minutes suggesting calcium mobilisation is cell dependent. Both studies found BOX-5 to inhibit WNT5a induced calcium and as such it would be interesting to see if BOX-5 can attenuate this response in BEAS-2B cells. Interestingly TGF-β1 cannot enhance the sensitivity of WNT5a-induced calcium release, this may be because WNT5a-related calcium molecules are not induced by TGF-β1 as suggested in the literature (Baarsma *et al*, 2016; Kumawat *et al*, 2013), however further work needs to be done to elucidate what mechanisms of WNT5a are induced by TGF-β1 (Katoh and Katoh, 2009; Kumawat *et al*, 2014).

The influence of WNT5a on SMAD-2/3 nuclear translocation was previously highlighted in the literature (Miyoshi *et al*, 2012). In our case we also observed this phenomenon to an extent. It is interesting to note that WNT5a has two isoforms that differ on the first 15 amino acids that represent the messenger signal and this may affect the downstream cross talk signalling pathway. Our data also compliments our hypothesis (figure 8) that WNT5a can promote wound closure/migration that is not influenced by proliferation.

Chapter 6:

Discussion and

Future work

6.1 Discussion

The key findings of the project reveal WNT ligands and receptors mRNA expression are differentially regulated in bronchial biopsies and epithelial brushings, albeit no significant difference was observed between health and asthmatics, which is in contrast to previous reports (Choy *et al*, 2011). Building upon this WNT5a protein can be detected by WNT5a 6F2 antibody in *ex vivo* and *in vitro* applications. This study is the first to visualize WNT5a protein expression pattern in health and asthmatic bronchial biopsies. Additionally, a significant difference in WNT5a protein expression was observed in the epithelium and submucosa in asthmatics when compared with healthy individuals. These data strongly correlated with TGF-β1 protein expression and co-localized bronchial biopsy tissue sections confirm dual staining for WNT5a and TGF-β1 protein. These data indicate that asthmatics have the capacity to express WNT5a protein even on steroids and may influence epithelial repair via cross talk with TGF-β1 suggested previously.

Examining the *in vitro* data, we can conclude that WNT5a similar to TGF-β1 can promote EMT and total-SMAD2/3 nuclear translocation highlighting a known cross talk network. Inhibition of WNT5a signal by BOX5 can attenuate WNT5a-induced total-SMAD2/3 nuclear translocation but not TGF-β1-induced total-SMAD2/3 nuclear translocation. Further WNT5a can induce intracellular calcium but not augment in the presence of TGF-β1, suggesting that WNT5a signaling complex is not amplified to increase calcium release and may not influence bidirectional cross talk with TGF-β1. Lastly, we show

WNT5a and TGF-β1 both promote BEAS-2B wound closure and is amplified by co-stimulation. Interestingly when BOX5 is applied only WNT5a-induced wound closure is inhibited and during co-stimulation wound closure is returned to TGF-β1 levels. Additionally, this wound closure effect is not influenced by proliferation. As a conclusion, these data are indicative that WNT5a protein when expressed in the epithelium can potentiate TGF-β1 via SMAD2/3 axis via unidirectional cross talk. However, as it promotes repair unwanted effects can promote airway wall remodeling by increasing ECM via EMT and migration promoting RBMT.

Asthma is heterogeneous disease displaying phenotypes at a clinical and molecular level. At a clinical level, asthma can be categorized with eosinophilic inflammation, either early or late onset or as non-eosinophilic (Haldar *et al*, 2008). Molecular phenotyping has deduced Th2-high asthma accounts for 50% of asthmatics. It is associated with various remodeling features most notably ECM deposition in the airway wall (Woodruff *et al*, 2009) and eosinophilia. Current treatments targeting the underlying inflammation do not reduce ECM deposition (Boulet *et al*, 2000; Bergeron, Al-Ramli and Hamid, 2009; Yamaguchi *et al*, 2008; de Kluijver *et al*, 2005). This is because once initiated inflammation and consequently remodeling become separate entities that occur in parallel. Evidence for this has been shown by (Saglani *et al*, 2009) in an allergic mice model, where inflammation and AHR subsided but collagen deposition persisted. This observation is seen in asthmatics after an allergen challenge, where cellular inflammation returned

to baseline after 24 hours but expression of collagen (I and III) remained elevated (Kariyawasam *et al*, 2007).

The functional consequence of ECM deposition still remains elusive however it correlates with disease severity and negatively correlates with lung function (Yamaguchi *et al*, 2008; Chetta *et al*, 1997). Deposition of ECM by structural cells can be induced by TGF-β1 (Kumawat *et al*, 2013; Hackett *et al*, 2009). However, targeting TGF-β1 is not viable due to its pleotropic nature. Recent evidence highlights WNT5a gene expression is increased in asthmatics particularly in ASM and epithelial cells. However, it is not known if this is true at the protein level and which, structural cell contributes to the pool of WNT5a. Here we investigate WNT5a protein expression in human bronchial biopsies embedded in GMA and lung structural cells. As such detection systems for WNT5a were optimized. As a conclusion, WNT5a 6F2 antibody has consistently stained positively for L-cells in immunohistochemistry, western blotting and immunofluorescence. Suggesting this monoclonal antibody has an affinity for WNT5a protein.

Furthermore, Choy *et al* recently demonstrated using a similar approach defining tissue inflammation by biopsy gene expression scores, that two types of airway inflammation in asthma Th2 vs Th17 may be reciprocally regulated, with the possible emergence of the Th17 phenotype in response to corticosteroid therapy (Choy *et al*., 2015). These observations suggest that inflammatory airway phenotypes may be highly plastic and responsive to therapy. A number of patients in the studies alluded to above could not be

easily categorised in to defined Th2, Th17 subgroups, suggesting that additional tissue phenotypes may be important in asthma pathogenesis.

Building upon these observations, Choy *et al* previously conducting a microarray on bronchial biopsy homogenates found multiple genes are differentially expressed in Th2-high asthma, particularly WNT5a. In our cohort, bronchial biopsy homogenates had previously undergone micro array analysis but found no significant elevation in WNT5a (or TGF-β1) as shown in table 6. One explanation for the discrepancy may be because patients in the Choy *et al* study were primarily mild asthmatics and had no CS treatment. As such other non-Th2 phenotypes such as Th17 that may be induced by CS therapy were unlikely to have been operational. Furthermore, the WNT5a gene expression signal may be diluted in bronchial homogenates as most of the pool of WNT5a was sourced in the epithelium particularly the Th-17 inflammatory phenotype.

In conclusion, the epithelium contains a large pool of WNT5a and is the first line of defence against the external milieu (Sumi and Hamid, 2007; Laitinen et al, 1985), however its role remained unexplored. Evidence of epithelial damage and shedding has been shown in asthmatics (Xiao et al, 2011; Puddicombe et al, 2000). Based on our observations we hypothesise that epithelial derived TGF-β1 induces EMT in basal epithelial cells, inducing WNT5a protein expression, directional polarised migration and a fibrotic response with cells secreting ECM proteins and MMPs (Hackett et al, 2009). Subsequently in disease such as asthma where the airway barrier may experience repetitive wounding, a vicious cycle of aberrant repair ensues

resulting in excessive deposition of ECM in the basement membrane and lamina propria. Mediating its effect through the planar cell polarity and Ca2+ pathway WNT5a can promote migration and basal apical cell polarity (Pourreyron et al, 2012; Witze et al, 2008; Yamamoto et al, 2015) as depicted in figure 68.

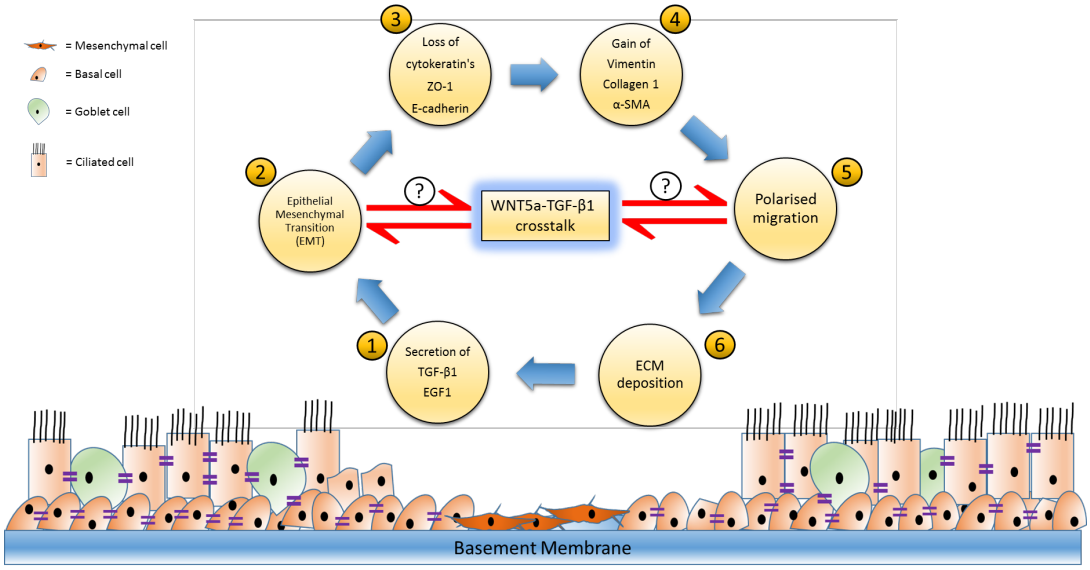


Figure 68: Depicting the epithelial repair process and the influence of WNT5a-TGF-β1 cross talk on these processes.

As a consequence, we set out to explore further the i*n vivo* effects of WNT5a and TGF-β1 protein expression signal in *in vitro*. Our data demonstrated that similar to TGF-β1, WNT5a could also mimic EMT changes in BEAS-2B cells, by reducing the expression of epithelial markers and altering the morphology. Additionally, WNT5a engaged the Ca^{2+} pathway, however the concentration at which intracellular calcium was induced was much higher at 4μg/ml with no evidence of induction at lower concentrations. Previous reports have shown induction of intracellular concentration at <1μg/ml and inhibition achieved by

pre-incubation with WNT5a competitive antagonist BOX5 (Prgomet *et al*, 2015; Jenei *et al*, 2009). As a result, this indicates that WNT5a calcium induction is concentration and cell type dependent. Furthermore, in our case, stimulation of BEAS-2B cells with TGF-β1 could not enhance WNT5a sensitivity of calcium induction either. This may in part be due to the effects of TGF-β1 could not enhance WNT5a signalling machinery.

Nevertheless, WNT5a and TGF-β1 could both promote wound closure, but more strikingly co-stimulation of WNT5a-TGF-β1 significantly enhanced wound closure. Inhibition with BOX5 during co-stimulation returned wound closure to TGF-β1 levels, suggesting that WNT5a-induced wound closure was only inhibited. Complimenting this we find that proliferation of BEAS-2B cells is unaltered at 24hrs and 48hrs suggesting that wound closure was primarily driven by cell migration.

To deduce the mechanism of WNT5a-TGF-β1 cross talk we evaluated epithelial microarrays from the Genentech cohort and found a significant correlation between WNT5a and SMAD2. Additionally, evidence from the literature shows WNT5a can potentiate the effects of TGF-β1 by promoting SMAD3 nuclear translocation in colonic organoids (intestinal epithelial cells) (Miyoshi *et al*, 2012). We show evidence that WNT5a to an extent can also promote total-SMAD2/3 into the nucleus, this can be further evaluated by observing total phosphorylated SMAD2/3. Interestingly WNT5a-induced SMAD2/3 nuclear translocation can be inhibited by BOX5 but has no effect on TGF-β1 induced SMAD2/3 nuclear translocation.

In conclusion, this is the first study looking at WNT5a protein expression distribution in asthma phenotypes, displaying an array of tissue compartments expressing the protein. Interestingly the epithelium, which is the first line of defence against the external milieu, contains a large pool of WNT5a. Exploring the effects of WNT5a in *in vitro* we find that it can similar to TGF--β1 promote morphological changes in BEAS-2B cell line. Furthermore, it can subsequently enhance wound closure in conjunction with TGF--β1 but not proliferation of BEAS-2B cells. As a consequence WNT5a can cross talk with TGF--β1 and may highlight a novel repair mechanism employed by the epithelium in asthma.

6.2 Future work

A targeted WNT array will be conducted obtained from Qiagen (these experiments will be facilitated by me and conducted by other members of the group). Changes in gene expression will help deduce important cross-talk molecules and pro-repair molecules. Building on this a new air liquid interface model for epithelial cultured will be established to induce different Th-inflammatory phenotypes and study the WNT5a-TGF--β1 repair mechanism. Lastly, the signal in the lamina propria also proposes an interesting avenue to explore as WNT5a could influence inflammatory cells migrating to the lung.

References

Bibliography

'Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC)', (1998) *The European respiratory journal,* 12(2), pp. 315-335.

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997) 'Beta-Catenin is a Target for the Ubiquitin-Proteasome Pathway', *The EMBO journal,* 16(13), pp. 3797-3804.

Aikawa, T., Shimura, S., Sasaki, H., Ebina, M. and Takishima, T. (1992) 'Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack', *Chest,* 101(4), pp. 916-921.

Akhabir, L. and Sandford, A.J. (2011) 'Genome-wide association studies for discovery of genes involved in asthma', *Respirology (Carlton, Vic.),* 16(3), pp. 396-406.

Akhurst, R.J. and Hata, A. (2012) 'Targeting the TGFbeta signalling pathway in disease', *Nature reviews.Drug discovery,* 11(10), pp. 790-811.

American Thoracic Society and European Respiratory Society (2005) 'ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005', *American journal of respiratory and critical care medicine,* 171(8), pp. 912-930.

Amin, K., Ludviksdottir, D., Janson, C., Nettelbladt, O., Bjornsson, E., Roomans, G.M., Boman, G., Seveus, L. and Venge, P. (2000) 'Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group', *American journal of respiratory and critical care medicine,* 162(6), pp. 2295-2301.

Araujo, B.B., Dolhnikoff, M., Silva, L.F., Elliot, J., Lindeman, J.H., Ferreira, D.S., Mulder, A., Gomes, H.A., Fernezlian, S.M., James, A. and Mauad, T. (2008) 'Extracellular matrix components and regulators in the airway smooth muscle in asthma', *The European respiratory journal,* 32(1), pp. 61-69.

Arthur, G.K., Duffy, S.M., Roach, K.M., Hirst, R.A., Shikotra, A., Gaillard, E.A. and Bradding, P. (2015) 'KCa3.1 K+ Channel Expression and Function in Human Bronchial Epithelial Cells', *PloS one,* 10(12), pp. e0145259.

Attisano, L. and Labbe, E. (2004) 'TGFbeta and Wnt pathway cross-talk', *Cancer metastasis reviews,* 23(1-2), pp. 53-61.

Auer, J. and Lewis, P.A. (1910) 'The Physiology of the Immediate Reaction of Anaphylaxis in the Guinea-Pig', *The Journal of experimental medicine,* 12(2), pp. 151-175.

Austen, K.F. (1974) 'Reaction mechanisms in the release of mediators of immediate hypersensitivity from human lung tissue', *Federation proceedings,* 33(11), pp. 2256-2262.

Baarsma, H.A., Konigshoff, M. and Gosens, R. (2013) 'The WNT signaling pathway from ligand secretion to gene transcription: molecular mechanisms and pharmacological targets', *Pharmacology & therapeutics,* 138(1), pp. 66-83.

Baarsma, H.A., Skronska-Wasek, W., Mutze, K., Ciolek, F., Wagner, D.E., John-Schuster, G., Heinzelmann, K., Gunther, A., Bracke, K.R., Dagouassat, M., Boczkowski, J., Brusselle, G.G., Smits, R., Eickelberg, O., Yildirim, A.O. and Konigshoff, M. (2016) 'Noncanonical WNT-5A signaling impairs endogenous lung repair in COPD', *The Journal of experimental medicine,* .

Baena-Cagnani, C., Rossi, G.A. and Canonica, G.W. (2007) 'Airway remodelling in children: when does it start?', *Current opinion in allergy and clinical immunology,* 7(2), pp. 196-200.

Barbee, R.A., Dodge, R., Lebowitz, M.L. and Burrows, B. (1985) 'The epidemiology of asthma', *Chest,* 87(1 Suppl), pp. 21S-25S.

Barczyk, A., Pierzchala, W. and Sozanska, E. (2003) 'Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine', *Respiratory medicine,* 97(6), pp. 726-733.

Barlow, J.L., Peel, S., Fox, J., Panova, V., Hardman, C.S., Camelo, A., Bucks, C., Wu, X., Kane, C.M., Neill, D.R., Flynn, R.J., Sayers, I., Hall, I.P. and McKenzie, A.N. (2013) 'IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction', *The Journal of allergy and clinical immunology,* 132(4), pp. 933-941.

Barnes, P.J., Jonsson, B. and Klim, J.B. (1996) 'The costs of asthma', *The European respiratory journal,* 9(4), pp. 636-642.

Barnett, S.B. and Nurmagambetov, T.A. (2011) 'Costs of asthma in the United States: 2002-2007', *The Journal of allergy and clinical immunology,* 127(1), pp. 145-152.

Barreto-Luis, A., Corrales, A., Acosta-Herrera, M., Gonzalez-Colino, C., Cumplido, J., Martinez-Tadeo, J., Carracedo, A., Villar, J., Carrillo, T., Pino-Yanes, M. and Flores, C. (2017) 'A pathway-based association study reveals variants from Wnt signalling genes contributing to asthma

susceptibility', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 47(5), pp. 618-626.

Bateman, E.D., Hurd, S.S., Barnes, P.J., Bousquet, J., Drazen, J.M., FitzGerald, M., Gibson, P., Ohta, K., O'Byrne, P., Pedersen, S.E., Pizzichini, E., Sullivan, S.D., Wenzel, S.E. and Zar, H.J. (2008) 'Global strategy for asthma management and prevention: GINA executive summary', *The European respiratory journal,* 31(1), pp. 143-178.

Bauer, M., Benard, J., Gaasterland, T., Willert, K. and Cappellen, D. (2013a) 'WNT5A encodes two isoforms with distinct functions in cancers', *PloS one,* 8(11), pp. e80526.

Bauer, M., Benard, J., Gaasterland, T., Willert, K. and Cappellen, D. (2013b) 'WNT5A encodes two isoforms with distinct functions in cancers', *PloS one,* 8(11), pp. e80526.

Beale, J., Jayaraman, A., Jackson, D.J., Macintyre, J.D., Edwards, M.R., Walton, R.P., Zhu, J., Ching, Y.M., Shamji, B., Edwards, M., Westwick, J., Cousins, D.J., Hwang, Y.Y., McKenzie, A., Johnston, S.L. and Bartlett, N.W. (2014) 'Rhinovirus-induced IL-25 in asthma exacerbation drives type 2 immunity and allergic pulmonary inflammation', *Science translational medicine,* 6(256), pp. 256ra134.

Benayoun, L., Druilhe, A., Dombret, M.C., Aubier, M. and Pretolani, M. (2003) 'Airway structural alterations selectively associated with severe asthma', *American journal of respiratory and critical care medicine,* 167(10), pp. 1360-1368.

Berair, R. and Brightling, C.E. (2014) 'Asthma therapy and its effect on airway remodelling', *Drugs,* 74(12), pp. 1345-1369.

Bergeron, C., Al-Ramli, W. and Hamid, Q. (2009) 'Remodeling in asthma', *Proceedings of the American Thoracic Society,* 6(3), pp. 301-305.

Bergeron, C., Hauber, H.P., Gotfried, M., Newman, K., Dhanda, R., Servi, R.J., Ludwig, M.S. and Hamid, Q. (2005) 'Evidence of remodeling in peripheral airways of patients with mild to moderate asthma: effect of hydrofluoroalkane-flunisolide', *The Journal of allergy and clinical immunology,* 116(5), pp. 983-989.

Bergstein, I., Eisenberg, L.M., Bhalerao, J., Jenkins, N.A., Copeland, N.G., Osborne, M.P., Bowcock, A.M. and Brown, A.M. (1997) 'Isolation of two novel WNT genes, WNT14 and WNT15, one of which (WNT15) is closely linked to WNT3 on human chromosome 17q21', *Genomics,* 46(3), pp. 450-458.

Berne, R.M., Levy, M.N., Koeppen, B.M. and Stanton, B.A. (2008) *Berne and Levy physiology.* 6th edn. Philadelphia, Pa.: Mosby/Elsevier.

Berthon, B.S., Gibson, P.G., Wood, L.G., MacDonald-Wicks, L.K. and Baines, K.J. (2017) 'A sputum gene expression signature predicts oral corticosteroid response in asthma', *The European respiratory journal,* 49(6), pp. 10.1183/13993003.00180-2017. Print 2017 Jun.

Bhakta, N.R. and Woodruff, P.G. (2011) 'Human asthma phenotypes: from the clinic, to cytokines, and back again', *Immunological reviews,* 242(1), pp. 220-232.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J. and Nusse, R. (1996) 'A new member of the frizzled family from Drosophila functions as a Wingless receptor', *Nature,* 382(6588), pp. 225-230.

Bhatt, P.M. and Malgor, R. (2014) 'Wnt5a: A player in the pathogenesis of atherosclerosis and other inflammatory disorders', *Atherosclerosis,* 237(1), pp. 155-162.

Borthwick, D.W., Shahbazian, M., Krantz, Q.T., Dorin, J.R. and Randell, S.H. (2001) 'Evidence for stem-cell niches in the tracheal epithelium', *American journal of respiratory cell and molecular biology,* 24(6), pp. 662-670.

Borthwick, L.A., Parker, S.M., Brougham, K.A., Johnson, G.E., Gorowiec, M.R., Ward, C., Lordan, J.L., Corris, P.A., Kirby, J.A. and Fisher, A.J. (2009) 'Epithelial to mesenchymal transition (EMT) and airway remodelling after human lung transplantation', *Thorax,* 64(9), pp. 770-777.

Boulet, L.P., Turcotte, H., Laviolette, M., Naud, F., Bernier, M.C., Martel, S. and Chakir, J. (2000) 'Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids', *American journal of respiratory and critical care medicine,* 162(4 Pt 1), pp. 1308-1313.

Bradding, P. and Brightling, C. (2007) 'Mast cell infiltration of airway smooth muscle in asthma', *Respiratory medicine,* 101(5), pp. 1045; author reply 1046-7.

Braman, S.S. (2006) 'The global burden of asthma', *Chest,* 130(1 Suppl), pp. 4S-12S.

Brightling, C.E., Ammit, A.J., Kaur, D., Black, J.L., Wardlaw, A.J., Hughes, J.M. and Bradding, P. (2005) 'The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle', *American journal of respiratory and critical care medicine,* 171(10), pp. 1103-1108.

Brightling, C.E., Bradding, P., Symon, F.A., Holgate, S.T., Wardlaw, A.J. and Pavord, I.D. (2002) 'Mast-cell infiltration of airway smooth muscle in asthma', *The New England journal of medicine,* 346(22), pp. 1699-1705.

British Thoracic Society Bronchoscopy Guidelines Committee, a Subcommittee of Standards of Care Committee of British Thoracic Society (2001) 'British Thoracic Society guidelines on diagnostic flexible bronchoscopy', *Thorax,* 56 Suppl 1, pp. i1-21.

Britten, K.M., Howarth, P.H. and Roche, W.R. (1993) 'Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies', *Biotechnic & histochemistry : official publication of the Biological Stain Commission,* 68(5), pp. 271-280.

Brown, H.M., Storey, G. and George, W.H. (1972a) 'Beclomethasone dipropionate: a new steroid aerosol for the treatment of allergic asthma', *British medical journal,* 1(5800), pp. 585-590.

Brown, H.M., Storey, G. and George, W.H. (1972b) 'Beclomethasone dipropionate: a new steroid aerosol for the treatment of allergic asthma', *British medical journal,* 1(5800), pp. 585-590.

Bucchieri, F., Puddicombe, S.M., Lordan, J.L., Richter, A., Buchanan, D., Wilson, S.J., Ward, J., Zummo, G., Howarth, P.H., Djukanovic, R., Holgate, S.T. and Davies, D.E. (2002) 'Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis', *American journal of respiratory cell and molecular biology,* 27(2), pp. 179-185.

Busse, W.W., Morgan, W.J., Gergen, P.J., Mitchell, H.E., Gern, J.E., Liu, A.H., Gruchalla, R.S., Kattan, M., Teach, S.J., Pongracic, J.A., Chmiel, J.F., Steinbach, S.F., Calatroni, A., Togias, A., Thompson, K.M., Szefler, S.J. and Sorkness, C.A. (2011) 'Randomized trial of omalizumab (anti-IgE) for asthma in inner-city children', *The New England journal of medicine,* 364(11), pp. 1005-1015.

Cakebread, J.A., Haitchi, H.M., Holloway, J.W., Powell, R.M., Keith, T., Davies, D.E. and Holgate, S.T. (2004) 'The role of ADAM33 in the pathogenesis of asthma', *Springer seminars in immunopathology,* 25(3-4), pp. 361-375.

Chakir, J., Shannon, J., Molet, S., Fukakusa, M., Elias, J., Laviolette, M., Boulet, L.P. and Hamid, Q. (2003) 'Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression', *The Journal of allergy and clinical immunology,* 111(6), pp. 1293-1298.

Chetta, A., Foresi, A., Del Donno, M., Bertorelli, G., Pesci, A. and Olivieri, D. (1997) 'Airways remodeling is a distinctive feature of asthma and is related to severity of disease', *Chest,* 111(4), pp. 852-857.

Chlumsky, J., Striz, I., Terl, M. and Vondracek, J. (2006) 'Strategy aimed at reduction of sputum eosinophils decreases exacerbation rate in patients with asthma', *The Journal of international medical research,* 34(2), pp. 129-139.

Cho, S.H., Seo, J.Y., Choi, D.C., Yoon, H.J., Cho, Y.J., Min, K.U., Lee, G.K., Seo, J.W. and Kim, Y.Y. (1996) 'Pathological changes according to the severity of asthma', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 26(10), pp. 1210-1219.

Choy, D.F., Hart, K.M., Borthwick, L.A., Shikotra, A., Nagarkar, D.R., Siddiqui, S., Jia, G., Ohri, C.M., Doran, E., Vannella, K.M., Butler, C.A., Hargadon, B., Sciurba, J.C., Gieseck, R.L., Thompson, R.W., White, S., Abbas, A.R., Jackman, J., Wu, L.C., Egen, J.G., Heaney, L.G., Ramalingam, T.R., Arron, J.R., Wynn, T.A. and Bradding, P. (2015) 'TH2 and TH17 inflammatory pathways are reciprocally regulated in asthma', *Science translational medicine,* 7(301), pp. 301ra129.

Choy, D.F., Modrek, B., Abbas, A.R., Kummerfeld, S., Clark, H.F., Wu, L.C., Fedorowicz, G., Modrusan, Z., Fahy, J.V., Woodruff, P.G. and Arron, J.R. (2011a) 'Gene expression patterns of Th2 inflammation and intercellular communication in asthmatic airways', *Journal of immunology (Baltimore, Md.: 1950),* 186(3), pp. 1861-1869.

Choy, D.F., Modrek, B., Abbas, A.R., Kummerfeld, S., Clark, H.F., Wu, L.C., Fedorowicz, G., Modrusan, Z., Fahy, J.V., Woodruff, P.G. and Arron, J.R. (2011b) 'Gene expression patterns of Th2 inflammation and intercellular communication in asthmatic airways', *Journal of immunology (Baltimore, Md.: 1950),* 186(3), pp. 1861-1869.

Chu, H.W., Halliday, J.L., Martin, R.J., Leung, D.Y., Szefler, S.J. and Wenzel, S.E. (1998) 'Collagen deposition in large airways may not differentiate severe asthma from milder forms of the disease', *American journal of respiratory and critical care medicine,* 158(6), pp. 1936-1944.

Chung, K.F. and Barnes, P.J. (1999) 'Cytokines in asthma', *Thorax,* 54(9), pp. 825-857.

Clark, C.C., Cohen, I., Eichstetter, I., Cannizzaro, L.A., McPherson, J.D., Wasmuth, J.J. and Iozzo, R.V. (1993) 'Molecular cloning of the human proto-oncogene Wnt-5A and mapping of the gene (WNT5A) to chromosome 3p14-p21', *Genomics,* 18(2), pp. 249-260.

Clark, T.J. (1972) 'Effect of beclomethasone dipropionate delivered by aerosol in patients with asthma', *Lancet,* 1(7765), pp. 1361-1364.

Copeland, N.G. and Jenkins, N.A. (2010) 'Harnessing transposons for cancer gene discovery', *Nature reviews.Cancer,* 10(10), pp. 696-706.

Corrigan, C.J. and Loke, T.K. (2007) 'Clinical and molecular aspects of glucocorticoid resistant asthma', *Therapeutics and clinical risk management,* 3(5), pp. 771-787.

Corris, P.A. and Dark, J.H. (1993) 'Aetiology of asthma: lessons from lung transplantation', *Lancet,* 341(8857), pp. 1369-1371.

Cosmi, L., Liotta, F., Maggi, E., Romagnani, S. and Annunziato, F. (2011) 'Th17 cells: new players in asthma pathogenesis', *Allergy,* 66(8), pp. 989-998.

Coster, A.D., Thorne, C.A., Wu, L.F. and Altschuler, S.J. (2017) 'Examining Crosstalk among Transforming Growth Factor beta, Bone Morphogenetic Protein, and Wnt Pathways', *The Journal of biological chemistry,* 292(1), pp. 244-250.

Crosby, L.M. and Waters, C.M. (2010) 'Epithelial repair mechanisms in the lung', *American journal of physiology.Lung cellular and molecular physiology,* 298(6), pp. L715-31.

Dao, D.Y., Yang, X., Chen, D., Zuscik, M. and O'Keefe, R.J. (2007) 'Axin1 and Axin2 are regulated by TGF- and mediate cross-talk between TGF- and Wnt signaling pathways', *Annals of the New York Academy of Sciences,* 1116, pp. 82-99.

David, M.D., Canti, C. and Herreros, J. (2010) 'Wnt-3a and Wnt-3 differently stimulate proliferation and neurogenesis of spinal neural precursors and promote neurite outgrowth by canonical signaling', *Journal of neuroscience research,* 88(14), pp. 3011-3023.

Davies, A. and Moores, C. (2010) *The respiratory system: basic science and clinical conditions.* 2nd edn. Edinburgh: Churchill Livingstone.

Davis, E.K., Zou, Y. and Ghosh, A. (2008) 'Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation', *Neural development,* 3, pp. 32-8104-3-32.

de Kluijver, J., Schrumpf, J.A., Evertse, C.E., Sont, J.K., Roughley, P.J., Rabe, K.F., Hiemstra, P.S., Mauad, T. and Sterk, P.J. (2005) 'Bronchial matrix and inflammation respond to inhaled steroids despite ongoing allergen exposure in asthma', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 35(10), pp. 1361-1369.

Dekkers, B.G., Maarsingh, H., Meurs, H. and Gosens, R. (2009) 'Airway structural components drive airway smooth muscle remodeling in

asthma', *Proceedings of the American Thoracic Society,* 6(8), pp. 683-692.

Demayo, F., Minoo, P., Plopper, C.G., Schuger, L., Shannon, J. and Torday, J.S. (2002) 'Mesenchymal-epithelial interactions in lung development and repair: are modeling and remodeling the same process?', *American journal of physiology.Lung cellular and molecular physiology,* 283(3), pp. L510-7.

Derynck, R. and Zhang, Y.E. (2003) 'Smad-dependent and Smad-independent pathways in TGF-beta family signalling', *Nature,* 425(6958), pp. 577-584.

Dietz, K., de Los Reyes Jimenez, M., Gollwitzer, E.S., Chaker, A.M., Zissler, U.M., Radmark, O.P., Baarsma, H.A., Konigshoff, M., Schmidt-Weber, C.B., Marsland, B.J. and Esser-von Bieren, J. (2017) 'Age dictates a steroid-resistant cascade of Wnt5a, transglutaminase 2, and leukotrienes in inflamed airways', *The Journal of allergy and clinical immunology,* 139(4), pp. 1343-1354.e6.

Dissanayake, S.K., Wade, M., Johnson, C.E., O'Connell, M.P., Leotlela, P.D., French, A.D., Shah, K.V., Hewitt, K.J., Rosenthal, D.T., Indig, F.E., Jiang, Y., Nickoloff, B.J., Taub, D.D., Trent, J.M., Moon, R.T., Bittner, M. and Weeraratna, A.T. (2007) 'The Wnt5A/protein kinase C pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors and initiation of an epithelial to mesenchymal transition', *The Journal of biological chemistry,* 282(23), pp. 17259-17271.

Doeing, D.C. and Solway, J. (2013) 'Airway smooth muscle in the pathophysiology and treatment of asthma', *Journal of applied physiology (Bethesda, Md.: 1985),* 114(7), pp. 834-843.

Doerner, A.M. and Zuraw, B.L. (2009) 'TGF-beta1 induced epithelial to mesenchymal transition (EMT) in human bronchial epithelial cells is enhanced by IL-1beta but not abrogated by corticosteroids', *Respiratory research,* 10, pp. 100-9921-10-100.

Duffy, D.L., Martin, N.G., Battistutta, D., Hopper, J.L. and Mathews, J.D. (1990) 'Genetics of asthma and hay fever in Australian twins', *The American Review of Respiratory Disease,* 142(6 Pt 1), pp. 1351-1358.

Duvernelle, C., Freund, V. and Frossard, N. (2003) 'Transforming growth factor-beta and its role in asthma', *Pulmonary pharmacology & therapeutics,* 16(4), pp. 181-196.

Elias, J.A., Zhu, Z., Chupp, G. and Homer, R.J. (1999) 'Airway remodeling in asthma', *The Journal of clinical investigation,* 104(8), pp. 1001-1006.

Ellul-Micallef, R., Borthwick, R.C. and McHardy, G.J. (1974) 'The time-course of response to prednisolone in chronic bronchial asthma', *Clinical science and molecular medicine,* 47(2), pp. 105-117.

Endo, M., Nishita, M., Fujii, M. and Minami, Y. (2015) 'Insight into the role of wnt5a-induced signaling in normal and cancer cells', *International review of cell and molecular biology,* 314, pp. 117-148.

Fahy, J.V. (2015) 'Type 2 inflammation in asthma--present in most, absent in many', *Nature reviews.Immunology,* 15(1), pp. 57-65.

Flood-Page, P., Menzies-Gow, A., Phipps, S., Ying, S., Wangoo, A., Ludwig, M.S., Barnes, N., Robinson, D. and Kay, A.B. (2003a) 'Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics', *The Journal of clinical investigation,* 112(7), pp. 1029-1036.

Flood-Page, P., Menzies-Gow, A., Phipps, S., Ying, S., Wangoo, A., Ludwig, M.S., Barnes, N., Robinson, D. and Kay, A.B. (2003b) 'Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics', *The Journal of clinical investigation,* 112(7), pp. 1029-1036.

Gao, C. and Chen, Y.G. (2010) 'Dishevelled: The hub of Wnt signaling', *Cellular signalling,* 22(5), pp. 717-727.

Gibson, P.G. (2009) 'Inflammatory phenotypes in adult asthma: clinical applications', *The clinical respiratory journal,* 3(4), pp. 198-206.

Ghosh, M.C., Collins, G.D., Vandanmagsar, B., Patel, K., Brill, M., Carter, A., Lustig, A., Becker, K.G., Wood, W.W.,3rd, Emeche, C.D., French, A.D., O'Connell, M.P., Xu, M., Weeraratna, A.T. and Taub, D.D. (2009) 'Activation of Wnt5A signaling is required for CXC chemokine ligand 12-mediated T-cell migration', *Blood,* 114(7), pp. 1366-1373.

Ghosh, M.C., Gorantla, V., Makena, P.S., Luellen, C., Sinclair, S.E., Schwingshackl, A. and Waters, C.M. (2013) 'Insulin-like growth factor-I stimulates differentiation of ATII cells to ATI-like cells through activation of Wnt5a', *American journal of physiology.Lung cellular and molecular physiology,* 305(3), pp. L222-8.

Global Initiative for Asthma, GINA 2018
Global Strategy for Asthma Management and Prevention, 2018 , GINA, www.ginasthma.org.

Global Initiative for Asthma, GINA (2004) *Global Burden of Asthma.* Available at: http://www.ginasthma.org/local/uploads/files/GINABurdenReport_1.pdf.

Gon, H., Fumoto, K., Ku, Y., Matsumoto, S. and Kikuchi, A. (2013) 'Wnt5a signaling promotes apical and basolateral polarization of single epithelial cells', *Molecular biology of the cell,* 24(23), pp. 3764-3774.

Green, R.H., Brightling, C.E., McKenna, S., Hargadon, B., Parker, D., Bradding, P., Wardlaw, A.J. and Pavord, I.D. (2002) 'Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial', *Lancet (London, England),* 360(9347), pp. 1715-1721.

Grumolato, L., Liu, G., Mong, P., Mudbhary, R., Biswas, R., Arroyave, R., Vijayakumar, S., Economides, A.N. and Aaronson, S.A. (2010) 'Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors', *Genes & development,* 24(22), pp. 2517-2530.

Hackett, N.R., Shaykhiev, R., Walters, M.S., Wang, R., Zwick, R.K., Ferris, B., Witover, B., Salit, J. and Crystal, R.G. (2011) 'The human airway epithelial basal cell transcriptome', *PloS one,* 6(5), pp. e18378.

Hackett, T.L., Warner, S.M., Stefanowicz, D., Shaheen, F., Pechkovsky, D.V., Murray, L.A., Argentieri, R., Kicic, A., Stick, S.M., Bai, T.R. and Knight, D.A. (2009) 'Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1', *American journal of respiratory and critical care medicine,* 180(2), pp. 122-133.

Haldar, P., Brightling, C.E., Singapuri, A., Hargadon, B., Gupta, S., Monteiro, W., Bradding, P., Green, R.H., Wardlaw, A.J., Ortega, H. and Pavord, I.D. (2014) 'Outcomes after cessation of mepolizumab therapy in severe eosinophilic asthma: a 12-month follow-up analysis', *The Journal of allergy and clinical immunology,* 133(3), pp. 921-923.

Haldar, P., Pavord, I.D., Shaw, D.E., Berry, M.A., Thomas, M., Brightling, C.E., Wardlaw, A.J. and Green, R.H. (2008) 'Cluster analysis and clinical asthma phenotypes', *American journal of respiratory and critical care medicine,* 178(3), pp. 218-224.

Halwani, R., Al-Muhsen, S., Al-Jahdali, H. and Hamid, Q. (2011) 'Role of transforming growth factor-beta in airway remodeling in asthma', *American journal of respiratory cell and molecular biology,* 44(2), pp. 127-133.

Halwani, R., Sultana, A., Vazquez-Tello, A., Jamhawi, A., Al-Masri, A.A. and Al-Muhsen, S. (2017) 'Th-17 regulatory cytokines IL-21, IL-23, and IL-6 enhance neutrophil production of IL-17 cytokines during asthma', *The Journal of asthma : official journal of the Association for the Care of Asthma,* , pp. 1-12.

Hamid, Q., Azzawi, M., Ying, S., Moqbel, R., Wardlaw, A.J., Corrigan, C.J., Bradley, B., Durham, S.R., Collins, J.V. and Jeffery, P.K. (1991) 'Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma', *The Journal of clinical investigation,* 87(5), pp. 1541-1546.

Hamid, Q., Tulic', M.K., Liu, M.C. and Moqbel, R. (2003) 'Inflammatory cells in asthma: mechanisms and implications for therapy', *The Journal of allergy and clinical immunology,* 111(1 Suppl), pp. S5-S12; discussion S12-7.

Han, T., Cao, Y., Tian, L., Feng, X. and Wang, Y. (2015) 'Enhanced expressions of Wnt5a/JNK signaling pathway-related molecules in the lung tissues of asthmatic rats', *Xi bao yu fen zi mian yi xue za zhi = Chinese journal of cellular and molecular immunology,* 31(3), pp. 325-7, 332.

Hansen, C.A., Barnett, A.G. and Pritchard, G. (2008) 'The effect of ambient air pollution during early pregnancy on fetal ultrasonic measurements during mid-pregnancy', *Environmental health perspectives,* 116(3), pp. 362-369.

Hargreave, F.E. and Nair, P. (2009) 'The definition and diagnosis of asthma', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 39(11), pp. 1652-1658.

Henderson, J., Sherriff, A., Farrow, A. and Ayres, J.G. (2008) 'Household chemicals, persistent wheezing and lung function: effect modification by atopy?', *The European respiratory journal,* 31(3), pp. 547-554.

Hilty, M., Burke, C., Pedro, H., Cardenas, P., Bush, A., Bossley, C., Davies, J., Ervine, A., Poulter, L., Pachter, L., Moffatt, M.F. and Cookson, W.O. (2010) 'Disordered microbial communities in asthmatic airways', *PloS one,* 5(1), pp. e8578.

Hislop, A., Muir, D.C., Jacobsen, M., Simon, G. and Reid, L. (1972) 'Postnatal growth and function of the pre-acinar airways', *Thorax,* 27(3), pp. 265-274.

Ho, H.Y., Susman, M.W., Bikoff, J.B., Ryu, Y.K., Jonas, A.M., Hu, L., Kuruvilla, R. and Greenberg, M.E. (2012) 'Wnt5a-Ror-Dishevelled signaling constitutes a core developmental pathway that controls tissue morphogenesis', *Proceedings of the National Academy of Sciences of the United States of America,* 109(11), pp. 4044-4051.

Hocevar, B.A., Mou, F., Rennolds, J.L., Morris, S.M., Cooper, J.A. and Howe, P.H. (2003) 'Regulation of the Wnt signaling pathway by disabled-2 (Dab2)', *The EMBO journal,* 22(12), pp. 3084-3094.

Hogan, S.P., Rosenberg, H.F., Moqbel, R., Phipps, S., Foster, P.S., Lacy, P., Kay, A.B. and Rothenberg, M.E. (2008) 'Eosinophils: biological properties and role in health and disease', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 38(5), pp. 709-750.

Holgate, S.T. (2010) 'A brief history of asthma and its mechanisms to modern concepts of disease pathogenesis', *Allergy, asthma & immunology research,* 2(3), pp. 165-171.

Holgate, S.T., Holloway, J., Wilson, S., Bucchieri, F., Puddicombe, S. and Davies, D.E. (2004) 'Epithelial-mesenchymal communication in the pathogenesis of chronic asthma', *Proceedings of the American Thoracic Society,* 1(2), pp. 93-98.

Holgate, S.T. and Polosa, R. (2006) 'The mechanisms, diagnosis, and management of severe asthma in adults', *Lancet,* 368(9537), pp. 780-793.

Holgate, S.T., Wilson, J.R. and Howarth, P.H. (1992) 'New insights into airway inflammation by endobronchial biopsy', *The American Review of Respiratory Disease,* 145(2 Pt 2), pp. S2-6.

Hoshino, M., Nakamura, Y., Sim, J., Shimojo, J. and Isogai, S. (1998) 'Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation', *The Journal of allergy and clinical immunology,* 102(5), pp. 783-788.

Hoshino, M., Nakamura, Y. and Sim, J.J. (1998) 'Expression of growth factors and remodelling of the airway wall in bronchial asthma', *Thorax,* 53(1), pp. 21-27.

Howell, J.E. and McAnulty, R.J. (2006) 'TGF-beta: its role in asthma and therapeutic potential', *Current Drug Targets,* 7(5), pp. 547-565.

Huang, J., Olivenstein, R., Taha, R., Hamid, Q. and Ludwig, M. (1999) 'Enhanced proteoglycan deposition in the airway wall of atopic asthmatics', *American journal of respiratory and critical care medicine,* 160(2), pp. 725-729.

Humbert, M., Durham, S.R., Ying, S., Kimmitt, P., Barkans, J., Assoufi, B., Pfister, R., Menz, G., Robinson, D.S., Kay, A.B. and Corrigan, C.J. (1996) 'IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity', *American journal of respiratory and critical care medicine,* 154(5), pp. 1497-1504.

James, A.L., Bai, T.R., Mauad, T., Abramson, M.J., Dolhnikoff, M., McKay, K.O., Maxwell, P.S., Elliot, J.G. and Green, F.H. (2009) 'Airway smooth

muscle thickness in asthma is related to severity but not duration of asthma', *The European respiratory journal,* 34(5), pp. 1040-1045.

James, A.L., Elliot, J.G., Jones, R.L., Carroll, M.L., Mauad, T., Bai, T.R., Abramson, M.J., McKay, K.O. and Green, F.H. (2012) 'Airway smooth muscle hypertrophy and hyperplasia in asthma', *American journal of respiratory and critical care medicine,* 185(10), pp. 1058-1064.

James, A.L., Maxwell, P.S., Pearce-Pinto, G., Elliot, J.G. and Carroll, N.G. (2002) 'The relationship of reticular basement membrane thickness to airway wall remodeling in asthma', *American journal of respiratory and critical care medicine,* 166(12 Pt 1), pp. 1590-1595.

Januskevicius, A., Vaitkiene, S., Gosens, R., Janulaityte, I., Hoppenot, D., Sakalauskas, R. and Malakauskas, K. (2016) 'Eosinophils enhance WNT-5a and TGF-beta1 genes expression in airway smooth muscle cells and promote their proliferation by increased extracellular matrix proteins production in asthma', *BMC pulmonary medicine,* 16(1), pp. 94-016-0254-9.

Jayaram, L., Pizzichini, M.M., Cook, R.J., Boulet, L.P., Lemiere, C., Pizzichini, E., Cartier, A., Hussack, P., Goldsmith, C.H., Laviolette, M., Parameswaran, K. and Hargreave, F.E. (2006) 'Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations', *The European respiratory journal,* 27(3), pp. 483-494.

Jeffery, P.K., Godfrey, R.W., Adelroth, E., Nelson, F., Rogers, A. and Johansson, S.A. (1992a) 'Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study', *The American Review of Respiratory Disease,* 145(4 Pt 1), pp. 890-899.

Jeffery, P.K., Godfrey, R.W., Adelroth, E., Nelson, F., Rogers, A. and Johansson, S.A. (1992b) 'Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study', *The American Review of Respiratory Disease,* 145(4 Pt 1), pp. 890-899.

Jenei, V., Sherwood, V., Howlin, J., Linnskog, R., Safholm, A., Axelsson, L. and Andersson, T. (2009a) 'A t-butyloxycarbonyl-modified Wnt5a-derived hexapeptide functions as a potent antagonist of Wnt5a-dependent melanoma cell invasion', *Proceedings of the National Academy of Sciences of the United States of America,* 106(46), pp. 19473-19478.

Jenei, V., Sherwood, V., Howlin, J., Linnskog, R., Safholm, A., Axelsson, L. and Andersson, T. (2009b) 'A t-butyloxycarbonyl-modified Wnt5a-derived hexapeptide functions as a potent antagonist of Wnt5a-dependent melanoma cell invasion', *Proceedings of the National*

Academy of Sciences of the United States of America, 106(46), pp. 19473-19478.

Jetten, A.M., Shirley, J.E. and Stoner, G. (1986) 'Regulation of proliferation and differentiation of respiratory tract epithelial cells by TGF beta', *Experimental cell research,* 167(2), pp. 539-549.

Jinnin, M., Ihn, H. and Tamaki, K. (2006) 'Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression', *Molecular pharmacology,* 69(2), pp. 597-607.

Jung, Y.S., Lee, H.Y., Kim, S.D., Park, J.S., Kim, J.K., Suh, P.G. and Bae, Y.S. (2013a) 'Wnt5a stimulates chemotactic migration and chemokine production in human neutrophils', *Experimental & molecular medicine,* 45, pp. e27.

Jung, Y.S., Lee, H.Y., Kim, S.D., Park, J.S., Kim, J.K., Suh, P.G. and Bae, Y.S. (2013b) 'Wnt5a stimulates chemotactic migration and chemokine production in human neutrophils', *Experimental & molecular medicine,* 45, pp. e27.

Kariyawasam, H.H., Aizen, M., Barkans, J., Robinson, D.S. and Kay, A.B. (2007) 'Remodeling and airway hyperresponsiveness but not cellular inflammation persist after allergen challenge in asthma', *American journal of respiratory and critical care medicine,* 175(9), pp. 896-904.

Katoh, M. (2001) 'Molecular cloning and characterization of human WNT3', *International journal of oncology,* 19(5), pp. 977-982.

Katoh, M. and Katoh, M. (2009a) 'Transcriptional mechanisms of WNT5A based on NF-kappaB, Hedgehog, TGFbeta, and Notch signaling cascades', *International journal of molecular medicine,* 23(6), pp. 763-769.

Katoh, M. and Katoh, M. (2009b) 'Transcriptional mechanisms of WNT5A based on NF-kappaB, Hedgehog, TGFbeta, and Notch signaling cascades', *International journal of molecular medicine,* 23(6), pp. 763-769.

Katoh, M. and Katoh, M. (2007) 'STAT3-induced WNT5A signaling loop in embryonic stem cells, adult normal tissues, chronic persistent inflammation, rheumatoid arthritis and cancer (Review)', *International journal of molecular medicine,* 19(2), pp. 273-278.

Kaur, D., Gomez, E., Doe, C., Berair, R., Woodman, L., Saunders, R., Hollins, F., Rose, F.R., Amrani, Y., May, R., Kearley, J., Humbles, A., Cohen, E.S. and Brightling, C.E. (2015) 'IL-33 drives airway hyper-

responsiveness through IL-13-mediated mast cell: airway smooth muscle crosstalk', *Allergy,* 70(5), pp. 556-567.

Kaur, D., Hollins, F., Saunders, R., Woodman, L., Sutcliffe, A., Cruse, G., Bradding, P. and Brightling, C. (2010) 'Airway smooth muscle proliferation and survival is not modulated by mast cells', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 40(2), pp. 279-288.

Kaur, D., Saunders, R., Berger, P., Siddiqui, S., Woodman, L., Wardlaw, A., Bradding, P. and Brightling, C.E. (2006) 'Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma', *American journal of respiratory and critical care medicine,* 174(11), pp. 1179-1188.

Kawano, Y. and Kypta, R. (2003) 'Secreted antagonists of the Wnt signalling pathway', *Journal of cell science,* 116(Pt 13), pp. 2627-2634.

KEENEY, E.L. (1964) 'The History of Asthma from Hippocrates to Meltzer', *The Journal of allergy,* 35, pp. 215-226.

Kenakin, T. (2011) 'Functional selectivity and biased receptor signaling', *The Journal of pharmacology and experimental therapeutics,* 336(2), pp. 296-302.

Kicic, A., Hallstrand, T.S., Sutanto, E.N., Stevens, P.T., Kobor, M.S., Taplin, C., Pare, P.D., Beyer, R.P., Stick, S.M. and Knight, D.A. (2010) 'Decreased fibronectin production significantly contributes to dysregulated repair of asthmatic epithelium', *American journal of respiratory and critical care medicine,* 181(9), pp. 889-898.

Kicic, A., Sutanto, E.N., Stevens, P.T., Knight, D.A. and Stick, S.M. (2006) 'Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma', *American journal of respiratory and critical care medicine,* 174(10), pp. 1110-1118.

Kikuchi, A., Yamamoto, H., Sato, A. and Matsumoto, S. (2012) 'Wnt5a: its signalling, functions and implication in diseases', *Acta physiologica (Oxford, England),* 204(1), pp. 17-33.

Klaus, A. and Birchmeier, W. (2008a) 'Wnt signalling and its impact on development and cancer', *Nature reviews.Cancer,* 8(5), pp. 387-398.

Klaus, A. and Birchmeier, W. (2008b) 'Wnt signalling and its impact on development and cancer', *Nature reviews.Cancer,* 8(5), pp. 387-398.

Konigshoff, M. and Eickelberg, O. (2010) 'WNT signaling in lung disease: a failure or a regeneration signal?', *American journal of respiratory cell and molecular biology,* 42(1), pp. 21-31.

Koopmans, T., Kumawat, K., Halayko, A.J. and Gosens, R. (2016) 'Regulation of actin dynamics by WNT-5A: implications for human airway smooth muscle contraction', *Scientific reports,* 6, pp. 30676.

Korn, T., Bettelli, E., Oukka, M. and Kuchroo, V.K. (2009) 'IL-17 and Th17 Cells', *Annual Review of Immunology,* 27, pp. 485-517.

Kumawat, K. and Gosens, R. (2016) 'WNT-5A: signaling and functions in health and disease', *Cellular and molecular life sciences : CMLS,* 73(3), pp. 567-587.

Kumawat, K., Menzen, M.H., Bos, I.S., Baarsma, H.A., Borger, P., Roth, M., Tamm, M., Halayko, A.J., Simoons, M., Prins, A., Postma, D.S., Schmidt, M. and Gosens, R. (2013) 'Noncanonical WNT-5A signaling regulates TGF-beta-induced extracellular matrix production by airway smooth muscle cells', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 27(4), pp. 1631-1643.

Kumawat, K., Menzen, M.H., Slegtenhorst, R.M., Halayko, A.J., Schmidt, M. and Gosens, R. (2014) 'TGF-beta-activated kinase 1 (TAK1) signaling regulates TGF-beta-induced WNT-5A expression in airway smooth muscle cells via Sp1 and beta-catenin', *PloS one,* 9(4), pp. e94801.

Laitinen, L.A., Heino, M., Laitinen, A., Kava, T. and Haahtela, T. (1985) 'Damage of the airway epithelium and bronchial reactivity in patients with asthma', *The American Review of Respiratory Disease,* 131(4), pp. 599-606.

Lands, A.M., Arnold, A., McAuliff, J.P., Luduena, F.P. and Brown, T.G.,Jr (1967) 'Differentiation of receptor systems activated by sympathomimetic amines', *Nature,* 214(5088), pp. 597-598.

Latzin, P., Roosli, M., Huss, A., Kuehni, C.E. and Frey, U. (2009) 'Air pollution during pregnancy and lung function in newborns: a birth cohort study', *The European respiratory journal,* 33(3), pp. 594-603.

Leandersson, K., Riesbeck, K. and Andersson, T. (2006) 'Wnt-5a mRNA translation is suppressed by the Elav-like protein HuR in human breast epithelial cells', *Nucleic acids research,* 34(14), pp. 3988-3999.

Levy, M.L. (2015) 'The national review of asthma deaths: what did we learn and what needs to change?', *Breathe (Sheffield, England),* 11(1), pp. 14-24.

Levy, M., Thomas, M., Small, I., Pearce, L., Pinnock, H. and Stephenson, P. (2009) 'Summary of the 2008 BTS/SIGN British Guideline on the management of asthma.', *Primary care respiratory journal: journal of the General Practice Airways Group,* 18, pp. S1-16.

Li, C., Xiao, J., Hormi, K., Borok, Z. and Minoo, P. (2002) 'Wnt5a participates in distal lung morphogenesis', *Developmental biology,* 248(1), pp. 68-81.

Lloyd, C.M. and Hessel, E.M. (2010) 'Functions of T cells in asthma: more than just T(H)2 cells', *Nature reviews.Immunology,* 10(12), pp. 838-848.

Loxham, M., Davies, D.E. and Blume, C. (2014) 'Epithelial function and dysfunction in asthma', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 44(11), pp. 1299-1313.

Lund, S., Walford, H.H. and Doherty, T.A. (2013) 'Type 2 Innate Lymphoid Cells in Allergic Disease', *Current immunology reviews,* 9(4), pp. 214-221.

LYONS, M.J. and MOORE, D.H. (1962) 'Purification of the mouse mammary tumour virus', *Nature,* 194, pp. 1141-1142.

Ma, J., Wang, R., Fang, X., Ding, Y. and Sun, Z. (2011) 'Critical role of TCF-1 in repression of the IL-17 gene', *PloS one,* 6(9), pp. e24768.

Macdowell, A.L. and Peters, S.P. (2007) 'Neutrophils in asthma', *Current allergy and asthma reports,* 7(6), pp. 464-468.

Mahesh, P.A. (2013) 'Unravelling the role of ADAM 33 in asthma', *The Indian journal of medical research,* 137(3), pp. 447-450.

Magnan, A., Frachon, I., Rain, B., Peuchmaur, M., Monti, G., Lenot, B., Fattal, M., Simonneau, G., Galanaud, P. and Emilie, D. (1994) 'Transforming growth factor beta in normal human lung: preferential location in bronchial epithelial cells', *Thorax,* 49(8), pp. 789-792.

Magnan, A., Retornaz, F., Tsicopoulos, A., Brisse, J., Van Pee, D., Gosset, P., Chamlian, A., Tonnel, A.B. and Vervloet, D. (1997) 'Altered compartmentalization of transforming growth factor-beta in asthmatic airways', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 27(4), pp. 389-395.

Marchetti, B. and Pluchino, S. (2013) 'Wnt your brain be inflamed? Yes, it Wnt!', *Trends in molecular medicine,* 19(3), pp. 144-156.

Masoli, M., Fabian, D., Holt, S., Beasley, R. and Global Initiative for Asthma (GINA) Program (2004) 'The global burden of asthma: executive summary of the GINA Dissemination Committee report', *Allergy,* 59(5), pp. 469-478.

Massague, J. (2000) 'How cells read TGF-beta signals', *Nature reviews.Molecular cell biology,* 1(3), pp. 169-178.

McGrath, K.W., Icitovic, N., Boushey, H.A., Lazarus, S.C., Sutherland, E.R., Chinchilli, V.M., Fahy, J.V. and Asthma Clinical Research Network of the National Heart, Lung, and Blood Institute (2012) 'A large subgroup of mild-to-moderate asthma is persistently noneosinophilic', *American journal of respiratory and critical care medicine,* 185(6), pp. 612-619.

McKenzie, A.N. (2014) 'Type-2 innate lymphoid cells in asthma and allergy', *Annals of the American Thoracic Society,* 11 Suppl 5, pp. S263-70.

Mead, J., Takishima, T. and Leith, D. (1970) 'Stress distribution in lungs: a model of pulmonary elasticity', *Journal of applied physiology,* 28(5), pp. 596-608.

Meetoo, D. (2008) 'Chronic diseases: the silent global epidemic', *British journal of nursing (Mark Allen Publishing),* 17(21), pp. 1320-1325.

Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M. and Simonet, W.S. (1998) 'Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless', *Genes & development,* 12(20), pp. 3156-3161.

Minami, Y., Oishi, I., Endo, M. and Nishita, M. (2010) 'Ror-family receptor tyrosine kinases in noncanonical Wnt signaling: their implications in developmental morphogenesis and human diseases', *Developmental dynamics : an official publication of the American Association of Anatomists,* 239(1), pp. 1-15.

Minshall, E., Chakir, J., Laviolette, M., Molet, S., Zhu, Z., Olivenstein, R., Elias, J.A. and Hamid, Q. (2000) 'IL-11 expression is increased in severe asthma: association with epithelial cells and eosinophils', *The Journal of allergy and clinical immunology,* 105(2 Pt 1), pp. 232-238.

Miyoshi, H., Ajima, R., Luo, C.T., Yamaguchi, T.P. and Stappenbeck, T.S. (2012) 'Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury', *Science (New York, N.Y.),* 338(6103), pp. 108-113.

Modena, B.D., Tedrow, J.R., Milosevic, J., Bleecker, E.R., Meyers, D.A., Wu, W., Bar-Joseph, Z., Erzurum, S.C., Gaston, B.M., Busse, W.W., Jarjour, N.N., Kaminski, N. and Wenzel, S.E. (2014) 'Gene expression in relation to exhaled nitric oxide identifies novel asthma phenotypes with unique biomolecular pathways', *American journal of respiratory and critical care medicine,* 190(12), pp. 1363-1372.

Moffatt, M.F., Gut, I.G., Demenais, F., Strachan, D.P., Bouzigon, E., Heath, S., von Mutius, E., Farrall, M., Lathrop, M., Cookson, W.O. and GABRIEL Consortium (2010) 'A large-scale, consortium-based genomewide association study of asthma', *The New England journal of medicine,* 363(13), pp. 1211-1221.

Moffatt, M.F., Kabesch, M., Liang, L., Dixon, A.L., Strachan, D., Heath, S., Depner, M., von Berg, A., Bufe, A., Rietschel, E., Heinzmann, A., Simma, B., Frischer, T., Willis-Owen, S.A., Wong, K.C., Illig, T., Vogelberg, C., Weiland, S.K., von Mutius, E., Abecasis, G.R., Farrall, M., Gut, I.G., Lathrop, G.M. and Cookson, W.O. (2007) 'Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma', *Nature,* 448(7152), pp. 470-473.

Molet, S., Hamid, Q., Davoine, F., Nutku, E., Taha, R., Page, N., Olivenstein, R., Elias, J. and Chakir, J. (2001) 'IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines', *The Journal of allergy and clinical immunology,* 108(3), pp. 430-438.

Moore, W.C., Meyers, D.A., Wenzel, S.E., Teague, W.G., Li, H., Li, X., D'Agostino, R.,Jr, Castro, M., Curran-Everett, D., Fitzpatrick, A.M., Gaston, B., Jarjour, N.N., Sorkness, R., Calhoun, W.J., Chung, K.F., Comhair, S.A., Dweik, R.A., Israel, E., Peters, S.P., Busse, W.W., Erzurum, S.C., Bleecker, E.R. and National Heart, Lung, and Blood Institute's Severe Asthma Research Program (2010) 'Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program', *American journal of respiratory and critical care medicine,* 181(4), pp. 315-323.

Morrisey, E.E. and Hogan, B.L. (2010) 'Preparing for the first breath: genetic and cellular mechanisms in lung development', *Developmental cell,* 18(1), pp. 8-23.

Mucenski, M.L., Wert, S.E., Nation, J.M., Loudy, D.E., Huelsken, J., Birchmeier, W., Morrisey, E.E. and Whitsett, J.A. (2003) 'beta-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis', *The Journal of biological chemistry,* 278(41), pp. 40231-40238.

Mukherjee, M., Stoddart, A., Gupta, R.P., Nwaru, B.I., Farr, A., Heaven, M., Fitzsimmons, D., Bandyopadhyay, A., Aftab, C., Simpson, C.R., Lyons, R.A., Fischbacher, C., Dibben, C., Shields, M.D., Phillips, C.J., Strachan, D.P., Davies, G.A., McKinstry, B. and Sheikh, A. (2016) 'The epidemiology, healthcare and societal burden and costs of asthma in the UK and its member nations: analyses of standalone and linked national databases', *BMC medicine,* 14(1), pp. 113-016-0657-8.

Murray, C.J., Vos, T., Lozano, R., Naghavi, M., Flaxman, A.D., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J.A., Abdalla, S., Aboyans, V., Abraham, J., Ackerman, I., Aggarwal, R., Ahn, S.Y., Ali, M.K., Alvarado, M., Anderson, H.R., Anderson, L.M., Andrews, K.G., Atkinson, C., Baddour, L.M., Bahalim, A.N., Barker-Collo, S., Barrero, L.H., Bartels, D.H., Basanez, M.G., Baxter, A., Bell, M.L., Benjamin, E.J., Bennett, D., Bernabe, E., Bhalla, K., Bhandari, B., Bikbov, B., Bin Abdulhak, A.,

Birbeck, G., Black, J.A., Blencowe, H., Blore, J.D., Blyth, F., Bolliger, I., Bonaventure, A., Boufous, S., Bourne, R., Boussinesq, M., Braithwaite, T., Brayne, C., Bridgett, L., Brooker, S., Brooks, P., Brugha, T.S., Bryan-Hancock, C., Bucello, C., Buchbinder, R., Buckle, G., Budke, C.M., Burch, M., Burney, P., Burstein, R., Calabria, B., Campbell, B., Canter, C.E., Carabin, H., Carapetis, J., Carmona, L., Cella, C., Charlson, F., Chen, H., Cheng, A.T., Chou, D., Chugh, S.S., Coffeng, L.E., Colan, S.D., Colquhoun, S., Colson, K.E., Condon, J., Connor, M.D., Cooper, L.T., Corriere, M., Cortinovis, M., de Vaccaro, K.C., Couser, W., Cowie, B.C., Criqui, M.H., Cross, M., Dabhadkar, K.C., Dahiya, M., Dahodwala, N., Damsere-Derry, J., Danaei, G., Davis, A., De Leo, D., Degenhardt, L., Dellavalle, R., Delossantos, A., Denenberg, J., Derrett, S., Des Jarlais, D.C., Dharmaratne, S.D., Dherani, M., Diaz-Torne, C., Dolk, H., Dorsey, E.R., Driscoll, T., Duber, H., Ebel, B., Edmond, K., Elbaz, A., Ali, S.E., Erskine, H., Erwin, P.J., Espindola, P., Ewoigbokhan, S.E., Farzadfar, F., Feigin, V., Felson, D.T., Ferrari, A., Ferri, C.P., Fevre, E.M., Finucane, M.M., Flaxman, S., Flood, L., Foreman, K., Forouzanfar, M.H., Fowkes, F.G., Fransen, M., Freeman, M.K., Gabbe, B.J., Gabriel, S.E., Gakidou, E., Ganatra, H.A., Garcia, B., Gaspari, F., Gillum, R.F., Gmel, G., Gonzalez-Medina, D., Gosselin, R., Grainger, R., Grant, B., Groeger, J., Guillemin, F., Gunnell, D., Gupta, R., Haagsma, J., Hagan, H., Halasa, Y.A., Hall, W., Haring, D., Haro, J.M., Harrison, J.E., Havmoeller, R., Hay, R.J., Higashi, H., Hill, C., Hoen, B., Hoffman, H., Hotez, P.J., Hoy, D., Huang, J.J., Ibeanusi, S.E., Jacobsen, K.H., James, S.L., Jarvis, D., Jasrasaria, R., Jayaraman, S., Johns, N., Jonas, J.B., Karthikeyan, G., Kassebaum, N., Kawakami, N., Keren, A., Khoo, J.P., King, C.H., Knowlton, L.M., Kobusingye, O., Koranteng, A., Krishnamurthi, R., Laden, F., Lalloo, R., Laslett, L.L., Lathlean, T., Leasher, J.L., Lee, Y.Y., Leigh, J., Levinson, D., Lim, S.S., Limb, E., Lin, J.K., Lipnick, M., Lipshultz, S.E., Liu, W., Loane, M., Ohno, S.L., Lyons, R., Mabweijano, J., MacIntyre, M.F., Malekzadeh, R., Mallinger, L., Manivannan, S., Marcenes, W., March, L., Margolis, D.J., Marks, G.B., Marks, R., Matsumori, A., Matzopoulos, R., Mayosi, B.M., McAnulty, J.H., McDermott, M.M., McGill, N., McGrath, J., Medina-Mora, M.E., Meltzer, M., Mensah, G.A., Merriman, T.R., Meyer, A.C., Miglioli, V., Miller, M., Miller, T.R., Mitchell, P.B., Mock, C., Mocumbi, A.O., Moffitt, T.E., Mokdad, A.A., Monasta, L., Montico, M., Moradi-Lakeh, M., Moran, A., Morawska, L., Mori, R., Murdoch, M.E., Mwaniki, M.K., Naidoo, K., Nair, M.N., Naldi, L., Narayan, K.M., Nelson, P.K., Nelson, R.G., Nevitt, M.C., Newton, C.R., Nolte, S., Norman, P., Norman, R., O'Donnell, M., O'Hanlon, S., Olives, C., Omer, S.B., Ortblad, K., Osborne, R., Ozgediz, D., Page, A., Pahari, B., Pandian, J.D., Rivero, A.P., Patten, S.B., Pearce, N., Padilla, R.P., Perez-Ruiz, F., Perico, N., Pesudovs, K., Phillips, D., Phillips, M.R., Pierce, K., Pion, S., Polanczyk, G.V., Polinder, S., Pope, C.A.,3rd, Popova, S., Porrini, E., Pourmalek, F., Prince, M., Pullan, R.L., Ramaiah, K.D., Ranganathan, D., Razavi, H., Regan, M., Rehm, J.T., Rein, D.B., Remuzzi, G., Richardson, K., Rivara, F.P., Roberts, T., Robinson, C., De Leon, F.R., Ronfani, L., Room, R., Rosenfeld, L.C., Rushton, L.,

Sacco, R.L., Saha, S., Sampson, U., Sanchez-Riera, L., Sanman, E., Schwebel, D.C., Scott, J.G., Segui-Gomez, M., Shahraz, S., Shepard, D.S., Shin, H., Shivakoti, R., Singh, D., Singh, G.M., Singh, J.A., Singleton, J., Sleet, D.A., Sliwa, K., Smith, E., Smith, J.L., Stapelberg, N.J., Steer, A., Steiner, T., Stolk, W.A., Stovner, L.J., Sudfeld, C., Syed, S., Tamburlini, G., Tavakkoli, M., Taylor, H.R., Taylor, J.A., Taylor, W.J., Thomas, B., Thomson, W.M., Thurston, G.D., Tleyjeh, I.M., Tonelli, M., Towbin, J.A., Truelsen, T., Tsilimbaris, M.K., Ubeda, C., Undurraga, E.A., van der Werf, M.J., van Os, J., Vavilala, M.S., Venketasubramanian, N., Wang, M., Wang, W., Watt, K., Weatherall, D.J., Weinstock, M.A., Weintraub, R., Weisskopf, M.G., Weissman, M.M., White, R.A., Whiteford, H., Wiebe, N., Wiersma, S.T., Wilkinson, J.D., Williams, H.C., Williams, S.R., Witt, E., Wolfe, F., Woolf, A.D., Wulf, S., Yeh, P.H., Zaidi, A.K., Zheng, Z.J., Zonies, D., Lopez, A.D., AlMazroa, M.A. and Memish, Z.A. (2012) 'Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010', *Lancet,* 380(9859), pp. 2197-2223.

Nemeth, M.J., Topol, L., Anderson, S.M., Yang, Y. and Bodine, D.M. (2007) 'Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation', *Proceedings of the National Academy of Sciences of the United States of America,* 104(39), pp. 15436-15441.

Newcomb, D.C., Boswell, M.G., Zhou, W., Huckabee, M.M., Goleniewska, K., Sevin, C.M., Hershey, G.K., Kolls, J.K. and Peebles, R.S.,Jr (2011) 'Human TH17 cells express a functional IL-13 receptor and IL-13 attenuates IL-17A production', *The Journal of allergy and clinical immunology,* 127(4), pp. 1006-13.e1-4.

Newcomb, D.C. and Peebles, R.S.,Jr (2013) 'Th17-mediated inflammation in asthma', *Current opinion in immunology,* 25(6), pp. 755-760.

Newman, D.R., Sills, W.S., Hanrahan, K., Ziegler, A., Tidd, K.M., Cook, E. and Sannes, P.L. (2016a) 'Expression of WNT5A in Idiopathic Pulmonary Fibrosis and Its Control by TGF-beta and WNT7B in Human Lung Fibroblasts', *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,* 64(2), pp. 99-111.

Newman, D.R., Sills, W.S., Hanrahan, K., Ziegler, A., Tidd, K.M., Cook, E. and Sannes, P.L. (2016b) 'Expression of WNT5A in Idiopathic Pulmonary Fibrosis and Its Control by TGF-beta and WNT7B in Human Lung Fibroblasts', *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,* 64(2), pp. 99-111.

Nichols, A.S., Floyd, D.H., Bruinsma, S.P., Narzinski, K. and Baranski, T.J. (2013) 'Frizzled receptors signal through G proteins', *Cellular signalling,* 25(6), pp. 1468-1475.

Nishita, M., Itsukushima, S., Nomachi, A., Endo, M., Wang, Z., Inaba, D., Qiao, S., Takada, S., Kikuchi, A. and Minami, Y. (2010) 'Ror2/Frizzled complex mediates Wnt5a-induced AP-1 activation by regulating Dishevelled polymerization', *Molecular and cellular biology,* 30(14), pp. 3610-3619.

Nishita, M., Yoo, S.K., Nomachi, A., Kani, S., Sougawa, N., Ohta, Y., Takada, S., Kikuchi, A. and Minami, Y. (2006) 'Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration', *The Journal of cell biology,* 175(4), pp. 555-562.

Nomachi, A., Nishita, M., Inaba, D., Enomoto, M., Hamasaki, M. and Minami, Y. (2008) 'Receptor tyrosine kinase Ror2 mediates Wnt5a-induced polarized cell migration by activating c-Jun N-terminal kinase via actin-binding protein filamin A', *The Journal of biological chemistry,* 283(41), pp. 27973-27981.

Noselli, S. and Perrimon, N. (2000) 'Signal transduction. Are there close encounters between signaling pathways?', *Science (New York, N.Y.),* 290(5489), pp. 68-69.

Nusse, R. and Varmus, H. (2012) 'Three decades of Wnts: a personal perspective on how a scientific field developed', *The EMBO journal,* 31(12), pp. 2670-2684.

Nusse, R. and Varmus, H.E. (1982) 'Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome', *Cell,* 31(1), pp. 99-109.

Okubo, T. and Hogan, B.L. (2004) 'Hyperactive Wnt signaling changes the developmental potential of embryonic lung endoderm', *Journal of biology,* 3(3), pp. 11.

Ordonez, C., Ferrando, R., Hyde, D.M., Wong, H.H. and Fahy, J.V. (2000) 'Epithelial desquamation in asthma: artifact or pathology?', *American journal of respiratory and critical care medicine,* 162(6), pp. 2324-2329.

Osler, W. (1895) *The principles and practice of medicine: designed for the use of practitioners and students of medicine.* , thoroughly rev edn. Edinburgh: Young J. Pentland.

Parmar, A. (2013) *Thymic stromal lymphopoietin and its downstream networks in severe asthma.* Leicester: University of Leicester.

Pavord, I.D., Brightling, C.E., Woltmann, G. and Wardlaw, A.J. (1999) 'Non-eosinophilic corticosteroid unresponsive asthma', *Lancet,* 353(9171), pp. 2213-2214.

Pavord, I.D., Pizzichini, M.M., Pizzichini, E. and Hargreave, F.E. (1997) 'The use of induced sputum to investigate airway inflammation', *Thorax,* 52(6), pp. 498-501.

Peters, M., Kohler-Bachmann, S., Lenz-Habijan, T. and Bufe, A. (2016) 'Influence of an Allergen-Specific Th17 Response on Remodeling of the Airways', *American journal of respiratory cell and molecular biology,* 54(3), pp. 350-358.

Pelaia, C., Vatrella, A., Bruni, A., Terracciano, R. and Pelaia, G. (2018) 'Benralizumab in the treatment of severe asthma: design, development and potential place in therapy', *Drug design, development and therapy,* 12, pp. 619-628.

Pini, L., Hamid, Q., Shannon, J., Lemelin, L., Olivenstein, R., Ernst, P., Lemiere, C., Martin, J.G. and Ludwig, M.S. (2007) 'Differences in proteoglycan deposition in the airways of moderate and severe asthmatics', *The European respiratory journal,* 29(1), pp. 71-77.

Plopper, C.G., Smiley-Jewell, S.M., Miller, L.A., Fanucchi, M.V., Evans, M.J., Buckpitt, A.R., Avdalovic, M., Gershwin, L.J., Joad, J.P., Kajekar, R., Larson, S., Pinkerton, K.E., Van Winkle, L.S., Schelegle, E.S., Pieczarka, E.M., Wu, R. and Hyde, D.M. (2007) 'Asthma/allergic airways disease: does postnatal exposure to environmental toxicants promote airway pathobiology?', *Toxicologic pathology,* 35(1), pp. 97-110.

Ponath, P.D., Qin, S., Ringler, D.J., Clark-Lewis, I., Wang, J., Kassam, N., Smith, H., Shi, X., Gonzalo, J.A., Newman, W., Gutierrez-Ramos, J.C. and Mackay, C.R. (1996) 'Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils', *The Journal of clinical investigation,* 97(3), pp. 604-612.

Pongracz, J.E. and Stockley, R.A. (2006) 'Wnt signalling in lung development and diseases', *Respiratory research,* 7, pp. 15.

Pourreyron, C., Reilly, L., Proby, C., Panteleyev, A., Fleming, C., McLean, K., South, A.P. and Foerster, J. (2012) 'Wnt5a is strongly expressed at the leading edge in non-melanoma skin cancer, forming active gradients, while canonical Wnt signalling is repressed', *PloS one,* 7(2), pp. e31827.

Prgomet, Z., Axelsson, L., Lindberg, P. and Andersson, T. (2015) 'Migration and invasion of oral squamous carcinoma cells is promoted by WNT5A, a regulator of cancer progression', *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology,* 44(10), pp. 776-784.

Puddicombe, S.M., Polosa, R., Richter, A., Krishna, M.T., Howarth, P.H., Holgate, S.T. and Davies, D.E. (2000) 'Involvement of the epidermal growth factor receptor in epithelial repair in asthma', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 14(10), pp. 1362-1374.

RACKEMANN, F.M. (1947) 'A working classification of asthma', *The American Journal of Medicine,* 3(5), pp. 601-606.

Ramsey, C.D. and Celedon, J.C. (2005) 'The hygiene hypothesis and asthma', *Current opinion in pulmonary medicine,* 11(1), pp. 14-20.

Rang, H.P. and Dale, M.M. (2011) *Rang and Dale's pharmacology.* 7th edn. Edinburgh: Elsevier Churchill Livingstone.

Redington, A.E., Roche, W.R., Holgate, S.T. and Howarth, P.H. (1998) 'Co-localization of immunoreactive transforming growth factor-beta 1 and decorin in bronchial biopsies from asthmatic and normal subjects', *The Journal of pathology,* 186(4), pp. 410-415.

Reuter, S., Martin, H., Beckert, H., Bros, M., Montermann, E., Belz, C., Heinz, A., Ohngemach, S., Sahin, U., Stassen, M., Buhl, R., Eshkind, L. and Taube, C. (2014) 'The Wnt/beta-Catenin Pathway Attenuates Experimental Allergic Airway Disease', *Journal of immunology (Baltimore, Md.: 1950),* 193(2), pp. 485-495.

Ricciardolo, F.L., Sorbello, V., Folino, A., Gallo, F., Massaglia, G.M., Favata, G., Conticello, S., Vallese, D., Gani, F., Malerba, M., Folkerts, G., Rolla, G., Profita, M., Mauad, T., Di Stefano, A. and Ciprandi, G. (2016) 'Identification of IL-17F/frequent exacerbator endotype in asthma', *The Journal of allergy and clinical immunology,* .

Roarty, K., Baxley, S.E., Crowley, M.R., Frost, A.R. and Serra, R. (2009) 'Loss of TGF-beta or Wnt5a results in an increase in Wnt/beta-catenin activity and redirects mammary tumour phenotype', *Breast cancer research : BCR,* 11(2), pp. R19.

Roarty, K. and Serra, R. (2007) 'Wnt5a is required for proper mammary gland development and TGF-beta-mediated inhibition of ductal growth', *Development (Cambridge, England),* 134(21), pp. 3929-3939.

Robinson, D., Hamid, Q., Bentley, A., Ying, S., Kay, A.B. and Durham, S.R. (1993) 'Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma', *The Journal of allergy and clinical immunology,* 92(2), pp. 313-324.

Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., Corrigan, C., Durham, S.R. and Kay, A.B. (1992) 'Predominant

TH2-like bronchoalveolar T-lymphocyte population in atopic asthma', *The New England journal of medicine,* 326(5), pp. 298-304.

Roche, W.R., Beasley, R., Williams, J.H. and Holgate, S.T. (1989) 'Subepithelial fibrosis in the bronchi of asthmatics', *Lancet,* 1(8637), pp. 520-524.

Saglani, S., Mathie, S.A., Gregory, L.G., Bell, M.J., Bush, A. and Lloyd, C.M. (2009) 'Pathophysiological features of asthma develop in parallel in house dust mite-exposed neonatal mice', *American journal of respiratory cell and molecular biology,* 41(3), pp. 281-289.

Sakula, A. (1985) 'Henry Hyde Salter (1823-71): a biographical sketch', *Thorax,* 40(12), pp. 887-888.

Sampson, A.P. (2000) 'The role of eosinophils and neutrophils in inflammation', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 30 Suppl 1, pp. 22-27.

Saunders, R., Siddiqui, S., Kaur, D., Doe, C., Sutcliffe, A., Hollins, F., Bradding, P., Wardlaw, A. and Brightling, C.E. (2009) 'Fibrocyte localization to the airway smooth muscle is a feature of asthma', *The Journal of allergy and clinical immunology,* 123(2), pp. 376-384.

Scadding, J.G. (1996) 'Essentialism and nominalism in medicine: logic of diagnosis in disease terminology', *Lancet,* 348(9027), pp. 594-596.

Schwartz, D.A. (2011) *Asthma: an epidemic caused by epigenetics?* London: Henry Stewart Talks.

Sen, M., Lauterbach, K., El-Gabalawy, H., Firestein, G.S., Corr, M. and Carson, D.A. (2000) 'Expression and function of wingless and frizzled homologs in rheumatoid arthritis', *Proceedings of the National Academy of Sciences of the United States of America,* 97(6), pp. 2791-2796.

Sharma, S., Tantisira, K., Carey, V., Murphy, A.J., Lasky-Su, J., Celedon, J.C., Lazarus, R., Klanderman, B., Rogers, A., Soto-Quiros, M., Avila, L., Mariani, T., Gaedigk, R., Leeder, S., Torday, J., Warburton, D., Raby, B. and Weiss, S.T. (2010) 'A role for Wnt signaling genes in the pathogenesis of impaired lung function in asthma', *American journal of respiratory and critical care medicine,* 181(4), pp. 328-336.

Shikotra, A., Choy, D.F., Ohri, C.M., Doran, E., Butler, C., Hargadon, B., Shelley, M., Abbas, A.R., Austin, C.D., Jackman, J., Wu, L.C., Heaney, L.G., Arron, J.R. and Bradding, P. (2012) 'Increased expression of immunoreactive thymic stromal lymphopoietin in patients with severe asthma', *The Journal of allergy and clinical immunology,* 129(1), pp. 104-11.e1-9.

Siddiqui, S., Hollins, F. and Brightling, C.E. (2008) 'What can we learn about airway smooth muscle from the company it keeps?', *The European respiratory journal,* 32(1), pp. 9-11.

Siddiqui, S., Mistry, V., Doe, C., Roach, K., Morgan, A., Wardlaw, A., Pavord, I., Bradding, P. and Brightling, C. (2008a) 'Airway hyperresponsiveness is dissociated from airway wall structural remodeling', *The Journal of allergy and clinical immunology,* 122(2), pp. 335-41, 341.e1-3.

Siddiqui, S., Mistry, V., Doe, C., Roach, K., Morgan, A., Wardlaw, A., Pavord, I., Bradding, P. and Brightling, C. (2008b) 'Airway hyperresponsiveness is dissociated from airway wall structural remodeling', *The Journal of allergy and clinical immunology,* 122(2), pp. 335-41, 341.e1-3.

Siddiqui, S., Mistry, V., Doe, C., Roach, K., Morgan, A., Wardlaw, A., Pavord, I., Bradding, P. and Brightling, C. (2008c) 'Airway hyperresponsiveness is dissociated from airway wall structural remodeling', *The Journal of allergy and clinical immunology,* 122(2), pp. 335-41, 341.e1-3.

Sobonya, R.E. (1984) 'Quantitative structural alterations in long-standing allergic asthma', *The American Review of Respiratory Disease,* 130(2), pp. 289-292.

Sullivan, P., Stephens, D., Ansari, T., Costello, J. and Jeffery, P. (1998) 'Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies', *The European respiratory journal,* 12(4), pp. 811-815.

Sumi, Y. and Hamid, Q. (2007) 'Airway remodeling in asthma', *Allergology international : official journal of the Japanese Society of Allergology,* 56(4), pp. 341-348.

Sutcliffe, A., Hollins, F., Gomez, E., Saunders, R., Doe, C., Cooke, M., Challiss, R.A. and Brightling, C.E. (2012) 'Increased nicotinamide adenine dinucleotide phosphate oxidase 4 expression mediates intrinsic airway smooth muscle hypercontractility in asthma', *American journal of respiratory and critical care medicine,* 185(3), pp. 267-274.

Syed, F., Huang, C.C., Li, K., Liu, V., Shang, T., Amegadzie, B.Y., Griswold, D.E., Song, X.Y. and Li, L. (2007) 'Identification of interleukin-13 related biomarkers using peripheral blood mononuclear cells', *Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals,* 12(4), pp. 414-423.

Takahashi, K., Hirose, K., Kawashima, S., Niwa, Y., Wakashin, H., Iwata, A., Tokoyoda, K., Renauld, J.C., Iwamoto, I., Nakayama, T. and Nakajima,

H. (2011) 'IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation', *The Journal of allergy and clinical immunology,* 128(5), pp. 1067-76.e1-6.

Thomson, N.C., Bicknell, S. and Chaudhuri, R. (2012) 'Bronchial thermoplasty for severe asthma', *Current opinion in allergy and clinical immunology,* 12(3), pp. 241-248.

Tomkowicz, A., Kraus-Filarska, M., Bar, J., Rabczynski, J., Jelen, M., Piesiak, P., Fal, A. and Panaszek, B. (2008) 'Bronchial hyper-responsiveness, subepithelial fibrosis, and transforming growth factor-beta(1) expression in patients with long-standing and recently diagnosed asthma', *Archivum Immunologiae et Therapiae Experimentalis,* 56(6), pp. 401-408.

Topol, L., Jiang, X., Choi, H., Garrett-Beal, L., Carolan, P.J. and Yang, Y. (2003a) 'Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation', *The Journal of cell biology,* 162(5), pp. 899-908.

Topol, L., Jiang, X., Choi, H., Garrett-Beal, L., Carolan, P.J. and Yang, Y. (2003b) 'Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation', *The Journal of cell biology,* 162(5), pp. 899-908.

Torday, J.S. and Rehan, V.K. (2007) 'The evolutionary continuum from lung development to homeostasis and repair', *American journal of physiology.Lung cellular and molecular physiology,* 292(3), pp. L608-11.

Trautmann, A., Schmid-Grendelmeier, P., Kruger, K., Crameri, R., Akdis, M., Akkaya, A., Brocker, E.B., Blaser, K. and Akdis, C.A. (2002) 'T cells and eosinophils cooperate in the induction of bronchial epithelial cell apoptosis in asthma', *The Journal of allergy and clinical immunology,* 109(2), pp. 329-337.

Tulic, M.K., Christodoulopoulos, P. and Hamid, Q. (2001) 'Small airway inflammation in asthma', *Respiratory research,* 2(6), pp. 333-339.

UniProt, U. (2014) *Protein Wnt-5a.* Available at: http://www.uniprot.org/uniprot/P41221#section_alternative.

Unwin, N. and Alberti, K.G. (2006) 'Chronic non-communicable diseases', *Annals of Tropical Medicine and Parasitology,* 100(5-6), pp. 455-464.

Usmani, O.S., Singh, D., Spinola, M., Bizzi, A. and Barnes, P.J. (2016) 'The prevalence of small airways disease in adult asthma: A systematic literature review', *Respiratory medicine,* 116, pp. 19-27.

van Amerongen, R. (2012) 'Alternative Wnt pathways and receptors', *Cold Spring Harbor perspectives in biology,* 4(10), pp. 10.1101/cshperspect.a007914.

van Amerongen, R., Fuerer, C., Mizutani, M. and Nusse, R. (2012a) 'Wnt5a can both activate and repress Wnt/beta-catenin signaling during mouse embryonic development', *Developmental biology,* 369(1), pp. 101-114.

van Amerongen, R., Fuerer, C., Mizutani, M. and Nusse, R. (2012b) 'Wnt5a can both activate and repress Wnt/beta-catenin signaling during mouse embryonic development', *Developmental biology,* 369(1), pp. 101-114.

Van Eerdewegh, P., Little, R.D., Dupuis, J., Del Mastro, R.G., Falls, K., Simon, J., Torrey, D., Pandit, S., McKenny, J., Braunschweiger, K., Walsh, A., Liu, Z., Hayward, B., Folz, C., Manning, S.P., Bawa, A., Saracino, L., Thackston, M., Benchekroun, Y., Capparell, N., Wang, M., Adair, R., Feng, Y., Dubois, J., FitzGerald, M.G., Huang, H., Gibson, R., Allen, K.M., Pedan, A., Danzig, M.R., Umland, S.P., Egan, R.W., Cuss, F.M., Rorke, S., Clough, J.B., Holloway, J.W., Holgate, S.T. and Keith, T.P. (2002) 'Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness', *Nature,* 418(6896), pp. 426-430.

Vignola, A.M., Chanez, P., Chiappara, G., Merendino, A., Pace, E., Rizzo, A., la Rocca, A.M., Bellia, V., Bonsignore, G. and Bousquet, J. (1997) 'Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis', *American journal of respiratory and critical care medicine,* 156(2 Pt 1), pp. 591-599.

Vuga, L.J., Ben-Yehudah, A., Kovkarova-Naumovski, E., Oriss, T., Gibson, K.F., Feghali-Bostwick, C. and Kaminski, N. (2009) 'WNT5A is a regulator of fibroblast proliferation and resistance to apoptosis', *American journal of respiratory cell and molecular biology,* 41(5), pp. 583-589.

Vyas, H. and Krishnaswamy, G. (2006) 'Paul Ehrlich's "Mastzellen"--from aniline dyes to DNA chip arrays: a historical review of developments in mast cell research', *Methods in molecular biology (Clifton, N.J.),* 315, pp. 3-11.

Wagner, E.M., Liu, M.C., Weinmann, G.G., Permutt, S. and Bleecker, E.R. (1990) 'Peripheral lung resistance in normal and asthmatic subjects', *The American Review of Respiratory Disease,* 141(3), pp. 584-588.

Wan, W.Y.H. (2013) *Impaired epithelium in asthma: mechanisms driving ciliary dysfunction.* Leicester: University of Leicester.

Wang, Z. and Chen, H. (2009) 'Amino acid limitation induces down-regulation of WNT5a at transcriptional level', *Biochemical and biophysical research communications,* 378(4), pp. 789-794.

Wardlaw, A.J., Dunnette, S., Gleich, G.J., Collins, J.V. and Kay, A.B. (1988) 'Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity', *The American Review of Respiratory Disease,* 137(1), pp. 62-69.

Wark, P.A., Johnston, S.L., Moric, I., Simpson, J.L., Hensley, M.J. and Gibson, P.G. (2002) 'Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma', *The European respiratory journal,* 19(1), pp. 68-75.

Welliver, R.C. and Duffy, L. (1993) 'The relationship of RSV-specific immunoglobulin E antibody responses in infancy, recurrent wheezing, and pulmonary function at age 7-8 years', *Pediatric pulmonology,* 15(1), pp. 19-27.

Wenzel, S.E. (2012) 'Asthma phenotypes: the evolution from clinical to molecular approaches', *Nature medicine,* 18(5), pp. 716-725.

Wenzel, S.E., Schwartz, L.B., Langmack, E.L., Halliday, J.L., Trudeau, J.B., Gibbs, R.L. and Chu, H.W. (1999) 'Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics', *American journal of respiratory and critical care medicine,* 160(3), pp. 1001-1008.

Wenzel, S.E., Szefler, S.J., Leung, D.Y., Sloan, S.I., Rex, M.D. and Martin, R.J. (1997) 'Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids', *American journal of respiratory and critical care medicine,* 156(3 Pt 1), pp. 737-743.

Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L. and Donaldson, D.D. (1998) 'Interleukin-13: central mediator of allergic asthma', *Science (New York, N.Y.),* 282(5397), pp. 2258-2261.

Wilson, J.W. and Li, X. (1997) 'The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology,* 27(4), pp. 363-371.

Winn, R.A., Marek, L., Han, S.Y., Rodriguez, K., Rodriguez, N., Hammond, M., Van Scoyk, M., Acosta, H., Mirus, J., Barry, N., Bren-Mattison, Y., Van Raay, T.J., Nemenoff, R.A. and Heasley, L.E. (2005) 'Restoration of Wnt-7a expression reverses non-small cell lung cancer cellular transformation through frizzled-9-mediated growth inhibition and promotion of cell differentiation', *The Journal of biological chemistry,* 280(20), pp. 19625-19634.

Witze, E.S., Litman, E.S., Argast, G.M., Moon, R.T. and Ahn, N.G. (2008) 'Wnt5a control of cell polarity and directional movement by polarized

redistribution of adhesion receptors', *Science (New York, N.Y.),* 320(5874), pp. 365-369.

Wong, H.C., Bourdelas, A., Krauss, A., Lee, H.J., Shao, Y., Wu, D., Mlodzik, M., Shi, D.L. and Zheng, J. (2003) 'Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled', *Molecular cell,* 12(5), pp. 1251-1260.

Woodman, L., Siddiqui, S., Cruse, G., Sutcliffe, A., Saunders, R., Kaur, D., Bradding, P. and Brightling, C. (2008a) 'Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1', *Journal of immunology (Baltimore, Md.: 1950),* 181(7), pp. 5001-5007.

Woodman, L., Siddiqui, S., Cruse, G., Sutcliffe, A., Saunders, R., Kaur, D., Bradding, P. and Brightling, C. (2008b) 'Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1', *Journal of immunology (Baltimore, Md.: 1950),* 181(7), pp. 5001-5007.

Woodruff, P.G., Boushey, H.A., Dolganov, G.M., Barker, C.S., Yang, Y.H., Donnelly, S., Ellwanger, A., Sidhu, S.S., Dao-Pick, T.P., Pantoja, C., Erle, D.J., Yamamoto, K.R. and Fahy, J.V. (2007) 'Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids', *Proceedings of the National Academy of Sciences of the United States of America,* 104(40), pp. 15858-15863.

Woodruff, P.G., Modrek, B., Choy, D.F., Jia, G., Abbas, A.R., Ellwanger, A., Koth, L.L., Arron, J.R. and Fahy, J.V. (2009) 'T-helper type 2-driven inflammation defines major subphenotypes of asthma', *American journal of respiratory and critical care medicine,* 180(5), pp. 388-395.

Xiao, C., Puddicombe, S.M., Field, S., Haywood, J., Broughton-Head, V., Puxeddu, I., Haitchi, H.M., Vernon-Wilson, E., Sammut, D., Bedke, N., Cremin, C., Sones, J., Djukanovic, R., Howarth, P.H., Collins, J.E., Holgate, S.T., Monk, P. and Davies, D.E. (2011) 'Defective epithelial barrier function in asthma', *The Journal of allergy and clinical immunology,* 128(3), pp. 549-56.e1-12.

Xue, H.H. and Zhao, D.M. (2012) 'Regulation of mature T cell responses by the Wnt signaling pathway', *Annals of the New York Academy of Sciences,* 1247, pp. 16-33.

Yamaguchi, M., Niimi, A., Matsumoto, H., Ueda, T., Takemura, M., Matsuoka, H., Jinnai, M., Otsuka, K., Oguma, T., Takeda, T., Ito, I., Chin, K. and Mishima, M. (2008) 'Sputum levels of transforming growth factor-beta1 in asthma: relation to clinical and computed tomography

findings', *Journal of investigational allergology & clinical immunology,* 18(3), pp. 202-206.

Yamaguchi, Y., Suda, T., Suda, J., Eguchi, M., Miura, Y., Harada, N., Tominaga, A. and Takatsu, K. (1988) 'Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors', *The Journal of experimental medicine,* 167(1), pp. 43-56.

Yamamoto, H., Awada, C., Matsumoto, S., Kaneiwa, T., Sugimoto, T., Takao, T. and Kikuchi, A. (2015) 'Basolateral secretion of Wnt5a in polarized epithelial cells is required for apical lumen formation', *Journal of cell science,* 128(5), pp. 1051-1063.

Yan, D., Wallingford, J.B., Sun, T.Q., Nelson, A.M., Sakanaka, C., Reinhard, C., Harland, R.M., Fantl, W.J. and Williams, L.T. (2001) 'Cell autonomous regulation of multiple Dishevelled-dependent pathways by mammalian Nkd', *Proceedings of the National Academy of Sciences of the United States of America,* 98(7), pp. 3802-3807.

Yasukawa, A., Hosoki, K., Toda, M., Miyake, Y., Matsushima, Y., Matsumoto, T., Boveda-Ruiz, D., Gil-Bernabe, P., Nagao, M., Sugimoto, M., Hiraguchi, Y., Tokuda, R., Naito, M., Takagi, T., D'Alessandro-Gabazza, C.N., Suga, S., Kobayashi, T., Fujisawa, T., Taguchi, O. and Gabazza, E.C. (2013) 'Eosinophils promote epithelial to mesenchymal transition of bronchial epithelial cells', *PloS one,* 8(5), pp. e64281.

Yu, Q., Sharma, A., Oh, S.Y., Moon, H.G., Hossain, M.Z., Salay, T.M., Leeds, K.E., Du, H., Wu, B., Waterman, M.L., Zhu, Z. and Sen, J.M. (2009) 'T cell factor 1 initiates the T helper type 2 fate by inducing the transcription factor GATA-3 and repressing interferon-gamma', *Nature immunology,* 10(9), pp. 992-999.

Yuan, Y., Niu, C.C., Deng, G., Li, Z.Q., Pan, J., Zhao, C., Yang, Z.L. and Si, W.K. (2011) 'The Wnt5a/Ror2 noncanonical signaling pathway inhibits canonical Wnt signaling in K562 cells', *International journal of molecular medicine,* 27(1), pp. 63-69.

Zhan, T., Rindtorff, N. and Boutros, M. (2017) 'Wnt signaling in cancer', *Oncogene,* 36(11), pp. 1461-1473.

Zijlstra, G.J., Ten Hacken, N.H., Hoffmann, R.F., van Oosterhout, A.J. and Heijink, I.H. (2012) 'Interleukin-17A induces glucocorticoid insensitivity in human bronchial epithelial cells', *The European respiratory journal,* 39(2), pp. 439-445.

Chapter 7.

Appendix

7.1 Appendix 1

Reagents for immunohistochemistry

0.05M Tris-Buffered Saline solution

The composition of 10X TBS, pH 7.6 is shown in the table below, made up to 1 litre with dH_2O in a volumetric flask. Further dilution of 1 in 10 with distilled water was done to obtain 1X TBS.

Stock Component	Amount Needed
Tris Base (VWR International, 28811.295)	6.05g
NaCl (Fisher Scientific, S/3120/60	80g
2M HCl	~17ml

Blocking Medium

The composition of blocking medium is show in the table below and stored at -20ºC.

Stock Component	Amount Needed
DMEM (Gibco)	80ml
20% FCS (Gibco)	20ml
1% BSA (Sigma)	1g

7.2 Appendix 2

1% pure collagen

Stock component	Amount
PureCol, Bovine Collagen (Cell systems, 5005-B)	500ul
DPBS (Sigma, D8662)	49.5ml

Seeding density and volume of culture vessels

Container/Surface	PureCol (1%) volume (ml)	HBSS volume (ml)	Number of cells to seed	Top up volume (ml)	volume (ml) for feeding
T75 Flask	10ml	10ml	>300k	10ml	10ml
T25 Flask	5ml	5ml	100k – 250k	5ml	5ml
6cm Petri dish	3ml	3ml	300k – 400k	3ml	2ml
6-well plate	2ml/well	2ml/well	100k – 200k	2ml/well	2ml/well
12-well plate	1ml/well	1ml/well	20k – 30k	1ml/well	1ml/well

24-well plate	1ml/well	1ml/well	15k – 25k	1ml/well	1ml/well
96-well plate	0.2ml/well	0.2ml/well	10k – 15k	0.2ml/well	0.2ml/well
8-well chamber slide	0.3ml/well	0.3ml/well	15k – 17k	0.3ml/well	0.3ml/well
T75 Flask	10ml	10ml	300k – 500k	10ml	10ml

7.3 Appendix 3

Reagents for western blotting;

0.5M Tris-Buffered Saline solution (TBS)

The composition of 10X TBS, pH 7.6 is shown in the table below, made up to 1 litre with dH_2O in a volumetric flask. Further dilution of 1in10 with distilled water was done to obtain 1X TBS. Next Tween-20 detergent (Sigma, P-1379) was added at 1:1000 in 1X TBS to give 1XTTBS.

Stock Component	Amount Needed
Tris Base (VWR International)	60.55g
NaCl (Fisher Scientific)	90g
6M HCl	~62ml

12% Acrylamide gel

The table below show the composition of 12% gel. Stacking gel was placed on top of the polymerised resolving gel.

(X2) Resolving Gel	Volume (ml) 12%
Proto-gel	**6ml**
4X resolving buffer	**3.75**
dH_2O	**5.09**
Ammonium Persulphate (APS)	**150ul**
TEMED	**15ul**
Total	**15ml**

(X2) Stacking Gel	Volume (ml)
Proto-gel	**1.3ml**
4X Pro-stacking gel	**2.5ml**
dH_2O	**6.1ml**
Ammonium Persulphate (APS)	**50ul**
TEMED	**10ul**
Total	**10ml**

1X Running Buffer

10X running buffer (Bio-Rad) was diluted 1in 10 with dH2O to obtain 1X running buffer.

1x Transfer Buffer

10X Transfer buffer (Bio-Rad) was diluted with one part 10X transfer buffer, two parts methanol and seven parts nano pure water to obtain 1X transfer buffer.

4x Laemmli Buffer composition

Stock Solutions	Per 50ml Total	Per 25ml total
0.5M Tris-HCL pH6.8	25ml	12.5ml
SDS	2g	1g
Bromophenol Blue (Sigma)	4mg	2mg
Glycerol	20ml	10ml
B-mercaptoethanol (Sigma) (14.3M in pure liquid solution)	5ml	2.5ml

7.4 Appendix 4

Reagents for Calcium ratiometric assay

Phosphate Saline Solution (PSS)

The solution was prepared by adding the following components;

Solute	Weight (g)	Molarity (mM)
Sodium Chloride (NaCl)	6.92g	118.4mM
Potassium Chloride (KCl)	0.35g	4.7mM
D-Glucose	2.0g	11.1mM
Hepes	2.38g	10mM
Magnesium dichloride ($MgCl_2$)	0.244g	1.2mM

The final solution was prepared in 1 litre of dH_2O and pH was equilibrated to 7-7.4 with NaOH. Before application solution aliquot was prepared with $CaCl_2$ at 2mM and warmed at 37°C.

7.5 Appendix 5

Table of genes selected for Qiagen PCR array

Gene Symbol	Alias	Refseq #	Official Full Name	RT2 Catalog Number
WNT5A	hWNT5A	NM_003392	Wingless-type MMTV integration site family, member 5A	PPH02410
WNT3A	-	NM_033131	Wingless-type MMTV integration site family, member 3A	PPH02772
ROR2	BDB/BDB1/NTRKR2	NM_004560	Receptor tyrosine kinase-like orphan receptor 2	PPH14408
NFATC1	NF-ATC/NFAT2/NFATc	NM_172390	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	PPH00277
LEF1	LEF-1/TCF10/TCF1ALPHA/TCF7L3	NM_016269	Lymphoid enhancer-binding factor 1	PPH02778
CTNNB1	CTNNB/MRD19/armadillo	NM_001904	Catenin (cadherin-associated protein), beta 1, 88kDa	PPH00643

RHOA	ARH12/ARHA/RHO12/RHOH12	NM_001664	Ras homolog gene family, member A	PPH00305
MAPK8	JNK/JNK-46/JNK1/JNK1A2/JNK21B1/2/PRKM8/SAPK1/SAPK1c	NM_002750	Mitogen-activated protein kinase 8	PPH00720
TGFB1	CED/DPD1/LAP/TGFB/TGFbeta	NM_000660	Transforming growth factor, beta 1	PPH00508
SMAD2	JV18/JV18-1/MADH2/MADR2/hMAD-2/hSMAD2	NM_005901	SMAD family member 2	PPH01949
SMAD3	HSPC193/HsT17436/JV15-2/LDS1C/LDS3/MADH3	NM_005902	SMAD family member 3	PPH01921
MAP3K7	MEKK7/TAK1/TGF1a	NM_003188	Mitogen-activated protein kinase kinase kinase 7	PPH00749
WNT1	BMND16/INT1/OI15	NM_005430	Wingless-type MMTV integration site family, member 1	PPH00984

ROCK1	P160ROCK/ROCK-I	NM_005406	Rho-associated, coiled-coil containing protein kinase 1	PPH01966
SMAD7	CRCS3/MADH7/MADH8	NM_005904	SMAD family member 7	PPH01905
FLNA	ABP-280/ABPX/CSBS/CVD1/FLN/FLN-A/FLN1/FMD/MNS/NHBP/OPD/OPD1/OPD2/XLVD/XMVD	NM_001456	Filamin A, alpha	PPH14154
ACTB	BRWS1/PS1TP5BP1	NM_001101	Actin, beta	PPH00073
GAPDH	G3PD/GAPD	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	PPH00150
18SrRNA		X03205	Human 18S ribosomal RNA	PPH05666

7.6 Appendix 6

Table displaying the adjusted p-values of bronchial biopsy homogenates WNT gene expression in health vs asthma

p_vals_adjusted	Gene_Name
0.643763453	FZD1
0.980440246	FZD10
0.695777614	FZD2
0.596859768	FZD3
0.596859768	FZD4
0.596859768	FZD5
0.596859768	FZD6
0.643763453	FZD7
0.980440246	FZD8
0.929873217	FZD9
0.961075791	LRP5
0.869985942	LRP6
0.980440246	ROR1
0.643763453	ROR2
0.603734458	WNT1

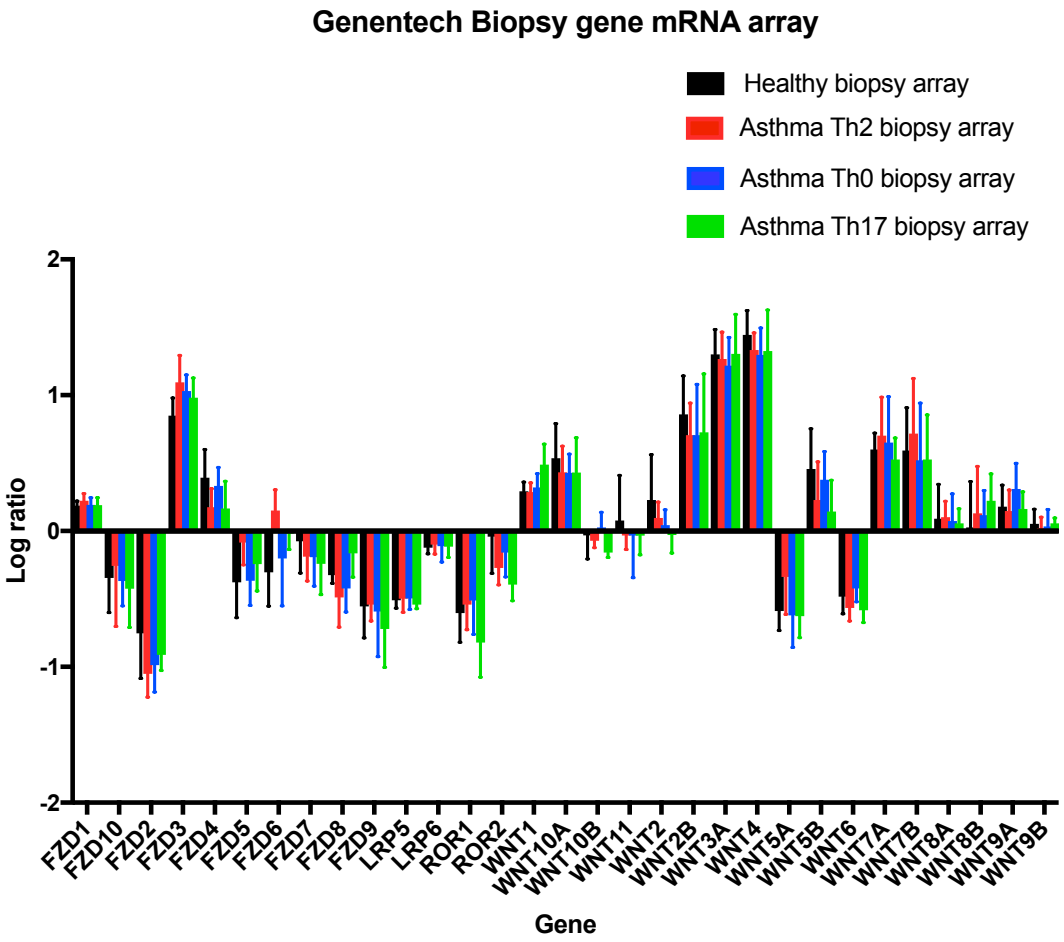
0.866525471	WNT10A
0.980440246	WNT10B
0.864859863	WNT11
0.596859768	WNT2
0.864859863	WNT2B
0.961075791	WNT3A
0.596859768	WNT4
0.961075791	WNT5A
0.596859768	WNT5B
0.96867038	WNT6
0.961075791	WNT7A
0.980440246	WNT7B
0.961075791	WNT8A
0.603734458	WNT8B
0.980440246	WNT9A
0.980440246	WNT9B

Table displaying the adjusted p-values of bronchial biopsy homogenates WNT gene expression in health vs asthma

p_vals_adjusted	Gene_Name
0.966459165	FZD1
0.924213003	FZD10
0.873938059	FZD2
0.965337497	FZD3
0.924213003	FZD4
0.924213003	FZD5
0.873938059	FZD6
0.741628927	FZD7
0.479093425	FZD8
0.924213003	FZD9
0.741628927	LRP5
0.927176826	LRP6
0.965337497	ROR1
0.924213003	ROR2
0.479093425	WNT1
0.925537988	WNT10A
0.479093425	WNT10B
0.924213003	WNT11
0.873938059	WNT2

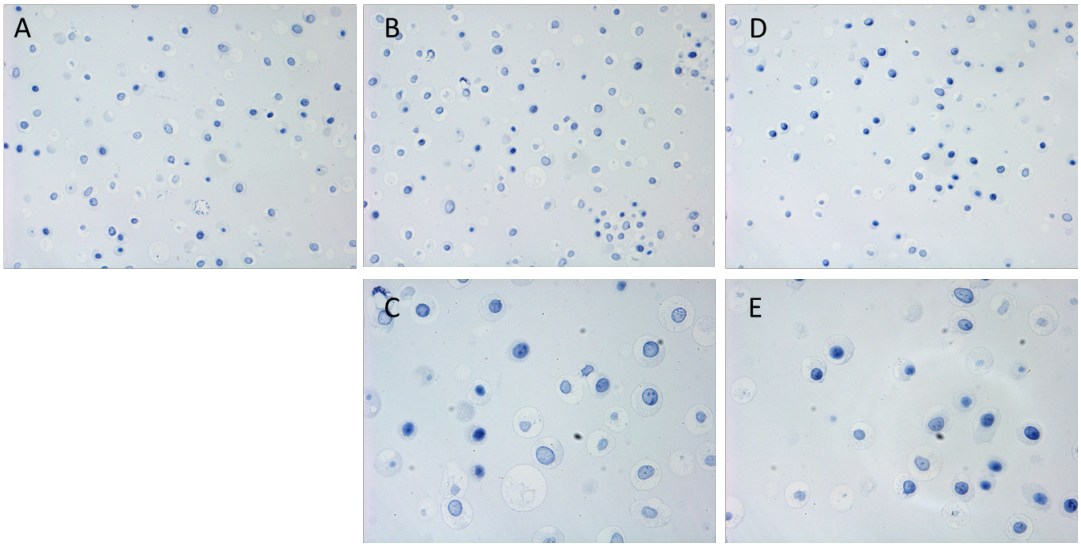
0.924213003	WNT2B
0.924213003	WNT3A
0.924213003	WNT4
0.924213003	WNT5A
0.5465792	WNT5B
0.864997438	WNT6
0.924213003	WNT7A
0.924213003	WNT7B
0.924213003	WNT8A
0.479093425	WNT8B
1	WNT9A
0.924213003	WNT9B

A graph displaying the expression of WNT related components in healthy and asthma phenotypes, analysed by 2-way ANOVA.

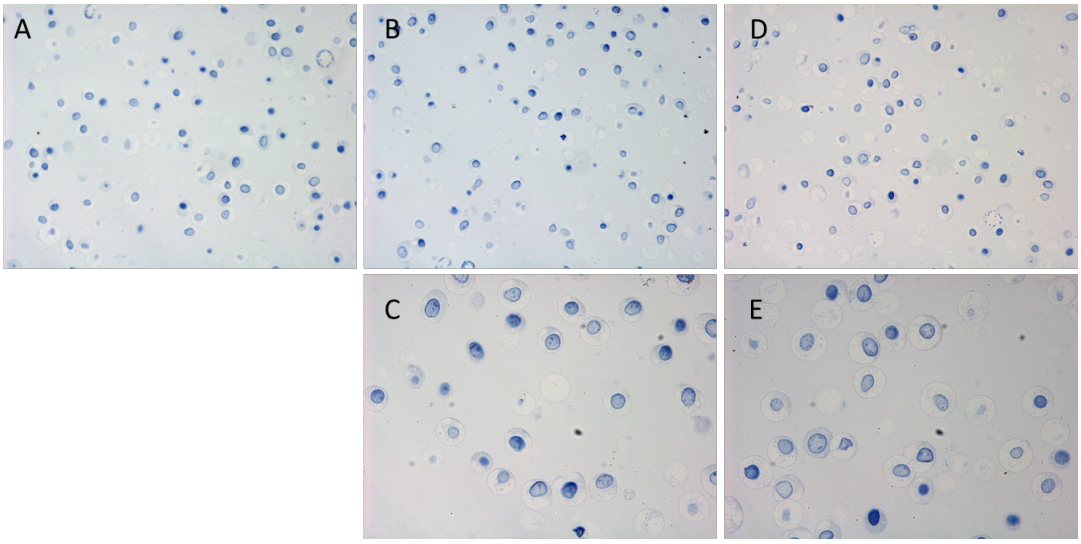


7.7 Appendix 7

Staining of WNT5a LS bio and WNT5a RnD in L-cells embedded in GMA.

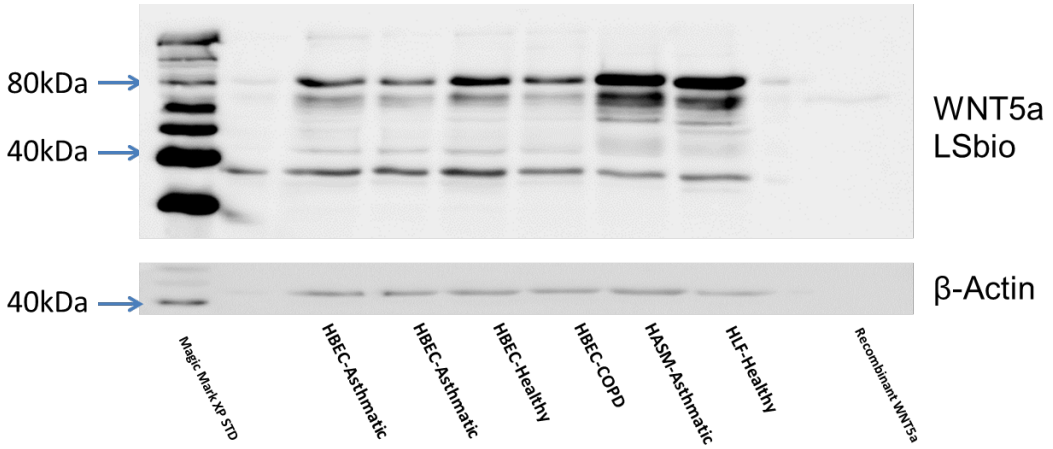


Photomicrograph sections in GMA. (A) No antibody, (B-C) rabbit IgG (isotype control) at 10µg/ml and (D-E) WNT5a LSbio antibody at 10µg/ml.



Photomicrograph of L-cells sections in GMA. (A) No antibody, (B-C) rat IgG2a (isotype control) at 20µg/ml and (D-E) WNT5a RnD antibody at 20µg/ml.

7.8 Appendix 8



Showing WNT5a protein expression in lung structural cells immunolabelled with WNT5a LSbio antibody. Equal loading of samples depicted by Beta-actin. (PVDF membrane wexposed for 5 minutes). HASM, Human airway smooth muscle; HBEC, human bronchial epithelial cells; HLF, human lung fibroblasts.

7.9 Appendix 9

List of genes identified and extracted from the Genentech epithelial microarray cohort, for bivariate correlation in the WNT-TGF pathway and Th-inflammatory pathway.

Gene ID			
AXIN1	SMAD4	FZD8	RHOA
BTRC	SMAD7	GSK3B	ROCK1
CAMK2A	SMURF1	IL8	ROR1
CDC42	TGFB1	LEF1	ROR2
CLCA1	TGFBR1	LRP5	RYK
CSF3	TGFBR2	LRP6	SERPINB2
CTNNB1	TGFBR3	MAP3K7	SMAD2
CXCL1	WNT1	MAPK11	SMAD3
CXCL2	WNT10A	MAPK12	PPP3CA
CXCL3	WNT10B	MAPK13	PRKCA

DVL2	WNT11	MAPK8	RAC1
EP300	WNT2B	NFATC1	WNT5A
FLNA	WNT3A	PLCB1	WNT5B
FZD2	WNT4	POSTN	ZFYVE9
FZD4	FZD6	FZD5	