REGULATION OF BASAL CELL PLASTICITY: IMPLICATIONS FOR AIRWAY EPITHELIAL REMODELING IN ASTHMA

by

Stephanie Mary Beth Warner

B.Sc., The University of British Columbia, Vancouver, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2014

Abstract

Asthma is characterized by reversible airflow limitation, airway inflammation and remodeling, which includes increased smooth muscle mass, sub-epithelial fibrosis, goblet cell metaplasia and loss of columnar epithelial cells. Basal cells are progenitor cells of the pseudostratified airway epithelium that undergo distinct phenotypic transitions to maintain epithelial homeostasis following damage. ***We hypothesized*** that differentiation of epithelial basal cells is defective in asthma, leading to impaired repair. We used three distinct *in vitro* models of human airway epithelial basal cell plasticity – epithelial-mesenchymal transition (EMT), repair of mechanical scratch wounds, and differentiation at air-liquid interface – which together provide a complete overview of basal cell function in epithelial repair. In ***Chapter 3*** we assessed the ability of transforming growth factor (TGF)-β_1 to induce molecular reprogramming indicative of EMT and found that basal cells from asthmatic and non-asthmatic patients undergo TGFβ_1-induced EMT. However, an expanded population of basal cells in differentiated epithelial cultures from asthmatic donors led to an increased EMT response. In ***Chapter 4*** we found that inhibition of ΔNp63α impaired restitution of scratch wounds in monolayer culture, due to decreased proliferation. Additionally, ΔNp63α regulated several genes known to be involved in epithelial repair, including β-catenin, epidermal growth factor receptor, and jagged1. In ***Chapter 5*** we found that basal epithelial cells from asthmatic donors with (+) and without (-) exercise-induced bronchoconstriction (EIB) were impaired in the transition to a ciliated but not goblet cell phenotype in an air-liquid interface (ALI) model of mucociliary differentiation. EIB(-) asthmatics also had an expansion of the basal cell population and shorter cilia**.** In ***Chapter 6,*** we used an unbiased RNA sequencing approach to identify aberrant expression of pathways

involved in actin cytoskeleton dynamics and cellular metabolism that were distinctly different in EIB(-) and EIB(+) asthma during mucociliary differentiation. We also identified a miRNA-mRNA network that regulates the epithelial transition from proliferation to differentiation. This thesis provides compelling evidence that lineage commitment and molecular reprogramming in basal cells are skewed in asthma to favour epithelial plasticity in response to TGFβ_1, rather than mucociliary differentiation, and that epithelial remodeling is more pronounced in the EIB(-) phenotype of asthma.

Preface

All projects and associated methods were approved by the University of British Columbia's Research Ethics Board [certificate # H13-02173].

Chapter 1: Introduction

I wrote chapter 1, with edits provided by Drs. Tillie-Louise Hackett, Darryl Knight and Peter Paré. This chapter includes some aspects of two previously-published manuscripts:

- **Warner SM**, Knight DA. Airway modeling and remodeling in the pathogenesis of asthma. Curr Opin Allergy Clin Immunol. 2008 Feb;8(1):44-8. I wrote the entire first draft and contributed to final edits of the review article.
- Hackett TL, **Warner SM**, Stefanowicz D and Knight DA. (2011) Epithelial Cells, in Inflammation and Allergy Drug Design (eds K. Izuhara, S. T. Holgate and M. Wills-Karp), Wiley-Blackwell, Oxford, UK. doi: 10.1002/9781444346688.ch10. I wrote sections of this book chapter, including development of the respiratory system, anatomy and function of the epithelium, and epithelial remodeling in asthma. I also created figures for the book chapter, which have been modified for inclusion here (as **Figure 1.5** and **Figure 1.6)**. All materials are used with permission of publishers.

Chapter 3: Induction of Epithelial-Mesenchymal Transition in Primary Airway Epithelial Cells from Patients with Asthma by Transforming Growth Factor-β1

I carried out approximately 30% of the experiments in the manuscript, including primary cell culture and immunoblot experiments and assisted in the preparation of figures for the

manuscript. A version of this chapter has been published and is reprinted here with permission of the American Thoracic Society.

- Cite: Hackett TL, **Warner SM**, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, Argentieri R, Kicic A, Stick SM, Bai TR, Knight DA. Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1. Am J Respir Crit Care Med. 2009 Jul 15;180(2):122-33.

Chapter 4: Transcription Factor p63 Regulates Key Genes and Wound Repair in Human Airway Epithelial Basal Cells

I performed all experiments, data collection and analysis, interpreted the data and wrote the manuscript. Dr. Darryl Knight conceived of the study and contributed to the manuscript. Dr. Tillie-Louise Hackett assisted with data analysis and manuscript preparation, and Teal Hallstrand contributed epithelial cells and edited the manuscript. Furquan Shaheen contributed immunohistochemistry assistance. A version of this chapter has been published and is reprinted here with permission of the American Thoracic Society.

- Cite: **Warner SM**, Hackett TL, Shaheen F, Hallstrand TS, Kicic A, Stick SM, Knight DA. Transcription Factor p63 Regulates Key Genes and Wound Repair in Human Airway Epithelial Basal Cells. Am J Respir Cell Mol Biol. 2013 Dec;49(6):978-88.

Chapter 5: Impaired Ciliary Differentiation of Airway Epithelial Cells from Asthmatics With and Without Exercise-Induced Bronchoconstriction

I designed the study, carried out all cell culture experiments, assisted with immunohistochemistry, carried out all image analyses, performed ELISA experiments, analyzed

and interpreted the data and wrote the chapter. Dr. Tillie-Louise Hackett contributed to the study design and data interpretation, assisted with the ELISA experiments, and contributed to writing the chapter. Dr. Darryl Knight assisted with experimental design and contributed to editing the chapter. Dr. Teal Hallstrand contributed to the experimental design and provided the primary human airway epithelial cells and biopsy tissues for the study. Furquan Shaheen assisted with immunohistochemistry (IHC) of biopsy and air-liquid interface cultures. Amrit Samra performed all Alcian blue staining. Dorota Stefanowicz assisted with cell culture and maintained the coding of samples during IHC work to ensure that I was blinded from sample identification. Dr. Peter Paré contributed final edits. Human tissues were collected under approval of the University of Washington Institutional Review Board and with written informed consent from all participants.

Chapter 6: Distinct Epithelial mRNA and miRNA Expression Profiles During Differentiation and Between EIB(+) and EIB(-) Asthma Phenotypes

I designed and carried out all cell culture experiments (as described for chapter 5), isolated and processed all RNA samples, performed pathway analyses, assessed the biological significance of findings, and wrote the chapter. Dr. Tillie-Louise Hackett contributed to study design, data interpretation, and assisted with writing and editing the chapter. Dr. Darryl Knight contributed to study design and edited the chapter. Sequencing and data analysis were carried out at Boston University, in collaboration with Dr. Avrum Spira of the Computational Bioinformatics group. Dr. Gang Liu prepared the sample libraries and performed the sequencing runs. Sequencing data was analyzed by Dr. Adam Gower, with input from Dr. Avrum Spira. Dr. Gower aligned raw sequencing reads to the genome, created libraries of normalized data, and performed negative binomial modeling and two-way ANOVA statistical analyses. Dr. Gower also carried out

hierarchical clustering, created heatmaps of differential expression and performed principal component analyses. Dr. Ma’en Obeidat provided critical input regarding statistical methodologies and interpretation of data. Yunlong Nie created correlation matrices for candidate gene co-expression and assisted with visualization of principal component analyses. Dr. Peter Paré contributed to the experimental design, data interpretation and provided final chapter edits.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A549	Alveolar adenocarcinoma cell line
AEC	Airway epithelial cell
ALI	Air-liquid interface
APC	Antigen presenting cell
α-SMA	Alpha smooth muscle actin
BHR	Bronchial hyperresponsiveness
BMP	Bone morphogenic protein
CK-	Cytokeratin (ie. CK-5, CK-14)
Cys-LT	Cysteinyl leukotriene
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIB	Exercise-induced bronchoconstriction
EMT	Epithelial – mesenchymal transition
EMTU	Epithelial – mesenchymal trophic unit
FDR	False discovery rate
FEV_1	Forced expiratory volume in one second
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FOXJ1	Forkhead box J1
FSP	Fibroblast specific protein

GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBEC6-KT	Human bronchial epithelial cells minimally immortalized with CDK4 and hTert
HDM	House dust mite
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-	Interleukin
LT	Leukotriene
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
ΔNp63	N-terminally truncated isoform of p63
P63	Tumour protein p63
PBS	Phosphate buffered saline
PC_{20}	Provocative concentration resulting in 20% reduction in FEV_1
PEF	Peak expiratory flow
pHAEC	Primary human airway epithelial cell
RISC	RNA-induced silencing complex
RR	Relative risk
SCF	Stem cell factor
SPDEF	SAM pointed domain-containing ETS transcription factor
STAT	Signal transducer and activator of transcription
TAp63	Transactivating isoform of p63

TBS	Tris buffered saline
TGFβ1	Transforming growth factor β_1
T_H	T helper
TSLP	Thymic stromal lymphopoetin

Acknowledgements

I owe great thanks to many people who have provided support throughout my degree. First, I thank Dr. Tillie-Louise Hackett for her immense help and support in the preparation of this thesis and all of the work contained herein. I would also like to thank my co-supervisor, Dr. Darryl Knight, for his support and guidance throughout my graduate degree. My supervisory committee members, Drs. Peter Paré, Kelly McNagny and Mike Allard, provided excellent guidance, both scientifically and professionally. Dr. Teal Hallstrand, at the University of Washington, provided extremely important clinical samples for this thesis work as well as critical review of the resulting data. At Boston University, Drs. Avi Spira, Gang Liu and Adam Gower were of immense help in obtaining and analyzing a very complex data set.

At the Centre for Heart Lung Innovation at St. Paul's Hospital, I am grateful to the many past and present members of the Knight and now Hackett labs, who have always been incredibly supportive and generous with their time and knowledge. I am thankful for Dorota Stefanowicz's insight and input on many aspects of my research, and for the encouragement of Tracee Wee and Hyun-Kyoung Koo. I owe Furquan Shaheen special thanks for his proficiency with laboratory matters and his assistance with immunohistochemistry. Amrit Samra provided excellent technical assistance in histology, and Dr. Samuel Wadsworth offered guidance for study design regarding air-liquid interface cultures.

My research has been supported by an Alexander Graham Bell Canada Graduate Scholarship from the Natural Sciences and Engineering Research Council, travel funding from the AllerGen Allergy, Genes and Environment Network, the Canadian Institutes for Health Research – Institute for Circulatory and Respiratory Health, as well as the University of British

Columbia, and operating grant funding obtained by Dr. Darryl Knight and Dr. Tillie Hackett from the Canadian Institutes of Health Research.

Personally, I thank my parents, Margaret and Rick Hartley and my brother Geoff Warner for their love and support. I am also continually inspired by my grandmother Beth Warner. My father, Stevan Warner, was the inspiration for me to pursue a PhD, and to make the most of every day and every opportunity. I will always be grateful for his love and support. I am especially grateful for the friendship and support of Kristin Bowden, Wendy Boivin, and Terna Daniel, and for the immeasurable support, patience and encouragement of Phillip Blundon.

Dedication

For my family

Chapter 1: Introduction

1.1 Overview of thesis

Asthma is a complex disease, with both genetic and environmental components contributing to its pathogenesis. Features of the disease generally include both immune sensitization and structural remodeling of the airways, including alterations to the epithelial cells that line the airways. In addition to acting as a physical barrier, the airway epithelium is known to contribute to immune modulation and repair responses. The overarching goal of this thesis is to further our understanding of the role that the airway epithelium plays in the pathophysiology of asthma. In particular, we will address several aspects of epithelial cell plasticity to determine whether aberrant differentiation may contribute to and potentiate the remodeling features that occur in the setting of disease.

1.2 Asthma

Asthma is an inflammatory disorder of the airways characterized by reversible airflow limitation and a variable degree of hyperresponsiveness of the airways to stimuli, manifesting as symptoms such as shortness of breath, wheeze and cough (Lougheed, Lemiere et al. 2010). The symptoms of asthma arise from the narrowing of the airways due to constriction of the surrounding airway smooth muscle, compounded by bronchial edema and mucus production (**Figure 1.1**). The resulting airflow limitation can be fatal.

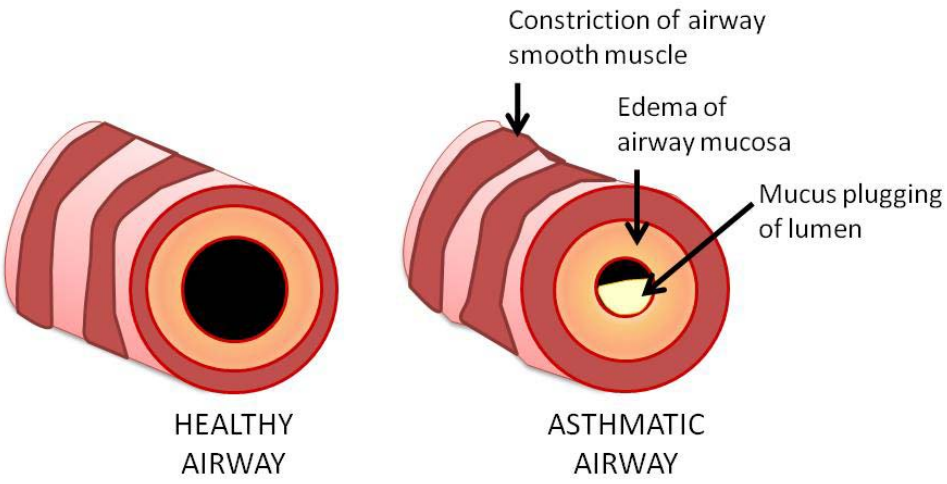


Figure 1.1 Schematic of airway pathology in asthmatic individuals.
Characteristic features of asthmatic airways include bronchial hyperresponsiveness leading to airway constriction, edema of airway mucosa, and mucus plugging of airway lumen.

Asthma is not a new disease; the word itself is derived from the word "aazein," meaning "to exhale with open mouth, to pant" in Greek, and can be found in Homer's Iliad (Marketos and Ballas 1982). Today there are approximately 300 million people worldwide who suffer from asthma (Global Initiative for Asthma 2012a), with the majority in developed countries. This includes Canada, which ranks in the top 10 countries for prevalence (Masoli, Fabian et al. 2004). In Canada alone there were over 2.3 million persons over the age of 12 who self-reported having the disease in 2012 – this is a striking 8.1% of the country's population. Importantly, following the 2004 report from the Global Initiative for Asthma, the prevalence of asthma continues to increase and it is estimated that by 2025, there will be approximately 400 million afflicted individuals worldwide (Masoli, Fabian et al. 2004). This increased prevalence parallels the

continuous urbanization of the global population, though the exact cause of the increase remains elusive (Masoli, Fabian et al. 2004).

Morbidity and mortality due to asthma are significant. Disability-adjusted life years lost per year to this disease total approximately 15 million around the world. Estimates are that worldwide, asthma accounts for 1 death out of every 250, for a cumulative 250,000 deaths annually (Global Initiative for Asthma 2012a). Between pharmaceutical treatments and acute medical care in instances of exacerbations, there is also a significant economic burden. In British Columbia alone, asthma-related health care cost $315.9 million between 2002 and 2007 (Bedouch, Marra et al. 2012). As the prevalence of the disease increases, there is an urgent need for advancements in our understanding of asthma, from both a medical and an economic standpoint.

1.3 Asthma diagnosis

Reversibility of airflow limitation is a defining feature of asthma. Bronchodilators, such as short-acting β2-agonists, cause relaxation of contracted airway smooth muscle. This reduces resistance to airflow and, in the setting of underlying smooth muscle tone, results in an increase in forced expiratory volume in one second (FEV_1). A diagnosis of asthma can be made by a ***post-bronchodilator*** increase in FEV_1 of greater than or equal to 12% and 200 mL (Tepper, Wise et al. 2012, Global Initiative for Asthma 2012b). Similarly, peak expiratory flow (PEF) measurements that improve by 60 L/min (or ≥ 20% of pre-bronchodilator PEF), or that vary more than 20% between days, are indicative of asthma (Global Initiative for Asthma 2012b).

If asthma is well-controlled by therapies, the magnitude of a post-bronchodilator improvement may be less significant. To further assess airway responsiveness, ***methacholine-***,

histamine- or ***exercise-challenge*** tests can be used. Methacholine is a derivative of acetylcholine that binds to specific muscarinic receptors, triggering bronchoconstriction. The concentration of inhaled methacholine that results in a 20% decrease in FEV_1 is known as the provocative concentration $(PC)_{20}$ value. In general, PC_{20} values of less than 4 mg/mL can be considered a positive test for bronchial hyperresponsiveness (BHR) (Crapo, Casaburi et al. 2000). ***Histamine*** challenge may also be used, and can produce bronchoconstriction at approximately equal concentrations as methacholine (Toelle, Peat et al. 1994). However, histamine can produce significant systemic inflammatory side effects, and may provide less reproducible measurements than methacholine (Juniper, Frith et al. 1978, Chatham, Bleecker et al. 1982, Higgins, Britton et al. 1988). ***Exercise*** can also trigger asthma symptoms in susceptible individuals, and as such exercise challenge can be a useful test for the diagnosis of this phenotype of asthma. Exercise challenge can be performed on a treadmill or a cycle ergometer, and is designed such that the patient will reach a target ventilation or heart rate for at least 4 minutes, usually over six to eight minutes of total exercise. A 15% decrease in FEV_1 after exercise challenge is considered indicative of exercise-induced bronchoconstriction (EIB) (Haby, Anderson et al. 1994, Miller, Hankinson et al. 2005).

1.4 Inflammation in the pathogenesis of asthma

Despite its global health burden, no cure for asthma exists. Symptoms of asthma are generally brought about by aberrant recruitment or activation of cells of the immune system, usually through mechanisms initiated by inappropriate skewing or sensitization of the adaptive immune system. This can lead to constriction of the airway smooth muscle surrounding the bronchi, as well as unchecked tissue inflammation and damage (**Figure 1.2**).

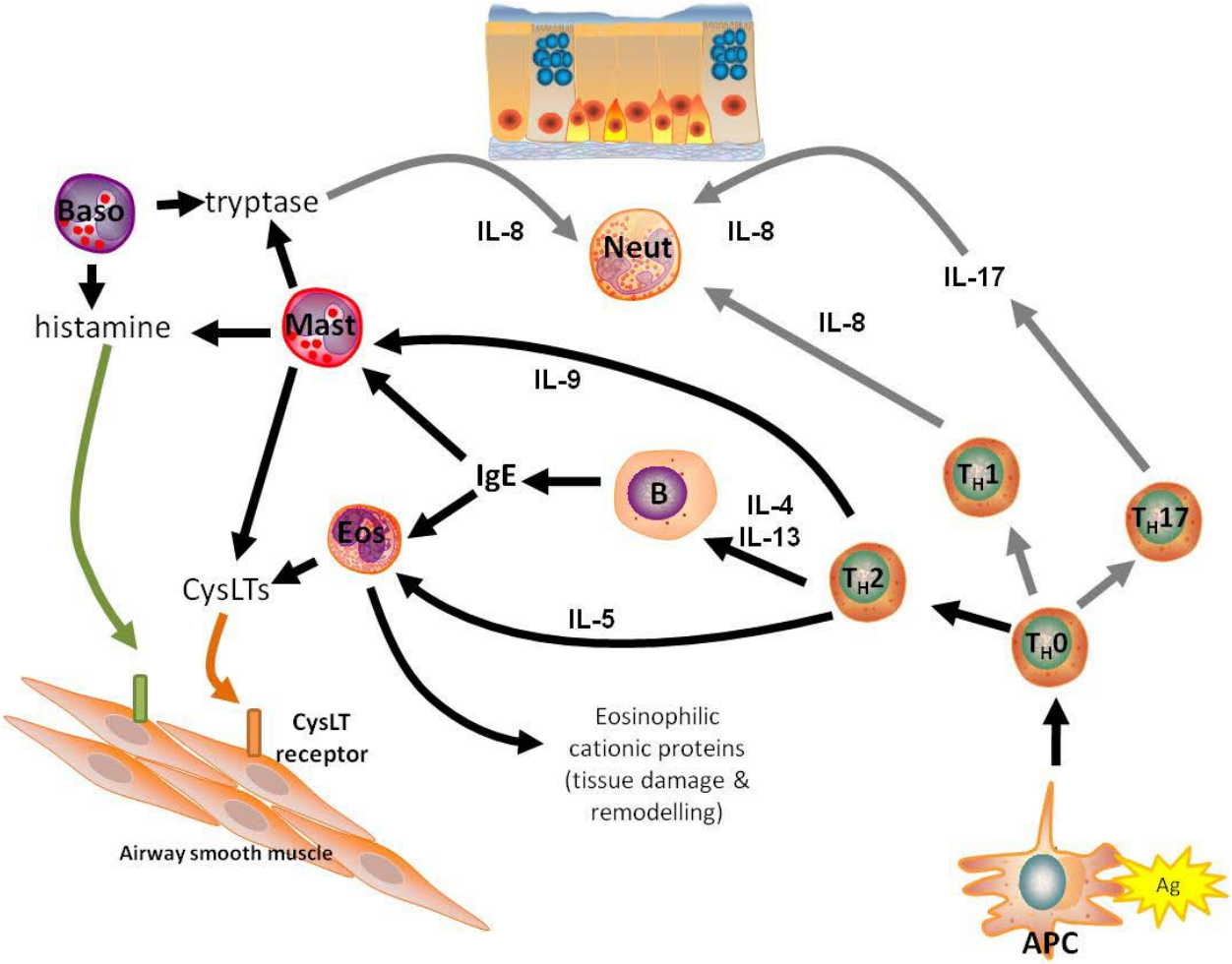


Figure 1.2 Immune and inflammatory cells in asthma.
Canonical T_H2 pathways are shown with black arrows, while pathways favouring other T helper cell polarizations are shown with grey arrows. T_H2 polarization is directed by interaction of APCs with an antigen. T_H2 cells direct B lymphocytes to begin production of specific IgE. Binding of antigen-crosslinked IgE to cell surface Fc receptors on mast cells, basophils and eosinophils induces degranulation and release of mediators such as histamine and tryptase from mast cells and basophils, and production of cysteinyl leukotrienes from both mast cells and eosinophils. Histamine and CysLTs act on bronchial smooth muscle to induce bronchoconstriction. T_H2 cytokines also induce migration and maturation of mast cells and eosinophils. The neutrophil chemoattractant IL-8 is released from epithelial cells activated by mast cell-derived tryptase or T_H17-derived IL-17 and directly from T_H1 cells.

1.4.1 Initiation of allergic sensitization

In the airways, inhaled allergens and particles encounter ***antigen presenting cells (APCs)*** whose function is to sample the tissue microenvironment and alert the immune system to the presence of infection. Macrophages and dendritic cells are the primary APCs of the airway. ***Macrophages*** are phagocytes responsible for engulfing and degrading extracellular bacteria and particles. Macrophages are often found residing in tissues at high risk of external exposure, such as the lung. ***Dendritic cells*** reside within the airway mucosa, and may encounter antigen that has traversed the airway epithelium, but may also extend long processes between the epithelial cells to enable direct encounters with inhaled antigens.

Once an antigen has been internalized and processed/degraded by APCs, it is then loaded onto major histocompatibility complex (MHC) molecules and trafficked to the APC cell surface. Antigen-activated APCs then migrate to lymph nodes, where they can interact with either T or B lymphocytes to induce adaptive immune responses. T-helper cells express CD4 on their surface, and are responsible for cell-mediated immunity and the activation of B cells. Naïve T helper cells (T_H0) can be stimulated by different cytokines to polarize into either T_H1 or T_H2 cells. Stimulation of T_H0 cells with IL-12 or IFNγ leads to ***T_H1*** cells, which produce IL-2, TNFβ and IFNγ, responsible for anti-bacterial cell-mediated immunity by maximizing the anti-microbial functions of macrophages and by promoting proliferation of $CD8^+$ T-cells. Conversely, polarization of T_H0 cells with T_H2 promoting cytokines IL-4, IL-5, or IL-13 will lead to the production of ***T_H2*** cells. T_H2 cells aid in the antibody response and B-cell mediated immunity, including antibody class switching to favour production of antibodies such as IgE.

1.4.2 T_H2 skewing

T_H2 responses are usually stimulated in response to parasitic infections, but in the vast majority of asthmatic patients, there is evidence of T_H2 skewing of the immune system in the absence of such external stimuli. This leads to increased antigen-specific IgE levels in the circulation (Lloyd and Saglani 2013), which has important implications for inflammatory cells such as mast cells and eosinophils. ***Mast cells*** are inflammatory cells that reside in high numbers beneath the airway epithelium. Mast cells are mainly recruited to the tissue by T_H2 cell-derived IL-9, and are more abundant in the airway mucosa of asthmatic individuals (Laitinen, Laitinen et al. 1993, Carroll, Mutavdzic et al. 2002). Mast cells contain abundant cytoplasmic granules with various pre-formed mediators, such as histamine and tryptase. Activation of mast cells occurs when cell-surface Fc receptors are cross-linked by the binding of antigen-crosslinked specific IgE molecules in sensitized individuals, leading to rapid degranulation. The released mediators trigger bronchoconstriction through activation of the histamine receptor H1 on airway smooth muscle cells and tissue remodeling by tryptase-dependent cleavage of protease-activated receptors, which subsequently leads to the production of pro-inflammatory cytokines such as interleukin (IL)-8 from the epithelium (Holgate 2002). In addition, cytokines such as IL-4, IL-5 and IL-13 can also be released from mast cells, which may further promote the T_H2 phenotype (Galli, Kalesnikoff et al. 2005).

Eosinophils are also prevalent in some types of T_H2-driven asthma. Eosinophilic granules contain high levels of cationic proteins, particularly major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin, which can all lead to tissue damage, fibrosis, and further induction of mast cell degranulation (Hogan, Rosenberg et al. 2008). In addition, eosinophils can produce pre-formed cytokines, including transforming growth

factor β (TGFβ), which has been linked to airway remodeling (Kay, Phipps et al. 2004). In fact, eosinophils may be functionally different in asthma, as eosinophils from asthmatic individuals produced the pro-fibrotic factor $TGF\beta_1$ in response to IL-17 and IL-23, whereas eosinophils from healthy individuals did not (Al-Muhsen, Letuve et al. 2013). Interleukin-5 (IL-5) is the primary cytokine responsible for eosinophil recruitment from the bone marrow (Collins, Marleau et al. 1995), and can also promote growth, activation, maturation and survival of eosinophils. The source of this IL-5 can be T_H2 lymphocytes, mast cells, eosinophils and type 2 innate lymphoid cells (ILC2) (Halim, Krauss et al. 2012).

Basophils function similarly to mast cells, releasing histamine and other mediators upon crosslinking of cell-surface Fc receptors with antigen-crosslinked IgE. A new and exciting role for the basophil in airway inflammation was recently described, wherein basophils expressed IL-4 in response to cysteine protease exposure (Motomura, Morita et al. 2014). The basophil-released IL-4 acted on natural helper cells, a subset of ILC2 cells, to induce expression of the T_H2 cytokines IL-5, IL-9 and IL-13, leading to allergen-induced eosinophilic airway inflammation (Motomura, Morita et al. 2014).

Once activated, mast cells and eosinophils can also generate and release arachidonic acid metabolites (eicosanoids) such as cysteinyl leukotrienes (Cys-LTs) (Laidlaw and Boyce 2012). Cys-LTs are metabolized from arachidonic acid via the 5-lipoxygenase pathway to generate leukotriene (LT)A_4, which is subsequently metabolized to the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4. The Cys-LTs bind to their receptors Cys-LT1 and Cys-LT2 to induce contraction of bronchial smooth muscle, increased permeability of blood vessels, mucus secretion, and the recruitment of leukocytes, which are hallmark features of asthma exacerbations.

When considering both clinical and pathological data, T_H2-predominant asthma includes three main clinical phenotypes (Wenzel, 2012). The most prevalent is ***early-onset allergic asthma***, which makes up the vast majority of asthma sufferers. Patients with this phenotype often present in childhood with co-morbidity associated with atopy and other diseases, such as atopic dermatitis and allergic rhinitis. These patients often exhibit specific IgE to allergens and increased T_H2 cytokines. This subset of patients is likely to have genetic contributors to disease, including a strong family history. ***Late-onset eosinophilic asthma*** is more severe and as suggested by the name, eosinophil-predominant and often adult-onset. Finally, a subset of asthmatic individuals experience airway hyperresponsiveness during exercise, known as ***exercise-induced asthma*** (Hallstrand, Kippelen et al. 2013). Exercise-induced asthma appears to be predominantly driven by aberrant mast cell activation.

There are also non-T_H2 asthma subtypes such as ***neutrophilic asthma***, which is generally more severe (Wenzel 2012). Neutrophils are phagocytic granulocytes that are numerous in the circulation. They are recruited in response to IL-8 and other mediators, such as leukotriene B_4 produced by other neutrophils and by macrophages, and can engulf microbes that have been opsonized. Once this occurs, the microbes are degraded in phagosomes by reactive oxygen species and enzymes, including myeloperoxidase and cathepsins. Neutrophilia may lead to tissue damage and inflammation, likely promoting tissue remodeling, in some asthma patients. These patients display notable sputum neutrophilia, increased production of the T_H1 cytokine IL-8, and activation of T_H17 inflammatory pathways (Brusselle, Vanderstichele et al. 2013). ***T_H17*** cell populations are a distinct subset of $CD4^+$ immune cells (Harrington, Hatton et al. 2005, Park, Li et al. 2005) that arise from stimulation with TGFβ, IL-6, IL-21 and IL-23. They produce

cytokines such as IL-17, IL-21 and IL-23. IL-17-producing cells may also make IL-4, and together these cytokines may recruit neutrophils to the airways (Wang, Voo et al. 2010).

1.4.3 The epithelial – immune interface

While the majority of the inflammatory and immune events seen in asthma are mediated by hematopoietic cell lineages, there is also a notable role of resident cells, and in particular the epithelium. As the interface with the inhaled environment, the epithelium is perfectly situated to modulate the immune response to external stimuli such as bacteria, allergens and viruses. In particular, there is a clear role for the epithelial cell in the T_H2 polarization of the immune system (**Figure 1.3**).

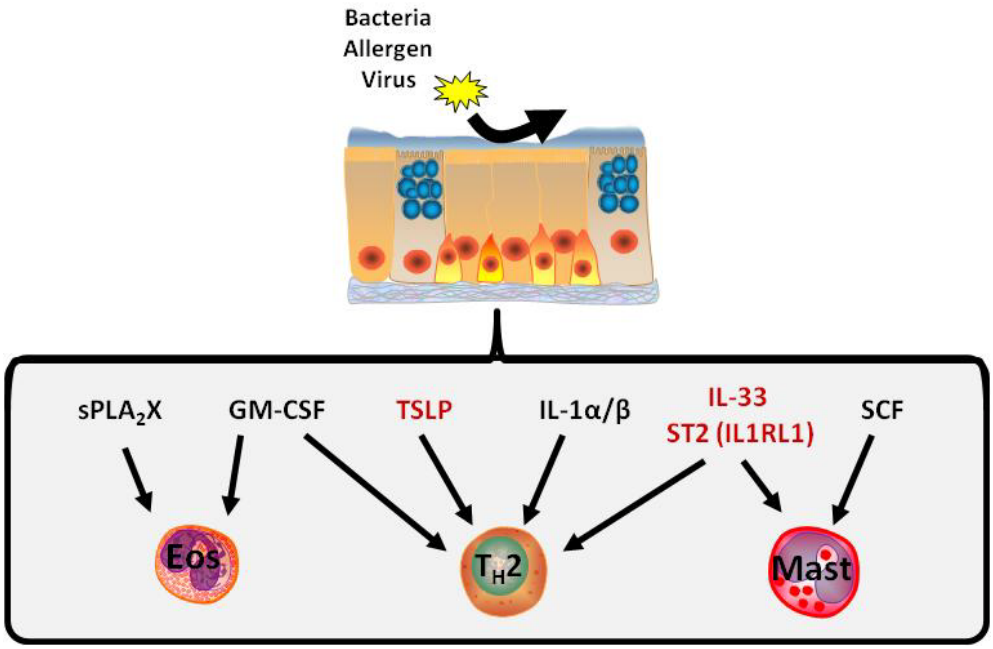


Figure 1.3 Important epithelial-derived mediators in asthma.
The epithelium is able to produce several immune-related cytokines and mediators. For example phospholipase A2 group 10 (sPLA2X) and GM-CSF are able to affect eosinophil survival and function, GM-CSF, TSLP, IL-1α and IL-33 are able to promote T_H2 polarization, and both IL-33 and Stem Cell Factor (SCF) are able to recruit mast cells. Furthermore, the epithelial-derived cytokines TSLP, as well as IL-33 and its receptor ST2 have been identified by genome-wide association studies as carrying significant association with asthma (denoted in red).

Many emerging pro-T_H2 cytokines are released by epithelial cells following stimulation of their pattern recognition receptors by allergens such as house dust mite, including thymic stromal lymphopoietin (TSLP), granulocyte/macrophage colony stimulating factor (GM-CSF), IL-1α, and IL-33 (Hammad, Chieppa et al. 2009, Phipps, Hansbro et al. 2009, Willart, Deswarte et al. 2012). These cytokines may also be induced in response to the T_H2 cytokines IL-4 and IL-13, for which the receptors exist on epithelial cells, indicating a feedback regulatory mechanism wherein the epithelial cell is centrally involved in perpetuating immune system polarization (Kato, Favoreto et al. 2007). IL-33 is of particular interest, as it is a member of the IL-1 innate cytokine family (Schmitz, Owyang et al. 2005) and is known to promote T_H2 inflammation, asthma and allergy (Saglani, Lui et al. 2013).

The epithelium is also able to attract and modulate several of the inflammatory cell types involved in the asthmatic response. For example, mast cells are recruited by epithelial-derived stem-cell factor (SCF) (Al-Muhsen, Shablovsky et al. 2004). CC-chemokine ligand 11, or eotaxin-1, is a chemoattractant for eosinophils that is produced by the airway epithelium (Garcia-Zepeda, Rothenberg et al. 1996). Furthermore, GM-CSF secreted by epithelial cells can prolong eosinophil survival (Uddin, Seumois et al. 2008). The epithelium can also influence production of eicosanoids by eosinophils via production of soluble phospholipase A_2 group 10 (sPLA$_2$-X), possibly enhancing asthma exacerbations (Hallstrand, Lai et al. 2012).

1.5 The epithelium is central to asthma susceptibility

Attempts to elucidate the reason for the predominant T_H2 skewing of the immune system in asthma have led to what is termed the ***hygiene hypothesis***. The predilection of the modern era is the minimization of dirt and dust in the home. In addition, the increased populations residing in

urban environments rather than rural, farming-type environments, decreases exposures to classic T_H1 antigens, such as bacterial lipopolysaccharide, which would have been more prevalent in less clean environments (Strachan 1989). This has led to the hypothesis that exposure to endotoxin may in fact have a protective effect against atopy in childhood (Braun-Fahrlander 2003). ***Exposure to allergens*** such as house dust mite, cockroach allergen, or pet dander may also lead to allergic sensitization. However, not all individuals develop asthma in the face of such environmental exposures, even among those who have developed allergic sensitization (atopy), indicating that some individuals display intrinsic asthma susceptibility.

The role of recurrent early-life ***viral infections*** in asthma development has been a topic of immense interest in recent years, particularly in the setting of patients who already have allergic sensitization (Holt and Sly 2012). Individuals who experience recurrent lower respiratory tract viral infections during the period when postnatal lung growth and differentiation are at their peak may be 10 to 30-fold more likely to develop severe and persistent asthma (Oddy, de Klerk et al. 2002). In line with this, children who had experienced viral infections and who were atopic at two years of age were significantly more likely to have persistent wheeze (Relative Risk (RR) 3.5) and asthma (RR 4.92) at 10 years (Kusel, Kebadze et al. 2012). These findings suggest that certain individuals are more susceptible to virus-induced wheeze, as allergic sensitization can increase the risk of viral infections leading to wheeze, but viral infection itself does not lead to allergic sensitization (Jackson, Evans et al. 2012). Early-life persistent wheeze has also been noted to carry with it significant features of airway remodeling even in the absence of atopy (Turato, Barbato et al. 2008), signifying the importance of susceptibility to multitrigger wheezing in asthma pathogenesis.

It is clear that an individual's ***genetic predisposition*** is an important component in the development of asthma. However, estimates of heritability range from 35 – 90% (Ober and Yao 2011). Genome-wide association studies have further highlighted a number of polymorphisms that are significantly associated with asthma, including filaggrin (FLG), TSLP, IL-33 and its receptor ST2 (IL1RL1), and the gasdermin family (GSDMA/B). Interestingly, many of these genes fall within the spectrum of the epithelium and its position at the interface between the immune system and the environment, emphasizing the importance of the epithelial tissue microenvironment in the initiation of asthma pathology.

Mutations in the epithelial barrier protein filaggrin have been found to carry significantly high risk for atopic dermatitis, and also for asthma in those individuals who already have atopic dermatitis (Palmer, Irvine et al. 2006). Filaggrin is important in epidermal differentiation and barrier formation; its mutation disrupts the epidermis. Interestingly, despite its association with asthma, filaggrin does not appear to be expressed in the bronchial epithelium (Ying, Meng et al. 2006). This may suggest that disruption of any epithelial barrier can lead to inappropriate immune sensitization.

TSLP is an epithelial-derived, T_H2-promoting cytokine; polymorphisms in the TSLP gene are significantly associated with asthma and airway hyperresponsiveness (He, Hallstrand et al. 2009). Asthma-associated polymorphisms were also found in the T_H2-promoting cytokine IL-33 and its receptor ST2 (IL1RL1), as well as the ORMDL3/GSDMB locus (specific to childhood-onset disease) (Moffatt, Gut et al. 2010). Polymorphisms at this locus on chromosome 17q21were significantly associated with whole-lung expression of GSDMA (Hao, Bosse et al. 2012). In this study, the authors demonstrated GSDMA protein expression in the airway epithelium. Interactions between genetic loci at 17q21, including variants of ORMDL3 and

GSDMB, and human rhinovirus infection have been described recently and implicated in the risk of developing asthma (Caliskan, Bochkov et al. 2013). These findings provide further evidence to support the idea that while both genetic and environmental factors are required for the development of asthma in susceptible individuals, the epithelium is central to this process.

1.6 Airway wall remodeling in asthma

While inflammation is clearly involved in asthma pathogenesis, there is also significant structural remodeling of the airways (James and Wenzel 2007). Airway remodeling in asthma can be defined as changes in cellular and extracellular composition and organization of the airway wall. Some of these changes include increased airway smooth muscle mass, increased angiogenesis, an expanded population of fibroblasts, thickened basement membrane, and of the utmost importance for the present body of work, an intrinsically altered epithelium (**Figure 1.4**). Many aspects of remodeling have been found to occur in early childhood, in mild disease, and in the absence of atopy, suggesting that remodeling may actually occur independently of excessive and repeated airway inflammation (Barbato, Turato et al. 2003, Payne, Rogers et al. 2003, Payne, Qiu et al. 2004, Barbato, Turato et al. 2006).

1.6.1 Increased airway smooth muscle

An ***increase in airway smooth muscle (ASM) mass***, caused by hyperplasia or hypertrophy is an important component of the remodeled airway wall in asthma (James 2005). Although the precise mechanisms behind the increased mass are unknown, a shift toward a synthetic phenotype with increased proliferation rates is believed to play a role in the development of ASM thickening (Halayko, Tran et al. 2006, Bai, Liu et al. 2007). Longitudinal studies

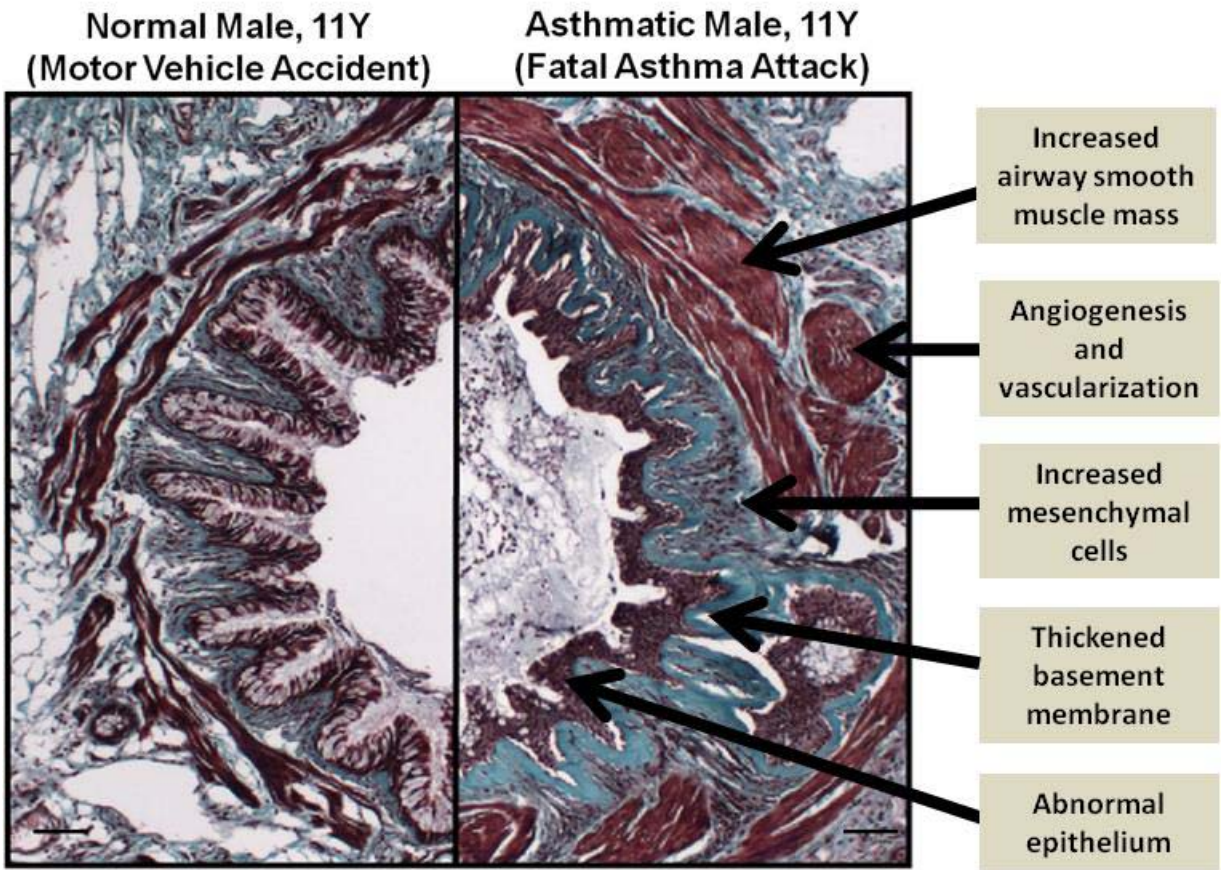


Figure 1.4 Histological features of airway remodeling in asthma.
FFPE sections of age-matched, sex-matched upper airways obtained from healthy (left) or asthmatic (right) human donor lungs deemed unsuitable for transplant. Masson's trichrome stain allows visualization of collagen (blue-green), keratin and muscle (red) and cytoplasm/intercellular space (light purple). Arrows indicate commonly remodeled components of human airways, including increased airway smooth muscle mass, increased vascularization, more mesenchymal cells, thickening of the basement membrane, and abnormal epithelium. There is also clear evidence of mucus plugging of the airway lumen (centre). Scale bar is 100 µm. Image courtesy of F. Shaheen and T.L. Hackett (Vancouver, Canada).

evaluating the contribution of ASM proliferation to asthma are lacking, but it is possible that ASM proliferation occurs very slowly *in vivo* or only occurs sporadically (James 2005). The increase in ASM mass may be present very early in disease progression, as biopsies from preschool wheezers who went on to develop asthma later in childhood were found to already

present this feature (O'Reilly, Ullmann et al. 2013). Recent work has provided compelling evidence that the mechanical properties of airway smooth muscle from asthmatic patients are actually very similar to that from non-asthmatics; the only differences in function were that at longer lengths, asthmatic ASM was stiffer, and was more resistant to the loss of force generation that usually occurs in response to length oscillation (Chin, Bosse et al. 2012). This indicates that the intrinsic properties of ASM may not in fact differ in asthmatic individuals, but rather that the inflammatory milieu in which the muscle exists *in vivo* is the driving force behind excessive bronchoconstriction. Increased smooth muscle mass, importantly, may lead to excessive airway narrowing during an acute exacerbation of asthma.

1.6.2 Angiogenesis and vascularization

Angiogenesis and vascularization are also features of airway remodeling. There is a reported increase in the number of blood vessels per unit area in asthma (Baluk, Lee et al. 2004), although vessel size is similar when analysis is based upon the outer diameter (Green, Butt et al. 2006). However, based on intimal thickening, asthmatic blood vessels were hypertrophic compared to control vessels and this abnormality correlated with disease duration. Barbato and colleagues have also reported a significant increase in vascularization in the airways of asthmatic children (Barbato, Turato et al. 2006). This was shown as the number of vessels per square millimeter of tissue, but was not reflected in the percentage area of the airway wall occupied by vessels. Interestingly, this increase in the numbers of vessels in the airway wall in asthmatics was not significantly in excess of that seen in atopy alone, suggesting that angiogenesis may in fact be mediated by the inflammatory response.

The factor most likely responsible for this angiogenic activity is vascular endothelial growth factor (VEGF). Elevated VEGF protein was detected in lavage fluid from atopic asthmatic adults compared to healthy individuals (Simcock, Kanabar et al. 2007) and in induced sputum of acute asthmatic children compared to normal children, suggesting an early activation of the angiogenic process (Abdel-Rahman, el-Sahrigy et al. 2006, Baren, Boudreaux et al. 2006). A direct link between allergen exposure and angiogenesis has been reported by Capetandes and colleagues who showed that exposure of lung epithelial cells to house dust mite allergen increases VEGF expression and release of mediators that stimulate fibroblasts to increase VEGF secretion (Capetandes, Zhuang et al. 2007). Increased angiogenesis and vascularization may exacerbate airway narrowing by promoting tissue edema and infiltration of inflammatory cells.

1.6.3 Mesenchymal alterations

In asthmatic airways, there is accumulation of sub-epithelial mesenchymal cells, including fibroblasts and myofibroblasts. Fibroblasts are spindle-shaped cells that lie directly beneath the basement membrane and can generally be identified by expression of vimentin and EDA-fibronectin, the intracellular variant of fibronectin. During wound repair, the fibroblast is highly synthetic and produces the provisional collagen matrix that is required for wound healing (McPherson 1988). When activated during wound repair, fibroblasts transition into myofibroblasts, which express α-smooth muscle actin through *de novo* synthesis of stress fibres, and which are able to remodel the extracellular matrix (ECM) (Hinz 2010). These properties are normally utilized during wound closure to draw wound edges together. Myofibroblasts can also secrete chemokines, cytokines, arachidonic acid metabolites and protease inhibitors, which enable tissue remodeling and trafficking of inflammatory cells into wounded regions. Once

wound healing is complete, myofibroblasts disappear through apoptosis (Darby, Skalli et al. 1990).

In asthma, the source of these excess fibroblasts and why they persist is unknown (Roche, Beasley et al. 1989). Previous data demonstrated a significant role for circulating, bone marrow-derived ($CD34^+$), collagen-producing cells called fibrocytes in remodeling of the lung and airways in asthmatic humans following allergen exposure and in the ovalbumin mouse model of allergic asthma (Schmidt, Sun et al. 2003). Similarly, proliferation and plasticity of resident fibroblasts may contribute to the increased fibroblast population in asthma. For example, mediators involved in allergy and inflammation, such as histamine, play a role in the induction of airway remodeling by increasing fibroblast proliferation and connective tissue growth factor (CTGF) expression in human fetal lung fibroblasts *in vitro* (Kunzmann, Schmidt-Weber et al. 2007). Recently, a role for the T_H2 cytokine IL-13 was described in initiating airway fibroblast invasion *in vitro* through a $TGF\beta_1$ and MMP-dependent mechanism (Ingram, Huggins et al. 2011). The TGFβ-induced activation could be blocked by inhibitors of TGFβ and MMPs, but only in cells obtained from asthmatic donors, indicating that fibroblasts from asthmatic patients are intrinsically altered. An important function of mesenchymal cells is the production of extracellular matrix proteins, which are incorporated into the ***basement membrane*** and surrounding mesenchymal stroma.

1.6.4 Thickening of the basement membrane

The basement membrane separates the underlying mesenchyme from the epithelium. It provides attachment points for epithelial cells, binds growth factors, hormones and ions, and is important for communications between epithelial and mesenchymal cells (Evans, Fanucchi et al. 2010).

The basement membrane when assessed by electron microscopy is actually made up of three distinct layers – the lamina lucida and lamina densa (that together make up the basal lamina), and lamina reticularis. The basal lamina is directly beneath the epithelium and is primarily composed of collagens, laminins, fibronectin, proteoglycans, stored growth factors, and glycoproteins. The lamina reticularis (or reticular basement membrane) is the layer that can be seen with the light microscope. It is primarily composed of collagen fibrils (primarily types I, III and V) crosslinked by smaller collagen fibres, fibronectin, tenascin (Laitinen, Altraja et al. 1997), and proteoglycans such as perlecan. This layer, and particularly perlecan, can store and modulate availability of many growth factors.

Thickening of the reticular basement membrane (RBM) is a common feature of airway remodeling in asthma. It occurs early in disease progression, with airways from pediatric asthmatics showing the same extent of RBM thickening as those from adults (Payne, Rogers et al. 2003). This thickening is common to both non-atopic and atopic confirmed wheezers, (Saglani, Payne et al. 2007), but as there was no difference in RBM thickness between those who did and did not develop asthma later in childhood, a thickened RBM may be a more general feature of airway inflammatory pathology (O'Reilly, Ullmann et al. 2013).

The most likely contributor to the increased basement membrane thickness are the fibroblasts and myofibroblasts, numbers of which correlate with the magnitude of subepithelial thickening (Gizycki, Adelroth et al. 1997), even in steroid-naïve mild asthmatics (Nihlberg, Larsen et al. 2006). The sources of stimuli for fibroblasts to synthesize excess or altered matrix are variable, however. In response to IL-13, fibroblasts from asthmatic patients produce increased amounts of collagen type 1 *in vitro* compared to fibroblasts from non-asthmatic patients (Firszt, Francisco et al. 2013). Allergic inflammation may also lead to increased

production of collagen III by lung fibroblasts, which is mediated by nerve growth factor and independent of TGFβ_1, in a murine model of asthma (Kilic, Sonar et al. 2011).

There has been some debate as to the nature of this thickened basement membrane and whether it is normal in composition or perhaps indicative of an aberrant repair response. Studies have shown increased deposition of collagens I, III and V, fibronectin, tenascin, lumican and biglycan in asthmatic airways (Roche, Beasley et al. 1989, Laitinen, Altraja et al. 1997, Wilson and Li 1997, Huang, Olivenstein et al. 1999, Karjalainen, Lindqvist et al. 2003, Fedorov, Wilson et al. 2005). The ratio of collagen fibrils to overall matrix did not differ significantly between asthmatic and normal patients in any age group studied (Saglani, Molyneux et al. 2006). However, later work in infant monkeys exposed to house dust mite demonstrated a reduction in the anchoring collagen filaments (Evans, Fanucchi et al. 2010). As techniques for observing the structure of the basement membrane evolve, this debate may begin to be resolved. However, what is clear is that alterations in the structure, thickness and composition of the basement membrane may have important repercussions for the cells that lie directly adjacent to this structure – namely the aforementioned fibroblasts and myofibroblasts, and the epithelium.

1.7 The airway epithelium

The airway epithelium forms the barrier between the inhaled environment and the body. It is a pseudostratified, columnar epithelium composed of several types of epithelial cells, whose nuclei do not lie in the same plane. In a healthy individual, the luminal surface of the airway epithelium is primarily comprised of ***columnar*** epithelial cells, which are perpendicular to the basement membrane. Of these, ***ciliated*** cells predominate (**Figure 1.5**). Ciliated cells each contain up to 300 cilia, which beat synchronously to catch the gel phase of the airway mucus layer and sweep

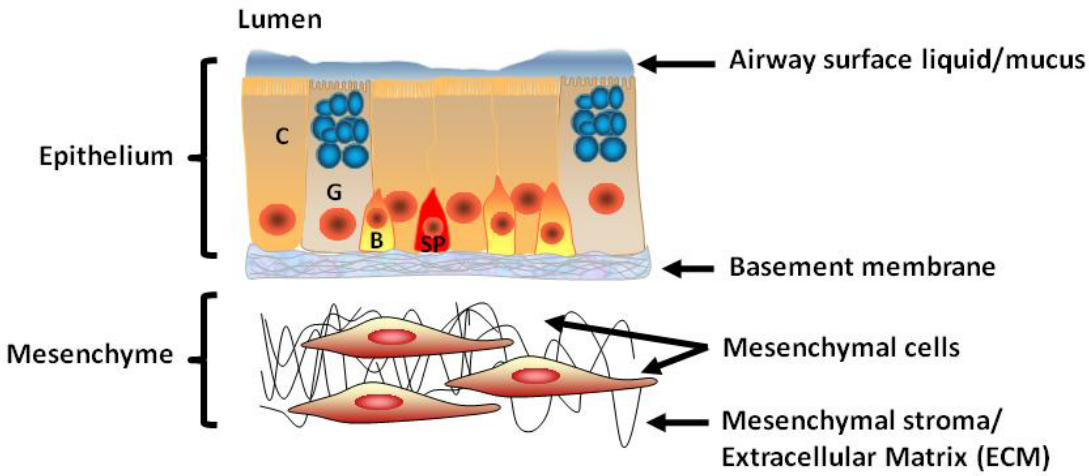


Figure 1.5 Structure of the airway epithelium and mesenchyme.
Epithelial cells in human airways larger than approximately 0.5 mm in diameter display ciliated cells (C), mucus-producing goblet cells (G) and basal cells (B). Some basal cells are also side population (SP) progenitor/stem cells, as will be discussed later in this chapter. Beneath the basement membrane lie the mesenchymal cells, which reside within the mesenchymal stroma.

trapped debris towards the esophagus. ***Goblet cells*** are unicellular paracrine glands that are specialized for production of the airway mucus layer. Goblet cells make up approximately 9% of cells in the trachea, but are generally not found in distal airways. Mucus also contains proteins secreted by ***serous cells*** of submucosal glands and small airways, including compounds with antimicrobial, anti-inflammatory, and antioxidant properties (Jeffery 1983, Basbaum, Jany et al. 1990, Finkbeiner 1999). ***Club (Clara) cells*** are tall, columnar, non-ciliated cells found in the terminal bronchioles of humans. Club cells secrete Club cell secretory protein (CCSP), a component of surfactant. Finally, ***basal cells*** are small, cuboidal cells defined by the parallel orientation of their nuclei to the basement membrane. Basal cells are found within the larger airways, where they constitute up to 30% of the total population of epithelial cells (Rock, Onaitis et al. 2009). As the airways decrease in diameter, the epithelium becomes a simple columnar

structure, and basal cells are found in clusters, before becoming individually distributed (Nakajima, Kawanami et al. 1998). In the respiratory bronchioles, airways of approximately 0.5 mm in diameter, the epithelium becomes a simple cuboidal layer, and basal cells are not observed.

1.7.1 Structural alterations of the airway epithelium in asthma

The asthmatic epithelium is far from normal, in many respects (**Figure 1.6**). Many studies have provided convincing evidence of structural fragility of the epithelium of asthmatic individuals, and this can occur even in early childhood. Biopsy samples showed a significantly higher percentage of epithelial loss in asthmatic children than either atopic or control children (Barbato, Turato et al. 2006). Interestingly, most often these sloughed cells were columnar epithelial cells, and only rarely was there concurrent loss of both basal and columnar cells.

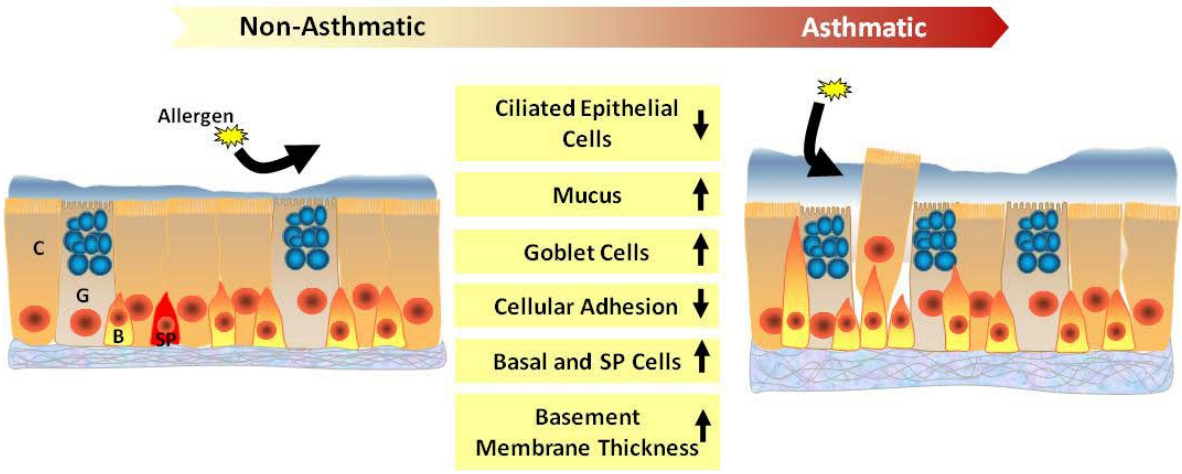


Figure 1.6 Schematic of airway epithelial alterations in asthma.
Schematic demonstrating the pseudotratified airway epithelium and consitituent epithelial cells including B, Basal; C, Ciliated; G, Goblet and SP, Side Population. Common features of epithelial remodeling in asthma are outlined in yellow boxes.

Excess mucus production is a common feature of asthmatic airways, particularly through the hyperplasia of goblet cells (Bai and Knight 2005). There is abundant evidence that this phenomenon is primarily dependent upon the *in vivo* inflammatory milieu, particularly T_H2 cytokines. In support of this, IL-13 and the repair-mediating epidermal growth factor receptor (EGFR) induce goblet cell hyperplasia in mouse airways (Tyner, Kim et al. 2006, Park, Korfhagen et al. 2007). Goblet cell differentiation is driven by transcription factors; both T_H2 cytokines and house dust mite allergen induce the expression of SAM pointed domain-containing ETS transcription factor (SPDEF) in club cells, which results in their differentiation into goblet cells (Park, Korfhagen et al. 2007). This process was found to be dependent upon STAT-6, which is a downstream signaling molecule of the T_H2 cytokines IL-13 or IL-4 (Park, Korfhagen et al. 2007). SPDEF is also regulated by the FOXM1 transcription factor (Ren, Shah et al. 2013). This finding of the dependence of goblet cell hyperplasia on inflammation has been confirmed in *in vitro* studies, wherein goblet cell numbers did not differ in air-liquid interface cultures of airway epithelial cells from asthmatic and non-asthmatic donors, despite numerous other inherent differences in phenotype (Hackett, Singhera et al. 2011).

In addition to alterations in columnar epithelial cells, we have also described a marked increase in the proportion of basal cells in the asthmatic epithelium (Hackett, Shaheen et al. 2008, Hackett, Warner et al. 2009). This expansion has potential implications for many aspects of airway epithelial maintenance and repair, including adhesion to the extracellular matrix, proliferative and progenitor potential, and susceptibility to external mediators, as will be discussed in the following sections.

1.7.2 Maintenance of epithelial barrier integrity

The average human adult takes 12 to 18 breaths per minute, each with an average volume of 500 mL, thus inhaling a vast quantity of air each day (Sherwood 2006). A multitude of potentially injurious stimuli such as viruses, fungi, particles, noxious gases, allergens and other environmental pollutants exist in the air we breathe. As the gatekeeper to the inside of the lung, the epithelium must possess enormous capacity to withstand these insults, and to reconstitute an appropriate barrier following injury.

The integrity of the epithelial barrier is maintained by the multiple adhesions that tether epithelial cells to one another and to the underlying matrix (**Figure 1.7**). The basal cells are of particular importance for the maintenance of structural integrity. Basal cells attach to the basal lamina via structures known as ***hemidesmosomes***, which are primarily composed of the integrin dimer α6β4, which links to the extracellular matrix protein laminin 5 and intracellularly to the intermediate filament network. Basal cells are the only epithelial cell type to exhibit this type of attachment to the matrix. Cell-cell contacts called ***desmosomes*** provide structural attachments between basal cells and columnar cells. Desmosomes consist of non-classical cadherins such as desmoplakin and plakoglobins that are anchored to intermediate filaments via an “adhesion plaque”. Basal cells thus provide concrete points of anchor for columnar cells at their lateral surfaces. This has been demonstrated by the finding that the number of basal cells significantly correlates with overall epithelial height, presumably through structural support of columnar cells (Evans and Plopper 1988).

Gap junctions, made up of connexins, provide direct accessibility as portholes between adjacent cells. ***Tight junctions*** regulate paracellular permeability and maintain apical-basal polarity of cells. Tight junctions are made up of transmembrane proteins such as junctional

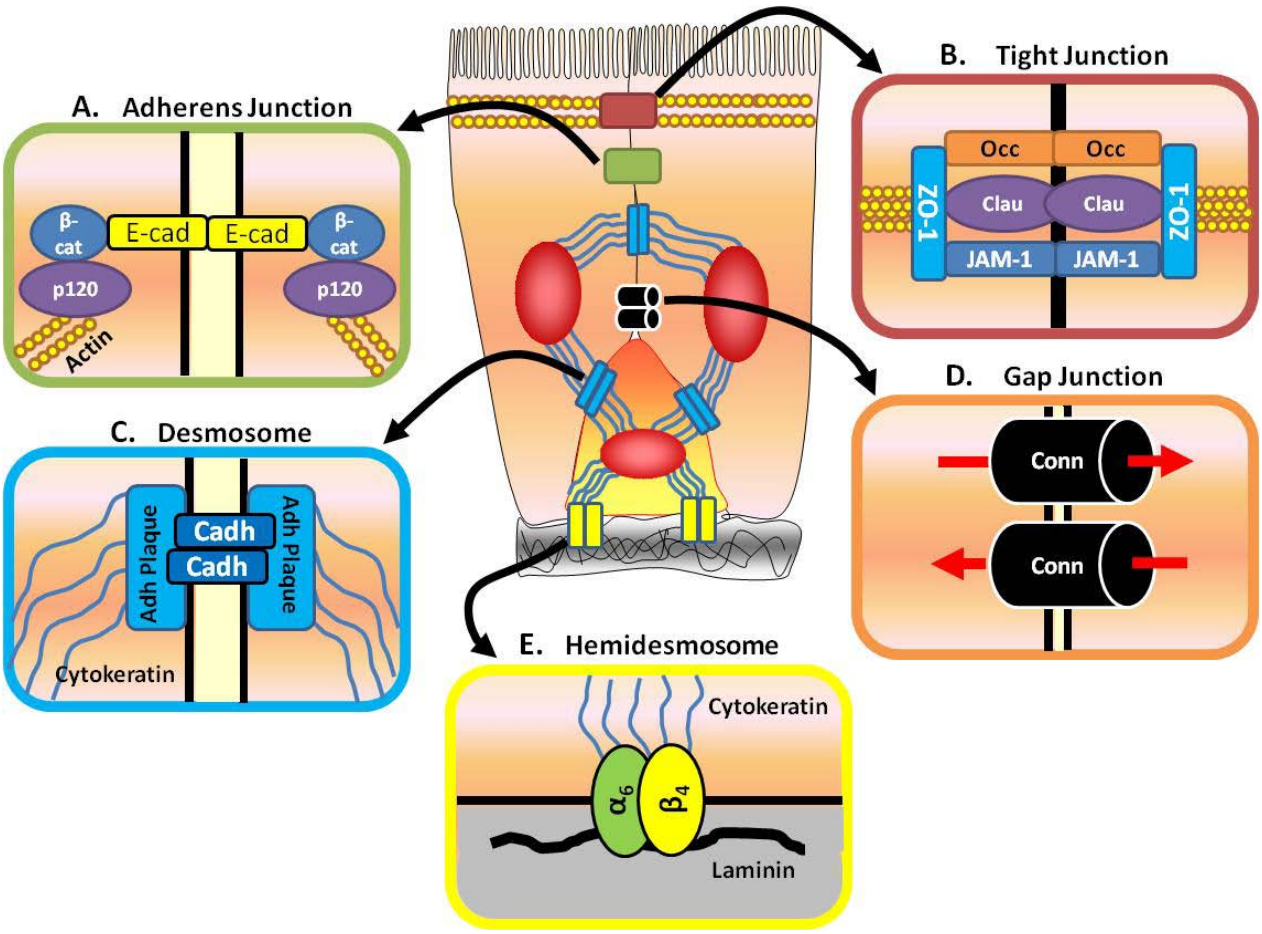


Figure 1.7 Epithelial junctional complexes.
(**A**) Adherens junctions are composed of several proteins (E-cad; E-cadherin, β-cat; β-catenin, p120; p120 catenin) that attach to the actin cytoskeleton (yellow). (**B**) Tight junctions include proteins such as Occludin (Occ), Claudin (Clau), Junctional Adhesion Molecule 1 (JAM-1) held together by the scaffold Zonula Occludens-1 (ZO-1), which is tethered to the actin cytoskeleton (yellow). (**C**) Desmosomes attach neighbouring cells via adhesion plaques (Adh plaque) comprised of cadherins (Cadh), desmoplakin and desmoglein tethered to the intermediate filament cytokeratins. (**D**) Gap junctions are made of connexins (Conn) that allow solute transport directly between cells. (**E**) Hemidesmosomes attach basal cells to the basement membrane using α6β4 integrins, which attach intracellular cytokeratin filaments to extracellular laminin.

adhesion molecules (JAMs), occludins and claudins. These anchor to the cytoskeleton by zonula occludens (ZO)-1, occludin-2, occludin-3, and cingulin. ***Adherens junctions*** mechanically connect adjacent cells, and also mediate the initiation of proliferation and differentiation of

epithelial cells. Adherens junctions are comprised of several proteins; transmembrane E-cadherin anchors to the actin cytoskeleton via catenins, including β- and α-catenin. The presence of adherens junctions provides an environment favourable for the formation of tight junctions; loss of adherens junction stability leads to delocalization of the tight junction proteins ZO-1, occludin and claudins (Jeffery, Wardlaw et al. 1989, Foresi, Bertorelli et al. 1990). In a healthy individual, junctions maintain the epithelial barrier.

In asthma, the maintenance of epithelial junctions appears to be compromised. There was a significant decrease in cadherins in biopsies from mild asthmatic donors (Trautmann, Kruger et al. 2005, Heijink, Kies et al. 2007). Our research group demonstrated a significant reduction in E-cadherin expression in the airway epithelium of asthmatic patients, both *in vivo* and when cultured at air-liquid interface *in vitro* (Hackett, Singhera et al. 2011). Associated with this, novel genes such as the adhesion molecule catenin alpha-like 1, which is linked to asthma susceptibility, may play a role in epithelial wound repair and proliferation (Xiang, Tan et al. 2008). Loss of E-cadherin-dependent intercellular adhesion promotes T_H2 cell recruitment through EGFR signalling, thereby further injuring the epithelium through inflammatory mechanisms (Heijink, Kies et al. 2007).

1.7.3 Restitution of the airway epithelium

Under normal circumstances, damage or loss of single epithelial cells or even small clusters of cells can occur without disrupting the integrity and permeability of the epithelium. In contrast, epithelial damage occurring in response to inflammatory insults, such as allergen challenge, causes widespread sites of epithelial damage (Erjefalt, Korsgren et al. 1997a, Erjefalt, Korsgren et al. 1997b). In such situations, the epithelium responds in a more robust fashion to repair and

reconstitute a functional barrier. The process of epithelial restitution is thought to follow four distinct steps, which were described elegantly in guinea pig studies from Erjefalt et al. (**Figure 1.8**). Following damage, cells surrounding the wound site de-differentiate, and then flatten and migrate rapidly to cover the denuded area. This occurs within hours *in vivo*, and the migrating cells move at a rate of several microns per minute. Proliferation then occurs to repair the denuded area, usually peaking between 15 and 30 hours after injury. In the following days, the cells re-differentiate so that after five days, the epithelium is populated by ciliated and secretory cells (Erjefalt, Erjefalt et al. 1995).

In vivo, the epithelium does not exist in isolation, and many other factors are involved in repair of mucosal tissues. Following injury, there is an accumulation of provisional matrix upon which the repairing epithelium can migrate and adhere. This is particularly important in settings where the basement membrane integrity has been compromised. The provisional matrix is primarily composed of plasma-derived fibrin, fibronectin and vitronectin, and fibroblast-derived fibronectin, collagens, proteoglycans and cytokines, but there is also production of fibronectin and collagen IV by epithelial cells (Sacco, Silvestri et al. 2004). In order to adhere to and migrate on this matrix, epithelial cells must repeatedly dissolve and rebuild intercellular junctional proteins, as well as transmembrane proteins such as integrins that attach intracellularly to the actin cytoskeleton and externally to the provisional wound repair matrix. In fact, integrin $\alpha 5\beta 1$ is rapidly upregulated following epithelial injury, and allows migration on provisional matrix (Pilewski, Latoche et al. 1997). Cellular migration and tissue remodeling is augmented by proteases released from the epithelium, such as matrix metalloproteinase (MMP)-7 and MMP-9, which cleave extracellular matrix proteins, including basement membrane components, and cell-cell junctions. MMP-7 is particularly important for airway repair, as it may lead to epithelial

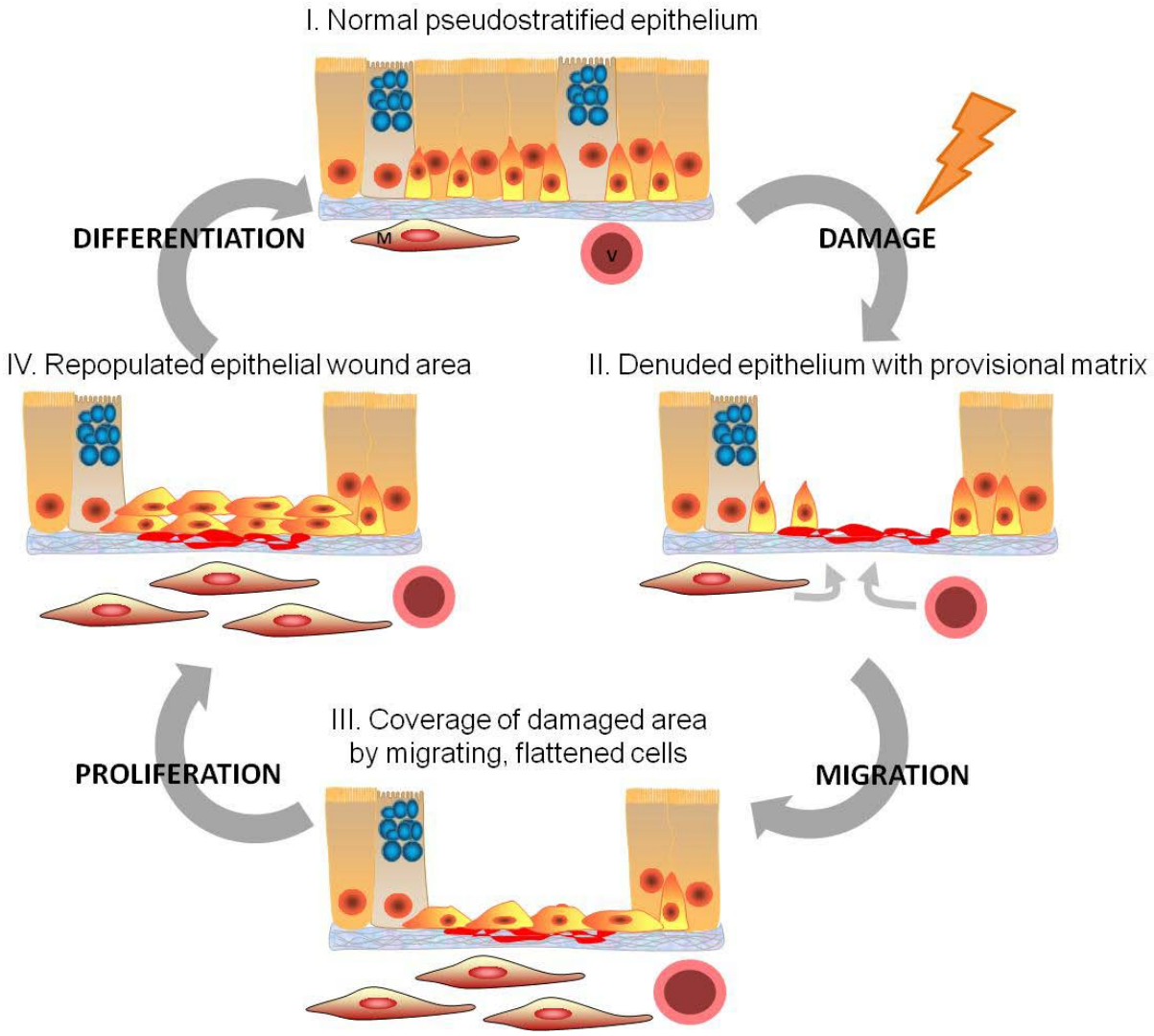


Figure 1.8 Epithelial restitution is a multi-step process.
Following damage, provisional matrix (red lines) is deposited in the injured area by plasma exudates, mesenchymal cells, and the epithelium. Epithelial cells bordering the injured area rapidly flatten and migrate to cover the denuded area, and neighbouring mesenchymal cells are recruited to the wound area and induced to proliferate. The epithelial cells proliferate to repopulate the wounded area, and finally, over a span of several days, re-differentiate to the various cell types required for normal tissue function. During this time, the provisional matrix is remodeled by mesenchymal cells, and excess mesenchymal cells are cleared by apoptosis to restore the tissue to its normal structure. C = Ciliated, G = Goblet, B = Basal, M = Mesenchymal (Fibroblast/Myofibroblast), V = Vessel.

migration by cleaving junctional proteins such as E-cadherin (Crosby and Waters 2010).

Shortly after the onset of the epithelial repair process, several factors cause mesenchymal cell proliferation (Erjefalt, Erjefalt et al. 1995). Fibronectin, in addition to providing a matrix attachment for epithelial migration and adherence via integrin α5β1, promotes fibroblast proliferation and migration (Sacco, Silvestri et al. 2004). Additionally, the injured epithelium releases several growth factors, such as TGFβ_2, insulin-like growth factor, basic fibroblast growth factor, platelet-derived growth factor and endothelin-1, which together augment the proliferation and migration of airway fibroblasts (Knight and Holgate 2003). Normally, once repair is complete, the provisional matrix is remodeled, and these additional mesenchymal cells undergo apoptosis. However, in settings of ongoing inflammation and injury, or if inappropriate repair mechanisms continue, mesenchymal cells persist and produce excess matrix proteins such as collagen, leading to airway fibrosis or remodeling. Complete epithelial restitution is required to halt the remodeling cycle that occurs during a wound repair response. Incomplete restitution may have multifaceted downstream effects on airway structure and function.

1.7.4 Resident epithelial progenitor cells in human airways

The mechanism of epithelial restitution requires progenitor cells with immense proliferative potential, self-renewal capacity and the ability to differentiate into specific lineages of cells, including basal, ciliated and goblet cells. Several airway progenitor cell types have been proposed, both circulating and tissue-resident, though the contribution of the different populations is likely affected by the degree and mechanism of injury. In settings of extensive injury, bone marrow-derived progenitor cells localize to various tissues and differentiate into tissue-specific cells. Circulating progenitor cells may express epithelial markers, for example

cytokeratin (CK)-5 (Gomperts, Belperio et al. 2006), or CCSP (Wong, Keating et al. 2009), or mesenchymal markers such as Flk-1 (Yan, Liu et al. 2007). Recent evidence from studies of intestinal repair suggests that rather than being permanently incorporated, circulating mesenchymal stem cells instead use paracrine signaling to promote resident epithelial progenitor activity *in situ* (Semont, Demarquay et al. 2013).

Several distinct tissue-resident progenitor cell populations and repair mechanisms have been identified in experimental models of airway injury. Airway progenitor cells include club cells, epithelial basal cells, cells residing in or near submucosal glands, at bronchoalveolar duct junctions (BADJs), and in neuroepithelial bodies (NEBs) (Breuer, Christensen et al. 1987, Reynolds, Giangreco et al. 2000, Hong, Reynolds et al. 2001, Hong, Reynolds et al. 2004a, Hong, Reynolds et al. 2004b). There is also some evidence that ciliated cells can contribute to wound repair (Erjefalt, Erjefalt et al. 1995), through rapid transdifferentiation to repair naphthalene-damaged tissue (Park, Wells et al. 2006). Turner et al. recently identified a goblet cell precursor expressing the ciliated cell marker FOXJ1 after IL-13 treatment, supporting the idea that ciliated cells can change phenotype in specific settings (Turner, Roger et al. 2011).

Many studies support the idea that club cells are resident progenitor cells of the murine airway epithelium (Phelps and Floros 1991, Van Winkle, Brown et al. 2004, Van Winkle, Fanucchi et al. 2004). In healthy or moderately injured GFP chimeric murine airways, single randomly-distributed progenitor club cells that comprised less than 1% of epithelial cells were able to maintain epithelial homeostasis (Giangreco, Arwert et al. 2009). Only when the airways were severely injured did stem cell “niches” such as the NEB and BADJ cells contribute to epithelial restitution. When club cells predominate, as they do in the majority of the murine respiratory tract, they clearly contribute to epithelial homeostasis and repair. In human airways,

club cells are primarily found only at the BADJ, and their reparative role is likely far less prevalent than in mice.

The most convincing evidence to date suggests that basal cells are the primary progenitor cells for maintenance and restitution of the pseudostratified epithelium (Engelhardt, Schlossberg et al. 1995, Borthwick, Shahbazian et al. 2001, Schoch, Lori et al. 2004, Hong, Reynolds et al. 2004a). In humans, a pseudostratified epithelium containing basal cells extends from the trachea to airways of approximately 0.5 mm in diameter, while in mice basal cells are only evident in the trachea and primary bronchi. In injured mouse airways, the pseudostratified epithelium of the trachea is largely repopulated by basal cells (Rawlins, Okubo et al. 2009). Multipotent progenitor activity of basal cells is supported by several studies that have shown that cells from the basal compartment have the capacity to generate a differentiated epithelium *in vitro* and in xenograft models *in vivo*, with basal, ciliated and goblet cells (Ford and Terzaghi-Howe 1992, Engelhardt, Schlossberg et al. 1995, Hajj, Baranek et al. 2007). However, the progenitor capacity of basal cells *in vitro* declines after passage three (Gray TE et al. 1996), and until recently it was unknown whether the basal cell population contains a subpopulation of true "stem" cells.

In 2008, our group identified a ***side population (SP)*** of airway epithelial stem cells. SP cells are characterized by the ability to actively efflux the DNA-binding dye Hoescht 33342 through expression of the channel protein BCRP-1, which is a marker of stem cells within the bone marrow (Hackett, Shaheen et al. 2008). As with other epithelial tissues, SP cells within the airways are rare, making up less than 0.1% of the total epithelial cell population (Smith and Chepko 2001, Alvi, Clayton et al. 2003, Bhattacharya, Jackson et al. 2003, Redvers, Li et al. 2006). We demonstrated that >95% of SP cells do not express markers of hematopoietic lineage (CD45-), but do express basal cell markers tissue factor (TF1), the transcription factor p63 and

intermediate filament protein CK5, suggesting they are not derived from bone marrow but are a tissue-resident population of stem cells (Hackett, Shaheen et al. 2008). Furthermore, SP cells exhibit several key features consistent with progenitor cell function, including sustained colony forming capacity over five passages, stable telomere length, and importantly, the ability to form a multi-layered differentiated epithelium consisting of basal, ciliated and goblet cells (Hackett, Shaheen et al. 2008).

We observed a 40-fold increase in the numbers of SP stem cells in airways from asthmatic compared to non-asthmatic subjects (Hackett, Shaheen et al. 2008). This indicates an immature epithelial phenotype with high proliferative potential. Indeed, the overall proliferative rate appears to be increased in the epithelium of adult asthmatics after allergen challenge (Ricciardolo, Di Stefano et al. 2003) and in adult asthmatics with severe disease (Cohen, E et al. 2007). However, more recent work has described an intrinsic defect in mitotic synchronicity in ALI cultures from asthmatic subjects, leading to defective repair (Freishtat, Watson et al. 2011). It appears that even when cultured at air-liquid interface *in vitro,* epithelial cells from asthmatic individuals maintain intrinsic defects in differentiation to a pseudostratified epithelium. Although the underlying reasons for these differentiation and repair defects in asthma remain elusive at this time, there is clear evidence to support basal cell involvement.

1.7.5 The basal cell transcription factor p63

Basal cells express several markers, including the intermediate filament protein CK-5, cell surface proteins CD151 and tissue factor, and the epithelial-restricted transcription factor p63. Of the described markers of epithelial basal cells, p63 is of particular interest for this thesis. P63 is the most evolutionarily ancient member of the p53 family of transcription factors and plays

significant roles in epithelial development. Mice lacking p63 exhibit a striking epidermal phenotype and die shortly after birth due to dehydration (Mills, Zheng et al. 1999, Yang, Schweitzer et al. 1999). In addition, the tracheobronchial epithelium of these mice at birth displays a highly-ordered, columnar, ciliated phenotype but completely lacks basal cells and mucus-producing cells, indicating that p63 plays a key role in development and maintenance the airway epithelium (Daniely, Liao et al. 2004).

In asthma, the number of cells expressing p63 in the airway epithelium is increased both *in vivo* and when grown at air-liquid interface *in vitro*, as described in **Chapter 3** of this thesis (Hackett, Warner et al. 2009). Importantly, p63 is commonly used as an immunohistochemical marker of unchecked, abnormal cell proliferation, as is observed in various carcinomas (Crook, Nicholls et al. 2000, Park, Lee et al. 2000, Yamaguchi, Wu et al. 2000, Massion, Taflan et al. 2003, Nobre, Albergaria et al. 2013). Expression of p63 in the bronchi has previously been described in basal cells of glandular structures (Di Como, Urist et al. 2002), nasal epithelium (Li, Shi et al. 2011) and bronchial epithelium (Araya, Cambier et al. 2007). The expanded population of p63-positive basal cells may have important implications for the epithelial abnormalities observed in asthma. However, the specific role that p63 plays in human airway epithelial cells is largely unknown. My contribution to the body of knowledge on p63 will form the basis of **Chapter 4** of this thesis.

1.8 Potential mechanisms underlying altered epithelial plasticity in asthma

Epithelial plasticity describes the ability of cells to reversibly change phenotype (Nieto 2013). Epithelial plasticity contributes to normal tissue function, such as during airway development, differentiation and repair, or may be pathological in nature, such as during tissue fibrosis. Some

specific mechanisms of epithelial plasticity that have implications for airway remodeling in asthma will be discussed below.

1.8.1 The developmental EMTU and its reactivation in asthma

Epithelial plasticity is an important component of embryonic lung development. Lung development occurs via budding from the foregut endoderm early in embryogenesis, followed by a process of branching morphogenesis (Ward 2006). The developmental process depends upon bi-directional interactions between endoderm-derived epithelial cells and mesoderm-derived mesenchymal cells. This is termed the ***epithelial - mesenchymal trophic unit***, or ***EMTU*** (Minoo and King 1994). Several families of growth factors are important for interactions within the developmental EMTU, such as mesenchymally-expressed fibroblast growth factor (FGF)-10, which initiates endodermal development through its endoderm-restricted receptor FGFR2 (Cardoso and Lu 2006). Mediators such as endodermally-expressed sonic hedgehog (Shh) and bone morphogenic protein (BMP)-4, which is expressed in both the epithelium and mesenchyme during development, are required for the formation of the airways (Abdala-Valencia, Earwood et al. 2007). There is also recent evidence supporting a key role for the Notch family of signaling molecules in directing epithelial progenitor cells to a secretory as opposed to ciliated lineage during development (Rock, Gao et al. 2011).

In the nonhuman primate airway, epithelial cell differentiation occurs in a wavelike manner that begins in the trachea and progresses toward the terminal airways (Plopper, Alley et al. 1986). Ciliated cells differentiate first, in late gestation, followed by non-ciliated cells containing secretory granules. Basal cells are the last cell type to appear, perhaps indicating that during embryonic development there is an intermediate progenitor prior to the formation of

mature basal cells. At the time of birth, the tracheal epithelium is not completely differentiated and thus the distal airways likely contain less differentiated cells. In a CCSP-GFP reporter mouse, secretory epithelial cells overlying cartilage rings differentiated first on the ventral side of the trachea (Perl, Wert et al. 2005), suggesting that the proximity to and robustness of the mesenchymal signals direct epithelial cell fate, in a dorsal-ventral-specific manner.

Many of the features of airway remodelling in asthma are reminiscent of the developmental EMTU. An intriguing hypothesis has been proposed wherein structural changes in the EMTU are necessary for allergen-induced T_H2 inflammation and the pathogenesis of chronic asthma (Holgate, Davies et al. 2000, Plopper, Smiley-Jewell et al. 2007). Indeed, a series of elegant experiments by Plopper and colleagues provided *in vivo* evidence for re-activation of the EMTU in response to allergen (Plopper, Smiley-Jewell et al. 2007). In these studies, exposure of infant rhesus monkeys to house dust mite allergen, a common household allergen, resulted in reduced airway number, epithelial cell hyperplasia, increased number of mucous cells, reorganization of the airway vascular system, changes to the orientation of airway smooth muscle bundles and a substantial alteration in the composition and arrangement of the basement membrane.

Many of the mediators of epithelial-mesenchymal crosstalk necessary for appropriate epithelial restitution following injury may be aberrantly expressed in asthma. In differentiated air-liquid interface cultures, airway epithelial cells from asthmatic children express more $TGF\beta_2$ than cells from atopic or healthy children, indicating their potential to mimic or stimulate a wound repair response (Lopez-Guisa, Powers et al. 2012). Endobronchial biopsies from adult asthmatics also had higher epithelial TGFβ2 expression than non-asthmatic donors (Chu, Balzar et al. 2004). Even in monolayer culture, airway epithelial cells from asthmatic children express

greater amounts of the pro-remodeling mediators TGFβ_1 and EGF (Kicic, Sutanto et al. 2006). Paradoxically, EGF signalling is required for epithelial differentiation and wound repair, both of which are impaired in asthma, indicating potential signalling defects in disease (Allahverdian, Harada et al. 2008). Kicic et al. demonstrated that epithelial cells from asthmatic children exhibit increased expression of plasminogen activator inhibitor-1, which may inhibit the degradation of ECM produced by fibroblasts, enhancing a pro-mesenchymal tissue microenvironment (Stevens, Kicic et al. 2008). Impaired fibronectin expression by epithelial cells in pediatric asthma may prevent appropriate epithelial-mesenchymal crosstalk and inhibit the ability of the epithelial cells to appropriately migrate following injury (Kicic, Hallstrand et al. 2010). It is evident that EMTU-like interactions, particularly in the setting of injury and repair, are altered or reactivated in asthma.

1.8.2 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a biological process whereby epithelial cells lose apical-basal polarity and intercellular contacts and transition to a non-polarized, motile, spindle-shaped mesenchymal phenotype (**Figure 1.9**). Hallmark features of EMT are the dissolution of epithelial adhesion junctions and transcriptional repression of adhesion proteins, particularly E-cadherin and ZO-1, and expression of mesenchymal genes and proteins, including fibronectin-EDA, vimentin, α-smooth muscle actin and fibroblast specific protein (FSP-1, also known as S100A4) (Kalluri 2009). The culmination of EMT is invasion of the extracellular matrix by the motile mesenchymal cell and secretion of ECM proteins such as collagen.

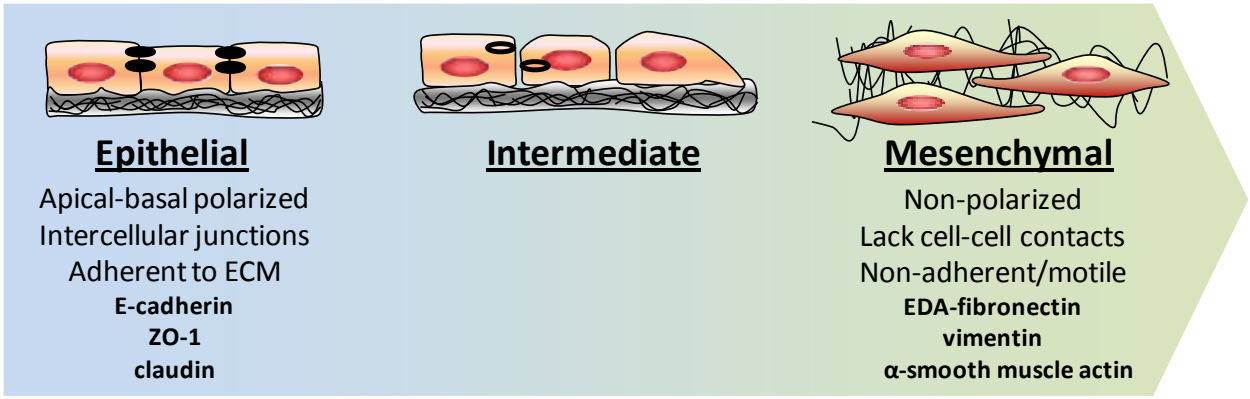


Figure 1.9 Schematic of epithelial-mesenchymal transition.
During the process of EMT, epithelial cell-cell contacts dissolve, leading to the loss of apical-basal polarity. The subsequent transcriptional repression of epithelial genes such as E-cadherin, ZO-1 and claudin, and the expression of mesenchymal markers such as EDA-fibronectin, vimentin and α-smooth muscle actin promote a non-polarized, migratory, mesenchymal cell phenotype. Cells in the midst of an epithelial-mesenchymal transition may have an intermediate phenotype that is neither fully epithelial nor mesenchymal. Figure modified from Kalluri 2009.

EMT can occur in several biological settings. During normal development, when there is seamless interaction between the epithelial and mesenchymal compartments, Type I EMT contributes to mesenchymal cell populations of the heart and neural crest (Acloque, Adams et al. 2009). Conversely, genetic or epigenetic alterations in cancer cells can also lead to a pathological manifestation called Type III EMT, enabling carcinoma *in situ* to become metastatic through the loss of polarity and migration of tumour cells to new tissue environments (Kalluri 2009). Epithelial plasticity is also a normal part of wound repair, as tissue reconstruction requires cell spreading, migration, and secretion of extracellular matrix. Wound repair-associated Type II EMT is induced by inflammation and tissue trauma, and is usually halted once repair is complete. However, if the inflammatory response is not halted, ongoing EMT can contribute to tissue remodeling or fibrosis, such as is seen in the kidney (LeBleu, Taduri et al. 2013). Type II EMT has been postulated to account for the excess fibroblast population and deposition of

extracellular matrix in the remodeled asthmatic airway (Hackett, Warner et al. 2009, Heijink, Postma et al. 2009).

Induction of EMT can be caused by a wide variety of stimuli, many of which are present in the airway tissue microenvironment in asthma. Reported inducers of EMT are the TGFβ_1/SMAD pathway, growth factors and receptor tyrosine kinases, the Wnt/β-catenin pathway, extracellular matrix proteins and inflammatory factors/NFκB (Hackett 2012, Bartis, Mise et al. 2014). Exposure to some allergens may also contribute to EMT through degradation of epithelial cell contacts, such as HDM serine protease Derp1 (Wan, Winton et al. 2001, Heijink, van Oosterhout et al. 2010) and pollen (Runswick, Mitchell et al. 2007), as well as increased histamine concentrations (Zabner, Winter et al. 2003). An intriguing study by Heijink et al. characterized the ability of HDM allergen to enhance TGFβ-induced EMT in immortalized human bronchial epithelial cells (Heijink, Postma et al. 2009). The authors suggested that TGFβ_1 facilitated the uncoupling of EGFR from E-cadherin in the presence of HDM proteases, leading to prolonged EGFR signaling (Heijink, Postma et al. 2009). Further, TGFβ_1 in combination with pro-inflammatory cytokines TNFα or IL-1β, but not IL-8, enhance EMT (Doerner and Zuraw 2009, Camara and Jarai 2010, Borthwick, Gardner et al. 2012). EMT-like responses have also been shown to occur in transformed airway epithelial cells in response to BMP4 (Molloy, Adams et al. 2008) and during wound repair in murine airway epithelial cells in culture (McCormack, Molloy et al. 2013). Taken together, these studies highlight that there are a multitude of signals present in the the airways in asthma that can induce and enhance epithelial-mesenchymal transition.

Importantly, increased protein levels of TGFβ have been observed in asthmatic airways, and its role in airway remodeling is well established (Holgate, Davies et al. 2003, Boxall,

Holgate et al. 2006). Our group has shown that in air-liquid interface conditions, basal cells are the only cell type to undergo TGFβ_1-induced EMT (Hackett, Warner et al. 2009). Intriguingly, in the asthmatic epithelium, there were proportionately more basal cells and these cultures were more susceptible to EMT compared to cultures from non-asthmatic donors. These data comprise **Chapter 3** of this thesis and support the idea that epithelial plasticity is altered in asthma.

While we did not observe EMT in human airways *in vivo*, there is evidence that both mouse and human airways undergo severe remodeling events in response to extensive injury. Features of EMT were reported in the small airways of lung transplant recipients with post-transplant bronchiolitis obliterans syndrome (Borthwick, Parker et al. 2009). Transgenic mice exposed to bleomycin or very high doses of TGFβ_1 (Wu, Yang et al. 2007) or to house-dust mite allergen (Johnson, Roos et al. 2011) also share features of EMT in their airways. These findings provide substantial evidence for the ability of the epithelium to undergo EMT-like phenotypic alterations *in vivo*.

1.8.3 MicroRNA regulation of epithelial plasticity

One endogenous mechanism controlling airway epithelial differentiation is through expression of ***microRNAs*** (miRNA). MiRNAs are small RNA molecules 21-23 nucleotides in length that are generally produced by transcription of non-protein-coding genes, and involved in regulation of many cellular functions (Blahna and Hata 2013). To mediate the inhibition of the target mRNA, mature miRNAs usually recognize and bind to sequences in the 3' UTR of the mRNA (**Figure 1.10**). The translation-inhibiting activity comes about either by blocking of translation, or by degradation of the target mRNA via the RNA-Induced Silencing Complex (RISC) (Gregory, Chendrimada et al. 2005). In some instances, such as during cell cycle arrest or in immature

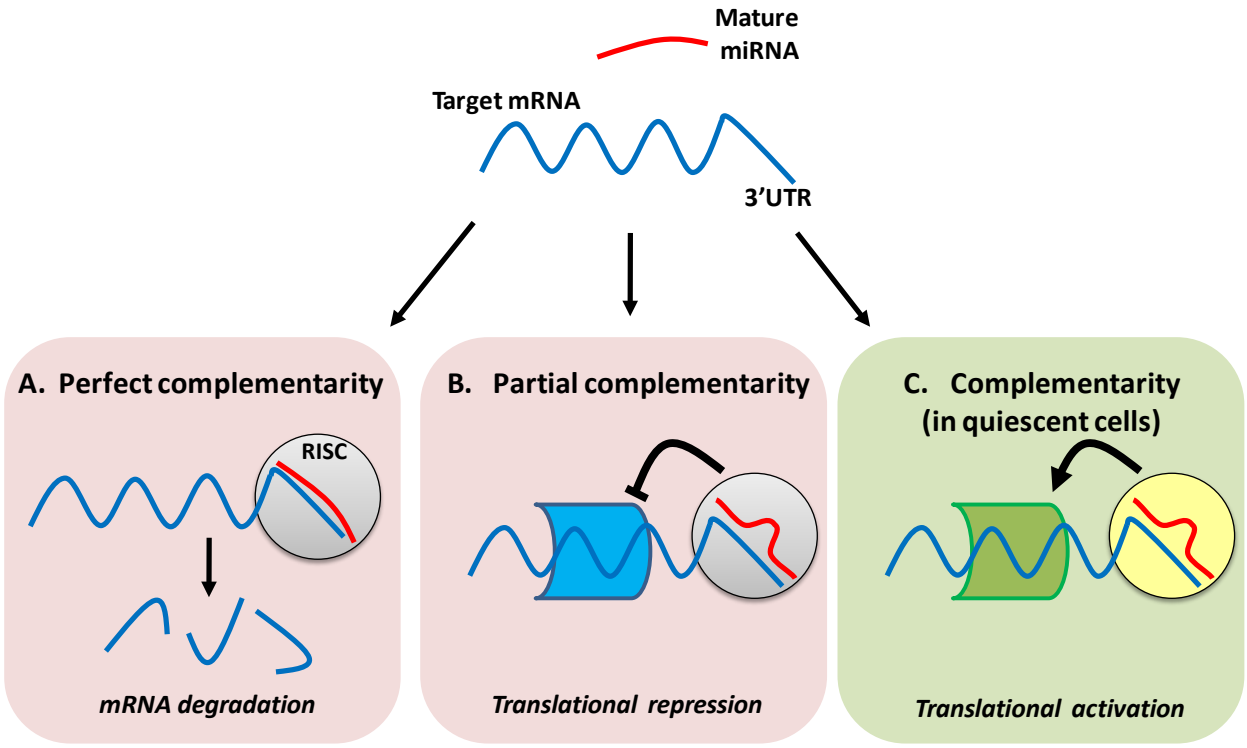


Figure 1.10 miRNA mechanisms of action on target mRNA sequences.
Mature miRNA in the cytoplasm can affect target mRNA translation by three distinct mechanisms. (**A**) In the case of perfect sequence complementarity between the miRNA and the 3' UTR of the target mRNA, the mRNA is degraded by the RNA-Induced Silencing Complex (RISC). (**B**) However, if the miRNA exhibits only partial complementarity, repression of the translational machinery can occur. **(C)** In some settings, such as in quiescent cells, miRNAs may also activate translation, potentially by increasing mRNA stability.

oocytes, miRNAs may also increase translation, possibly by increasing mRNA stability (Vasudevan, Tong et al. 2007, Mortensen, Serra et al. 2011).

A number of miRNAs are differentially expressed in monolayer as compared to differentiated cultures of human airway epithelial cells. One such miRNA is ***miR-449a***, which is upregulated by several orders of magnitude throughout the process of mucociliary differentiation in airway epithelial cells (Lize, Herr et al. 2010). MiR-449a functions by repressing the Delta/Notch pathway and promotes differentiation to the ciliated cell lineage (Marcet, Chevalier

et al. 2011) (**Figure 1.11**). In the epidermis, ***miR-203*** plays a pivotal role in differentiation. MiR-203 is induced in suprabasal cells upon epidermal differentiation, and is a direct repressor of p63 (Yi, Poy et al. 2008). Expression of miR-203 causes terminal differentiation of the epidermis by restricting the "stemness" of p63-positive cells to the basal cell layer (Lena et al. 2008). Intriguingly, miR-203 directly represses caveolin-1 in mouse breast tissue during caloric restriction (Orom, Lim et al. 2012). These findings are relevant to epithelial integrity. For example, caveolin-1 is decreased in asthma, causing loss of E-cadherin in epithelial adherens junctions and increased production of the pro-T_H2 cytokine TSLP (Hackett, de Bruin et al. 2013).

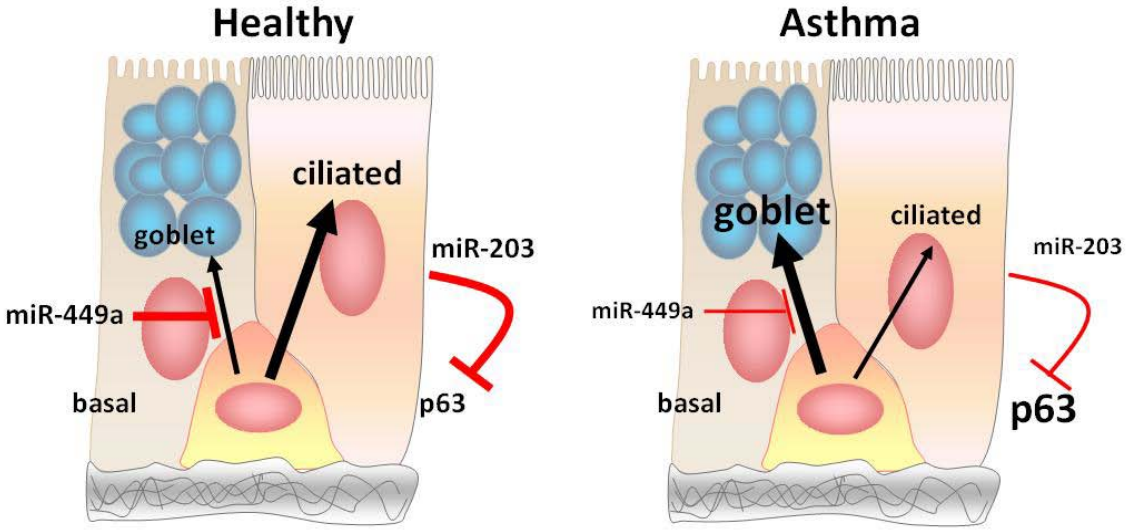


Figure 1.11 Implications of altered epithelial miRNA expression in asthma.
In healthy epithelial cells, miR-449a and miR-203 are induced during differentiation. MiR-449a represses the Notch pathway, promoting ciliated cell fates, while miR-203 restricts the pro-proliferative p63 to the basal cell layer. In asthma, decreased miR-449a may promote secretory cell fate decisions, while decreased miR-203 may allow expansion of the $p63^+$ basal cell population.

Expression of several epithelial miRNAs is altered in asthma, including those that regulate epithelial differentiation. In a comprehensive analysis, Solberg et al. used miRNA microarrays to demonstrate that the miR-34/449 family is under-represented in bronchial epithelial cells from adult asthmatics compared to controls (Solberg, Ostrin et al. 2012). These same miRNAs were found to be repressed by IL-13 in cultured epithelial cells, providing additional insight into how the inflammatory environment may lead to aberrant epithelial differentiation and repair. In a different study comparing miRNA in bronchial epithelial cells from adult asthmatic vs. non-asthmatic donors, 66 of the 165 detected miRNAs were differentially expressed by at least 1.5-fold (Jardim, Dailey et al. 2012). This study found that let-7f, miR-487b and miR-181c* were increased in cells from asthmatic donors. MiR-203 was significantly decreased, a finding that may have implications for the expanded population of p63-positive cells in the asthmatic epithelium (Jardim, Dailey et al. 2012). While miR-203 may play a role in repressing p63, the authors identified a novel miR-203 target, aquaporin 4 (AQP4), which is upregulated in asthma, indicating the complexity and possible redundancy of cellular regulation by miRNAs.

Altered control of miRNA processing and activity are important components of disease pathogenesis, as their normal function is as much a part of tissue maintenance and remodeling as is canonical transcription and protein trafficking. MiRNAs are regulated at multiple levels, as pre-miRNA sequences contain transcription factor binding sites (Piriyapongsa, Jordan et al. 2011). Furthermore, the processing of miRNAs is regulated by growth factors such as TGFβ and BMP4, leading to increased expression of mature miRNAs that may influence EMT (Davis, Hilyard et al. 2008). Several miRNAs were implicated in TGFβ-induced EMT in human keratinocytes, including increased expression of miR-21, -32, -137 and -346, and loss of miR-

136, -192, -210 and -211 (Zavadil, Narasimhan et al. 2007). Of these, miR-21 has been confirmed to be responsive to TGFβ (Pan, Wang et al. 2010), and was also responsible for T_H1 polarization and severity of delayed-type hypersensitivity in an ovalbumin-challenge mouse model of asthma (Lu, Hartner et al. 2011). RNA-binding proteins influence processing of miRNA by increasing catalytic activity of miRNA-processing enzymes, though this level of regulation is just beginning to be understood (Apponi, Corbett et al. 2011).

While it is clear that miRNAs function in a cell-autonomous manner, they can also be exported from cells within extracellular vesicles known as exosomes that have both functional and diagnostic significance (Valadi, Ekstrom et al. 2007). Recently, differential miRNA content of exosomes from bronchoalveolar lavage fluid from adult asthmatic patients as compared to non-asthmatic adults was identified (Levanen, Bhakta et al. 2013). A subset of 16 differentially expressed miRNAs, including decreased let-7, miR-200 and miR-34 families, were detected in asthmatic compared to healthy individuals despite the fact that these patients had mild, asymptomatic disease. Though several promising initial targets were identified, much remains to be explored in the field of mRNA and miRNA regulation of airway differentiation and repair in order to understand the molecular complexities that cause epithelial dysregulation in asthma.

1.9 Current therapeutic agents do not effectively target airway remodeling

Maintenance therapies for asthma are primarily aimed at easing airway narrowing by targeting airway smooth muscle contraction and ongoing inflammation. Although there are many such therapies with proven clinical benefit, evidence of their effect on features of airway remodeling is scarce and frequently contradictory. Longitudinal studies evaluating aspects of airway

remodeling in asthma are difficult to perform for reasons that are both ethical and practical, and repeated sampling of airways of young asthmatics is invasive and not without risk.

Inhaled corticosteroids are widely-used and extremely effective anti-inflammatory agents used to control long-term inflammation. In asthmatic individuals, epigenetic modifications including histone acetylation of inflammatory-related genes render them transcriptionally "active". Corticosteroids function by activating histone deacetylase 2 (HDAC2), which removes histone modifications from chromatin, thereby transcriptionally deactivating the inflammatory genes, breaking the cycle of continuous inflammation. Although corticosteroids were shown to suppress mucus production by decreasing the number of goblet cells in patients with mild asthma (de Kluijver, Schrumpf et al. 2005, Rogers and Barnes 2006), a more recent study showed no such effect (Broekema, Timens et al. 2011). Inhaled fluticasone did not reduce basement membrane thickness in adults with mild or moderate asthma after six weeks of therapy (Baraket, Oliver et al. 2012), a finding that contradicts a previous report by Olivieri and colleagues (Olivieri, Chetta et al. 1997). In nasal polyposis, a syndrome with epithelial hyperplasia and expansion of p63-expressing basal cells, treatment with the oral corticosteroid prednisone for 10 days restored p63 expression to baseline levels (Li, Shi et al. 2011). Whether corticosteroid treatment ameliorates epithelial basal cell hyperplasia in asthma is unknown. Unfortunately, corticosteroids do not affect disease progression; early use of inhaled corticosteroids before age two in children with asthma had no effect on asthma 8 years later (Devulapalli, Lodrup Carlsen et al. 2007) and did not prevent lung function decline later in life (Guilbert, Morgan et al. 2006). Taken together, though there is evidence that current therapeutics improve symptoms by modulating the inflammatory response, airway remodeling and disease pathogenesis persist.

Development of more targeted asthma therapies will undoubtedly be aided by the identification of novel candidate genes/miRNAs, and a greater understanding of the role of those that have already been identified (such as GSDMA/B, IL-33/ST2, TSLP and let-7). Experiments using relevant tissues/cells are still necessary to confirm these genetic and epigenetic associations, and this is an exciting avenue of discovery with great therapeutic potential. Let-7 miRNAs decreased in the lungs of mice in response to allergen challenge-induced inflammation, and exogenous let-7 administration reduced IL-13 expression and resolved many of the features of airway remodeling (Kumar, Ahmad et al. 2011). Recent work has identified an E3 ubiquitin ligase that is increased in the bronchial epithelium following house dust mite allergen exposure in a murine model, and inhibition of this enzyme prevented the accumulation of immune cells and goblet cell hyperplasia (Collison, Hatchwell et al. 2013). These findings emphasize the potential benefits that exist beyond the global anti-inflammatory action of corticosteroids, and highlight the epithelium as an attractive target for future therapeutic efforts.

1.10 Synopsis of research questions

This introduction has presented abundant evidence that the epithelium is a crucial tissue for overall lung function, and that it is positioned to orchestrate inflammation and repair. The basal cell is a progenitor cell of the pseudostratified epithelium, and is able to undergo distinct phenotypic transitions to maintain epithelial homeostasis and respond to stimuli in the tissue microenvironment. ***We hypothesize*** that differentiation of epithelial basal cells is impaired in asthma, leading to impaired repair.

Aim 1: Airway remodeling in asthma includes features such as subepithelial fibrosis and increased numbers of mesenchymal cells. In **Chapter 3**, we hypothesized that primary human

bronchial epithelial cells can undergo epithelial-mesenchymal transition (EMT) and contribute to the aforementioned fibrotic components of airway remodeling. We set out to characterize the features of TGFβ_1-induced EMT in primary human airway epithelial cells, and to determine whether epithelial cells from asthmatic individuals are more or less susceptible to this process than those from non-asthmatic donors. **Outcomes:** This study was the first to demonstrate that TGFβ_1 induces an EMT phenotype in primary human airway epithelial cells, through activation of the SMAD3 signalling pathway. We observed that only basal cells underwent TGFβ_1-induced EMT, and that there was an expanded population of basal cells in the epithelium of asthmatic subjects both *in vivo* and *in vitro*. Though the expanded population of basal cells led to a greater EMT response *in vitro*, we did not observe expression of mesenchymal markers in the airway epithelim of asthmatic patients *in vivo*.

Aim 2*:* As the basal cell is central to epithelial homeostasis and repair, we next turned our attention to a transcription factor that is specifically expressed in basal cells. In **Chapter 4**, we hypothesized that p63 regulates basal cell functions such as epithelial repair and expression of repair-related genes in primary human airway epithelial cells. Our aims were to identify which isoforms of p63 are expressed in pHAECs, and to determine the effect of altered p63 expression on 1) the expression of putative target genes and 2) the process of epithelial repair. **Outcomes:** We described for the first time that in monolayer culture, airway epithelial basal cells from non-asthmatic and asthmatic donors express equivalent amounts of p63, and that this was primarily the ΔNp63α isoform. Furthermore, we demonstrated that loss of ΔNp63α resulted in decreased expression of several genes, including EGFR, β-catenin and jagged1, and inhibition of epithelial repair. This work highlighted the importance of ΔNp63α for appropriate airway epithelial basal cell function.

Aim 3: It is unknown whether the expanded epithelial basal cell population in asthma is due to an aberrant and ongoing repair phenotype, or whether it arises from an inherent defect in differentiation. In **Chapter 5**, we hypothesized that mucociliary differentiation would be impaired in epithelial basal cells from asthmatic compared to non-asthmatic donors and that there would be differences in epithelial phenotype between asthmatic donors with (+) and without (-) the exercise-induced bronchoconstriction (EIB) disease subtype. Our specific goals were to compare the epithelial phenotype in the disease subgroups by quantifying: 1) basal, ciliated and goblet cell populations, 2) cilia length and 3) release of inflammatory cytokines IL-6 and IL-8 up to day 20 in ALI culture, and 4) to compare the basal and goblet cell populations in each disease subgroup *in vivo,* using endobronchial biopsies. **Outcome:** This chapter describes our finding of a striking lack of ciliated cells in both the EIB(-) and EIB(+) subgroups *in vitro,* and that this was accompanied by shorter cilia length and an expanded basal cell population in the EIB(-) subgroup only. We also observed phenotype-specific differences in IL-8 and IL-6 release, suggesting distinct epithelial differences in subgroups of asthmatic donors.

Aim 4: In **Chapter 6**, we hypothesized that transcriptional and epigenetic differences are responsible for the aforementioned phenotypic features of the epithelium in asthma. To this end, we carried out whole-transcriptome RNA sequencing to assess expression of mRNA and miRNA at days 0, 5, 11 and 20 of ALI culture of epithelial cells from EIB(+), EIB(-) and healthy control donors. We first set out to examine expression of candidate genes that are either involved in developmental control of epithelial differentiation or are expressed in specific epithelial cell types, in a hypothesis-driven approach. Secondly, we used hypothesis-generating statistical modeling to identify mRNAs, miRNAs and biological pathways that are differentially expressed in epithelial cells from different disease subgroups. Finally, we set out to identify key miRNA

regulators of gene expression in our culture model. **Outcome:** We identified several metabolic and signaling pathways that are differentially expressed during differentiation between healthy, EIB(-) asthmatic and EIB(+) asthmatic donors. Aberrant expression of oxidative phosphorylation, transcription, actin cytoskeleton, insulin signaling and MAPK signaling pathways was distinct between the EIB (+) and EIB(-) phenotypes, but genes related to ciliogenesis were equally downregulated in both asthmatic subgroups. We also describe a miRNA-mRNA regulatory network that regulates the transition between proliferation and differentiation, but found no miRNAs that were differentially-expressed between groups during ALI culture.

As a whole, this thesis identifies several alterations in epithelial basal cell plasticity that result in aberrant mucociliary differentiation and altered susceptibility to external mediators in asthma. These findings enhance our understanding of airway epithelial plasticity and advance the field of research regarding the pathophysiology of airway remodeling in asthma.

Chapter 2: General Methods

2.1 Epithelial cell isolation from human lungs

Human donor lungs deemed unsuitable for transplantation and donated for medical research were obtained through the International Institute for the Advancement of Medicine (Edison, NJ). The ethics committees of the involved institutions approved this study. Primary human airway epithelial cells (pHAECs) were isolated by protease digestion of human airways as previously described, with minor modifications (Karp et al., 2002). Briefly, after surgical removal, specimens were washed in Custodiol® HTK (Histidine-Tryptophan-Ketoglutarate) solution and packed on ice for transportation. Upon arrival at the University of British Columbia, the trachea and bronchi to the 3rd generations were blunt dissected (**Figure 2.1**). Segments of trachea, primary bronchus and secondary bronchus of approximate width 3-5 mm were placed in tissue cassettes in formalin for paraffin-embedding. Remaining airway segments were placed in PBS without Ca^{2+} and Mg^{2+} (Thermo Scientific, Waltham, MA, USA). Excess tissue was removed by blunt dissection, and the airways were equilibrated in PBS at 4°C for one hour to completely remove blood and mucus plugs. The epithelium on intact segments of trachea and bronchi (2-4cm long) was then dissociated with 0.11 g pronase enzyme (Roche Diagnostics, Indianapolis, USA) per 100ml of Bronchial Epithelial Basal Media (BEBM; Lonza, Basel, Switzerland) at 4°C for 16 hours.

After digestion the tracheal/bronchial tubes were rinsed by manual pipetting to dissociate cells. Dissociated cell clumps were strained through a 70 μm nylon mesh (Becton-Dickinson, Franklin Lakes, NJ, USA). To neutralize pronase activity, 10% FBS (Gibco, Burlington, Canada)

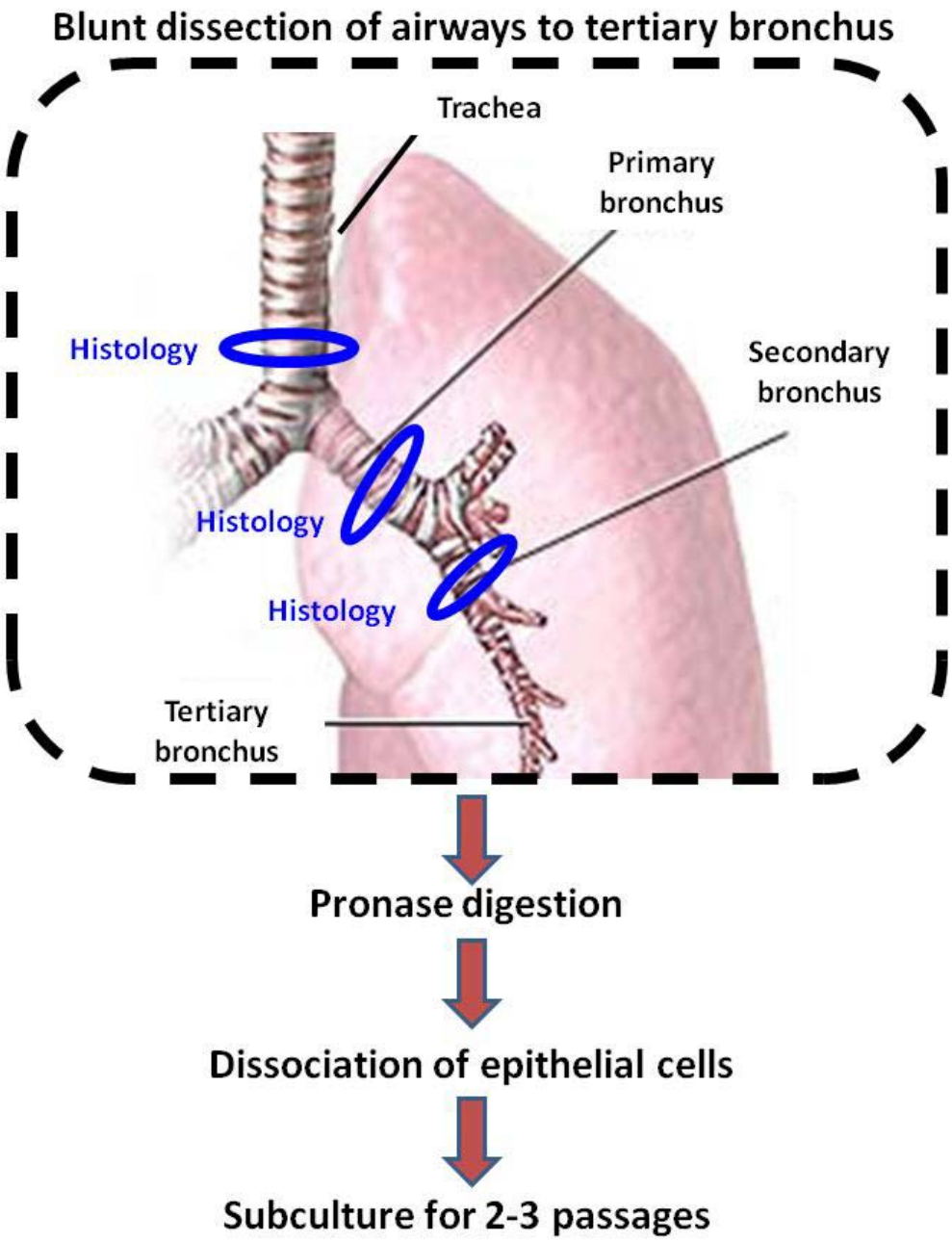


Figure 2.1 Schematic of isolation of primary airway epithelial cells from donor tissue.
Airways to the third generation bronchi were blunt dissected from intact human lungs. Tissue sections were set aside for histological preparation from each of the areas denoted with blue circles. Airways were equilibrated in PBS and then incubated with pronase solution overnight at 4°C. Airway epithelial cells were dissociated by washing of the airways and straining through a 70 μm nylon mesh. Cells were then subcultured on plastic for 2-3 passages.

was added and primary human airway epithelial cells (pHAEC) were collected by centrifugation at room temperature. Cell pellets were resuspended in approximately 5 mL of bronchial epithelial growth media (BEGM, Lonza) supplemented with 1x antibiotic/anti-mycotic (Gibco). Approximately 20 x 10^6 epithelial cells were recovered from each donor lung. After counting, cells were diluted such that 2 x 10^6 cells were seeded in each 25 cm^2 flask (T25; Sarstedt, Thermo Fisher Scientific, Ottawa, ON, Canada) in 3 mL BEGM supplemented with antibiotic/antimycotic.

Cells were cultured for 10-12 days, until they reached 80-90% confluent, and were then sub-cultured into T25 flasks using trypsin-EDTA (0.25%; Lonza). Cells were also cryo-preserved in Pro-Freeze (Lonza) according to manufacturer's instructions. At passages 2 or 3, cells were seeded into 6-well tissue culture plates (Falcon; Thermo Fisher Scientific) or air-liquid interface conditions for experiments.

2.2 Immunoblot

Protein was extracted from cell cultures using a modified RIPA buffer containing inhibitors of proteases (Protease Inhibitor Cocktail; Sigma-Aldrich, Oakville, ON, Canada) and phosphatases (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich). Cells were rinsed with PBS and then overlaid with an appropriate volume of protein extraction buffer (100-120 µL in a 6-well plate) and scraped to collect protein. Protein lysates were stored at -80°C until used for immunoblot.

Lysates were thawed on ice and then centrifuged at 13,000 rpm for 5 minutes at 4°C to exclude membrane lipids. Lysate supernatant was mixed with a glycerol-containing SDS loading buffer, boiled for 5 minutes to denature proteins, cooled on ice and centrifuged briefly to collect any condensate. Samples were loaded ont 7.5-12.5% SDS-polyacrylamide gels for SDS-PAGE.

A molecular weight standard ladder (SeeBlue Plus 2 Prestained Standard; Invitrogen, Carlsbad, CA, USA) was loaded in one lane of each gel. Electrophoresis was carried out at 100 V for 10-15 minutes to align samples at the separating gel, and then at 150 V until adequate separation was attained.

Separated proteins were transferred to nitrocellulose membranes in methanol-containing transfer buffer, on ice, at 35 V overnight followed by 15 minutes at 100V (for detection of large proteins, such as fibronectin-EDA) or at 100 V for 2-3 hours (small to mid-size proteins, such as p63). Transfer efficiency was visually assessed by Ponceau staining, followed by washing in TBS to remove Ponceau stain. Non-specific binding of antibodies was blocked using 1x TBS/Casein Buffer (BioRad, Mississauga, ON, Canada) at room temperature for two hours. Once blocking buffer was removed, primary antibodies were applied in TBS/Casein blocking buffer supplemented with 0.1% Tween-20 (Fisher). Primary antibodies were generally applied overnight at 4 °C, but on occasion four hours incubation at room temperature was substituted. The antibodies used in each thesis chapter will be detailed in that chapter's methods section.

Primary antibodies were removed, and membranes washed three times for ten minutes each in TBS/0.1% Tween-20 wash buffer. Secondary antibodies were either goat anti-mouse IR-800 (1/2500; Vector Laboratories, Burlingame, CA, USA) or goat anti-rabbit Alexa 680 (1/2500; Invitrogen). Secondary antibodies were applied in TBS/Casein with 0.1% Tween-20 and 0.02% SDS, at room temperature, for 45 minutes. Membranes were then washed three times for 10 minutes in TBS/0.1% Tween-20 and placed in TBS prior to imaging on the LI-COR Odyssey system (LI-COR Biosciences, Lincoln, NE, USA). Imaging was generally carried out at an intensity of 5.0 in both the infrared 700 and 800 channels. Densitometry was performed using the LI-COR Odyssey software, by applying a rectangular shape around bands and determining the

digital intensity of the signal in the appropriate channel. The same size of analysis rectangle was used for each band within a given blot. Target protein signals were normalized to a loading control, usually β-tubulin.

2.3 Immunohistochemistry (IHC) and immunofluorescence (IF)

Formalin-fixed paraffin-embedded (FFPE) blocks were sectioned at a thickness of 5-6 µm and placed on SuperFrost slides (Fisher Scientific). Slides were de-paraffinized in two changes of xylene for five minutes each. Slides were rehydrated through graded ethanol solutions (100%, 95% and 70% in dH_20) for three minutes each, and then placed in TBS for 3 minutes. Depending on the primary antibody, different antigen retrieval methods were used. Heat-induced epitope retrieval was carried out by autoclaving slides for 15 minutes at 120psi in either a 1x Citrate Buffer or a high-pH buffer (both Dako Cytomation). Once cooled to room temperature, slides were washed in TBS and then incubated with 3% hydrogen peroxide in dH_20 (made fresh each time) for 10 minutes to quench endogenous peroxidase activity. Slides were washed again in TBS for 10 minutes and non-specific binding was blocked with appropriate blocking solution (based on the source species of the secondary antibody) for 20 minutes at room temperature. Blocking solution was removed and primary antibody was immediately applied and allowed to bind overnight at 4°C. Slides were washed three times for 10 minutes each in TBS. Biotinylated secondary antibodies were applied for two hours at room temperature, washed as above to remove unbound antibody, and a streptavidin-tagged HRP (Dako Cytomation) was applied for 20 minutes at room temperature to allow binding to the secondary antibody. Following washing, a diaminobenzidine (DAB; Dako Cytomation) substrate was added for 10 to 20 minutes at room temperature. Slides were then washed with TBS for 10 minutes and placed in Harris

Hematoxylin for five seconds to counterstain nuclei, followed by washing in tap water and then distilled water for one minute each. Slides were dehydrated through graded ethanol (70%, 95% and 100%) for three minutes each, and then allowed to air-dry for 45 minutes prior to applying coverslips using Cytoseal non-aqueous mounting medium. Once dried, slides were photographed and images were analyzed as described in individual chapters.

Immunofluorescence was carried out in the same manner, except that the hydrogen peroxidase quenching was omitted, and the secondary antibody was fluorescently-tagged rather than biotinylated; no tertiary chemistry was required. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1/5000), and then dehydration and coverslipping was carried out as above.

2.4 Gene expression analysis

Cells were rinsed with PBS and then overlaid with buffer RLT (QIAGEN, Valencia, CA, USA). Lysate was collected by scraping and then stored at -80°C until extraction could be performed. RNA was extracted according to manufacturer's instructions using the RNeasy Mini Kit (QIAGEN), using the QiaShredder method of homogenization. One microgram total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Ontario, CA) using the random primer method.

Gene expression was determined by real-time quantitative polymerase chain reaction (RT-qPCR) using the Taqman Universal PCR Master Mix (Applied Biosystems) and pre-developed Taqman Gene Expression Assays as per manufacturer's instructions. Briefly, cDNA was aliquotted into 384-well plates, and then a master mix of 2x Universal Master Mix without UNG, assay-specific primers, and dNTPs was added to each well. Plates were sealed using

Optical Adhesive Film (Applied Biosystems) and centrifuged at 1600 rpm for 5 minutes prior to PCR. Plates were loaded into the thermocycler (ABI 7900HT or ViiA7, both from Applied Biosystems) and cDNA amplified for up to 35 cycles. Gene expression levels were calculated as normalized to GAPDH using the equation: expression = 10000 x $2^{CtGAPDH - CtGene}$. Any samples in which the threshold cycle was greater than 35 cycles were considered to be negative for target amplification.

2.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out on cell culture supernatants to assess content of specific secreted proteins such as IL-6 and IL-8. See **Table 2.1** for specific reagents. Briefly, the capture antibody was diluted to the working concentration in PBS and 100 µL of this was added to each well of a 96-well plate and allowed to bind overnight at room temperature. The following day, capture antibody was removed and wells were washed three times with wash buffer.

ELISA Kit	IL-6 (R&D Systems DY206)	IL-8 (R&D Systems DY208)
Capture antibody (working concentration)	Mouse anti-human IL-6 (2.0 µg/mL)	Mouse anti-human IL-8 (4.0 µg/mL)
Wash buffer	PBS + 0.05% Tween-20	PBS + 0.05% Tween-20
Reagent diluent (also blocking solution)	1% BSA in PBS	0.1% BSA, 0.05% Tween-20 in TBS
Highest standard concentration	600 pg/mL	2000 pg/mL
Detection antibody (working concentration)	Biotinylated goat anti-IL-6 (50 ng/mL)	Biotinylated goat anti-IL-8 (20 ng/mL)
Substrate solution	1:1 H_2O_2 and Tetramethylbenzidine (TMB)	1:1 H_2O_2 and Tetramethylbenzidine (TMB)
Stop solution	2N H_2SO_4	2N H_2SO_4

Table 2.1 ELISA Kits and reagents used.

After the last wash, the plate was blotted firmly against on paper towel to remove all traces of solution. Wells were blocked by addition of 300 µL reagent diluent for one hour at room temperature, followed by three washes as above. Samples were diluted 1:1 in reagent diluent by addition of 50 uL each of the supernatant and reagent diluents. Each sample was assayed in duplicate. Serial dilutions of assay standards were also prepared in appropriate reagent diluents, with maximum standard concentrations for each assay listed in **Table 2.1**, and 100 uL was added to the plate in duplicate. Plates were sealed with parafilm and incubated at room temperature for two hours to allow binding to capture antibody. Plates were washed three times as above, and 100 uL of detection antibody was added to each well and incubated for two hours at room temperature. Plates were washed again as above, and 100 uL of streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature, protected from light. Plates were washed three times in wash buffer and finally 100 uL TMB/peroxide substrate solution was added to each well for up to 20 minutes. Assays were stopped by addition of 50 uL stop solution to each well, and mixed gently by tapping. Optical density was determined at 450 nm with a correction wavelength of 540 nm, using a microplate reader (Tecan Group AG, Männedorf, Switzerland).

Chapter 3: Induction of Epithelial-Mesenchymal Transition in Primary Airway Epithelial Cells from Patients with Asthma by Transforming Growth Factor-β_1[1]

3.1 Introduction

A characteristic feature of the underlying pathology of asthma is airway remodeling, which contributes to the clinical symptoms of the disease and can occur independently of inflammation (Bai and Knight 2005, Martinez 2007). Chronic airway remodeling is characterized by structural changes within the airway wall including smooth muscle hypertrophy, basement membrane thickening, submucosal fibrosis, mucus cell metaplasia, epithelial shedding and angiogenesis (Gizycki, Adelroth et al. 1997). In particular, features such as a thickened basal lamina, epithelial damage, accumulation of smooth muscle and angiogenesis have now been shown to occur in asthmatic children often before the diagnosis of asthma (Payne, Rogers et al. 2003, Fedorov, Wilson et al. 2005, Barbato, Turato et al. 2006). Mechanisms underlying the expansion of the mesenchymal cell populations and excessive deposition of extra-cellular matrix are unknown, although expansion of the resident fibroblast population and recruitment of fibrocytes from the circulation have both been implicated (Nihlberg, Larsen et al. 2006). Abnormal responses of the epithelial-mesenchymal trophic unit to environmental challenges have been proposed to play a central role in the airway pathology and physiology in asthma (Holgate, Davies et al. 2000).

[1]A version of this chapter has been published. Reprinted with permission of the American Thoracic Society. © 2013 American Thoracic Society. Cite: Hackett TL, **Warner SM**, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, Argentieri R, Kicic A, Stick SM, Bai TR, Knight DA. Am J Respir Crit Care Med. 2009 Jul 15;180(2):122-33. Official journal of the American Thoracic Society.

Epithelial cells contribute to local fibroblast pools through a process of molecular reprogramming called epithelial-mesenchymal transition (EMT) (Kalluri and Neilson 2003, Liu 2004).

EMT is a biological process whereby epithelial cells lose apical-basal polarity and intercellular contacts and transition to a non-polarized, motile, spindle-shaped mesenchymal-like cell (see **Chapter 1.8.2**, page 36). EMT has long been known to play a role in cellular differentiation during development and tumor invasion (Greenburg and Hay 1982, Thiery 2002, Nawshad, Lagamba et al. 2005). While dysregulated EMT has been linked to tumor metastasis and progression, in other settings it is recognized as an important process in epithelial tissues in response to stress/injury contributing to fibrosis in the kidney (Forino, Torregrossa et al. 2006), liver (Kaimori, Potter et al. 2007) and lung (Kim, Kugler et al. 2006, Willis and Borok 2007) both in human and animal models. However, whether EMT occurs in multilayered and differentiated airway epithelium is unknown. Additionally, whether this process is dysregulated in asthma remains unanswered.

Transforming growth factor-beta ($TGF\beta_1$) is established as a central mediator involved in tissue repair and the progression of fibrosis as well as inducing EMT in multiple organs (Willis and Borok 2007). Several elegant studies have provided evidence of $TGF\beta_1$-induced EMT in experimental animal models of fibrosis and human alveolar epithelium and in these studies phosphorylation of SMAD 2/3 was essential for the induction of multiple EMT-related processes (Sato, Muragaki et al. 2003, Saika, Kono-Saika et al. 2004, Zavadil, Cermak et al. 2004, Kasai, Allen et al. 2005, Valcourt, Kowanetz et al. 2005). However, we and others have shown that $TGF\beta_1$ also has effects that are independent of SMAD signaling and involve activation of MAPK-dependant pathways (Yu, Hebert et al. 2002, Pechkovsky, Scaffidi et al. 2008). For

example, it has been demonstrated that TGFβ_1-induced p38 MAPK and ERK1/2 activation is involved in EMT of human keratinocytes (Davies, Robinson et al. 2005), hepatocytes (Kojima, Takano et al. 2008) and some mammary carcinomas (Gal, Sjoblom et al. 2008). Whether TGFβ_1-induced EMT occurs in primary human airway epithelial cells and the signaling pathways involved remain unclear.

The aim of the present study was to determine whether EMT occurs in primary cultures of human airway epithelial cells following TGFβ_1 exposure. We investigated the signaling mechanisms and cell types involved using monolayer and differentiated, air-liquid interface (ALI) cultures. Some of these data were reported in the form of an abstract (Hackett T-L 2008).

3.2 Methods and materials

3.2.1 Airway epithelial cell isolation and culture conditions

See **Chapter 2.1**. The clinically relevant information for each of the subjects is listed in **Table 3.1**. Prior to experiments monolayers were placed in bronchial epithelial basal medium (BEBM, Cambrex) without supplements for 24 hours and then incubated with either 10 μM of the MEK/ERK1/2 inhibitor UO126, 5 μM of the p38 MAPK inhibitor SB203580 (both Calbiochem) or vehicle control for 1hour prior to the addition of recombinant (rh)TGFβ_1 (10 or 50 ng/mL, Peprotech, Rocky Hill, USA) and every 24 hours of the 48 and 72 hour experiments in a total volume of 1 mL BEBM containing 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

3.2.2 SMAD3 siRNA treatment

AEC monolayers grown to 70% confluence in six well plates were transfected with human SMAD3-specific chimera-RNAi (SMAD3 siRNA, Abnova Corp, Taipei City, Taiwan) and non-silencing control siRNA (Qiagen, Ontario, Canada) at a final concentration of 10 or 1 nM using HiPerFect transfection reagent (Qiagen) as instructed by the manufacturer. AEC monolayer cultures were then incubated with BEGM media for a further 72 hours, with or without TGFβ_1. Cells were then lysed and protein expression analyzed by immunoblot as described below to confirm protein knock-down and inhibition of EMT.

Patient ID	Gender	Age (yrs)	Disease	Medication	Cell Source
aN1*	Male	20	None	None	Donor Lung
aN2*	Male	21	None	None	Donor Lung
aN3*	Female	22	None	None	Donor Lung
aN4*	Male	18	None	None	Donor Lung
aN5*	Male	24	None	None	Donor Lung
pN6*	Female	4	None	None	Donor Lung
pN7*	Male	14	None	None	Donor Lung
pN8	Female	8	None	None	Bronchial Brushing
pN9	Male	12	None	None	Bronchial Brushing
pN10	Female	11	None	None	Bronchial Brushing
pA1*	Female	8	Asthma	Albuterol, Singulair	Donor Lung
pA2*	Male	11	Asthma	Albuterol	Donor Lung
aA3*	Female	21	Asthma	Albuterol, Advair	Donor Lung
pA4*	Female	15	Asthma	Albuterol, Advair	Donor Lung
pA5*	Male	14	Asthma	Albuterol	Donor Lung
pA6	Male	6	Asthma	None	Bronchial Brushing
pA7	Female	13	Asthma	None	Bronchial Brushing
pA8	Male	8	Asthma	None	Bronchial Brushing

Table 3.1 Patient demographics used in chapter 3.
Patients are identified as adult (a) or pediatric (p) and by disease status denoted as either none (N) or asthmatic (A). * denotes patients used in all experiments.

3.2.3 SDS-PAGE and immunoblot

See general methods, **Chapter 2.2**. For antibodies used, see **Table 3.2**. The results are expressed as a phosphorylated protein/non-phosphorylated protein density ratio or protein/β-tubulin density ratio.

Epitope	Host	Catalog #	Company	Uses
α-smooth muscle actin (SMA)	Mouse	ab5694	Abcam, Cambridge, UK	WB, IHC
β-tubulin	Mouse	05-661	EMD Millipore Corporation, Billerica, USA	WB
Cytokeratin 5 (D5/16 B4)	Mouse	M7237	Dako, Glostrup, Denmark	IHC
Cytokeratin 18	Mouse	sc-32329	Santa Cruz Biotechnology, Dallas, USA	IHC
E-cadherin	Mouse	sc-8426	Santa Cruz Biotechnology	WB, IF, IHC
EDA-Fibronectin	Mouse	MAB1940	Chemicon International, Temecula, USA	WB, IF
Phospho-ERK1/2	Mouse	612358	BD Biosciences, Mississauga, Canada	WB
ERK1/2	Rabbit	06-182	EMD Millipore Corporation	WB
Mucin5AC	Mouse	ab24070	Abcam	IHC
Phospho-p38 (Thr180/Tyr182)	Mouse	9216	Cell Signalling Technology, Danvers, USA	WB
p38	Rabbit	9212	Cell Signalling Technology	WB
p63(4A4)	Mouse	sc-8341	Santa Cruz Biotechnology	IHC, IF
Phospho-SMAD3 (Ser423/425)	Rabbit	1880-1	Epitomics, Burlingame, CA	WB
SMAD2/3	Mouse	610843	BD Biosciences, Mississauga, Canada	WB
Vimentin	Rabbit	Ab45939	Abcam	WB, IHC
ZO-1	Rabbit	Ab59720	Abcam	WB

Table 3.2 List of all primary antibodies used in chapter 3

3.2.4 Immunofluorescent staining

AEC monolayers were grown on chamber slides (Falcon; Becton Dickson, Franklin Lakes, NJ), incubated with or without TGFβ_1 (10 ng/mL) and fixed in 4% paraformaldehyde (Fisher) for 20 minutes. Chamber slides were then washed with phosphate buffered saline (PBS) followed by blocking with 20% normal goat serum (Invitrogen) in PBS with 0.1% saponin for 20 minutes. Slides were then incubated with antibodies for either EDA-fibronectin or E-cadherin (see **Table 3.2**) in phosphate buffered saline (PBS) with 0.1% saponin for 2 hours at room temperature. Following washing with PBS containing 0.1% saponin and 0.1% Tween 20, slides were incubated with secondary antibodies conjugated with either goat anti-mouse IgG Alexa Fluor 488 or goat anti-rabbit IgG Alexa Fluor 594 (Invitrogen, Oregon, USA), were incubated for 2 hours at room temperature. Following a final wash, cells were incubated with DAPI (1 μg/mL) and visualized with a Nikon fluorescent microscope and imaged with a C-spot camera (Nikon Instruments, Melville, NY, USA).

3.2.5 RNA isolation and RT-qPCR

RNA was isolated from AEC cultures using RNeasy Plus Mini-Kits (QIAGEN, Valencia, CA). Purified RNA was treated with DNase and reverse transcribed into cDNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Gene expression was determined by real-time PCR using the Taqman® Universal PCR Master Mix (Applied Biosystems) and pre-developed Taqman Gene Expression Assays designed by Applied Biosystems, as per manufacturer's instructions. Fold induction of gene expression was determined. Gene expression was calculated using Ct values for gene of interest and housekeeping genes as described in **Chapter 2.4**.

3.2.6 Confocal microscopy of ALI-AEC cultures

Following treatment with TGFβ_1, inhibitor, or vehicle, ALI-AEC cultures were fixed in 4% paraformaldehyde for 1 hour on ice, and stained with antibodies for cytokeratin-5, E-cadherin, EDA-fibronectin, or p63 (see **Table 3.2**) in 0.1% saponin in PBS for 2 hours at room temperature. Following washing in PBS with 0.1% saponin and 0.1% Tween 20, ALI-AEC cultures were incubated with fluorescently-tagged secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG and/or Alexa Fluor® 594 goat anti-rabbit IgG for 2 hours at room temperature. Following final washes, ALI-AEC cultures were incubated with 1 ng/mL DAPI and then the membrane was excised and mounted on a glass slide using Secure-seal^{TM} imaging spacers (Size 20 mm; Sigma, Oakville, ON). Confocal images of fluorescently labelled ALI-AEC cultures were acquired with a Leica AOBS SP2 laser scanning confocal microscope (Leica, Heidelberg, Germany) using a Leica 63X/1.4 Plan-Apochromat oil immersion objective. The acquisition software was Leica Confocal Software TCS SP2. The laser lines used were 405 (for Hoechst), 488 (for Alexa 488) and 594 nm (for Alexa 594) and these excitation beams were produced by UV diode, Ar and HeNe lasers (Leica AOBS SP2 module) respectively. Emission signals were captured sequentially to minimize signal bleed-through and the confocal pinhole was set at one Airy disk. Images (8 bit) were frame-averaged 3-4 times to minimize the noise, and the pixel dimensions used in this image acquisition process satisfied the Nyquist sampling criteria. The images were overlaid and the contrast enhancements were performed on the images using Volocity software (Improvisions, Boston, USA).

Volocity Classification was used to perform comparative measurements of the number of brightest fluorescent spots per cell in the samples stained with DAPI, EDA-fibronectin (Alexa 488-conjugated secondary) or E-cadherin (Alexa 594-conjugated secondary). A classifier was

created by choosing a minimal “intensity” (brightness) threshold. The program uses these thresholds to identify the pixels with these characteristics in every slice of the Z-stack processed. A total of 250 slices within a Z-stack were taken per ALI-AEC and every 10 sequential slices were averaged to obtain an average classifier value/10 Z-stack slices. The classifier was validated by demonstrating the pixels counted in the 3D-rendered image and confirming that the visible pixels were selected.

3.2.7 Immunohistochemical staining

Please see **Chapter 2.3** for detailed procedures used. Antigens were retrieved by autoclaving (15 minutes at 120˚C and 30 psi) for 20 minutes in citrate target retrieval solution (Dako, Mississauga, ON). Antibodies directed against human cytokeratin-5, p63, cytokeratin-18, mucin 5AC, α-smooth muscle actin or vimentin (see **Table 3.2**) were added overnight at 4°C in 25% goat serum.

3.2.8 Statistics

Data are presented as the mean ± SEM of 12 or more independent experiments. Student’s unpaired t-test was used for pair-wise comparisons and ANOVA with Dunnett’s post-test for comparison of group data. Statistical analysis was performed using commercial statistical software Prism Version 4.0 (GraphPad, Software Inc., San Diego, CA). P values less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 TGFβ_1 induces EMT in primary airway epithelial cells

Monolayer cultures of pHAECs obtained from non-asthmatic donors were incubated with TGFβ_1 (10 and 50 ng/mL) for 48 and 72 hours. As shown in **Figure 3.1A**, pHAECs underwent TGFβ_1-induced EMT characterized by loss of epithelial markers E-cadherin and Zonula Occludins-1 (ZO-1), with concurrent expression of mesenchymal proteins EDA-fibronectin, α-smooth muscle actin (α-SMA) and vimentin when analyzed by immunoblot. As demonstrated by the phase contrast images, the changes in molecular phenotype analyzed by immunoblot were also associated with changes in morphology from a cobblestone epithelial to spindle-shaped appearance, characteristic of mesenchymal cells (**Figure 3.1B** and **C**). As demonstrated by immunofluorescence staining we demonstrated that changes in morphology were also associated with loss of expression of the epithelial marker E-cadherin (red staining, **Figure 3.1D** and **E**) and gain in expression of EDA-fibronectin (green staining, **Figure 3.1F** and **G**). We found no differences in the ability of epithelial cells to undergo EMT whether obtained from bronchial brushings or pronase extraction from whole airways, as demonstrated by the densitometric analysis for α-smooth muscle actin expression (**Figure 3.2**, n=18).

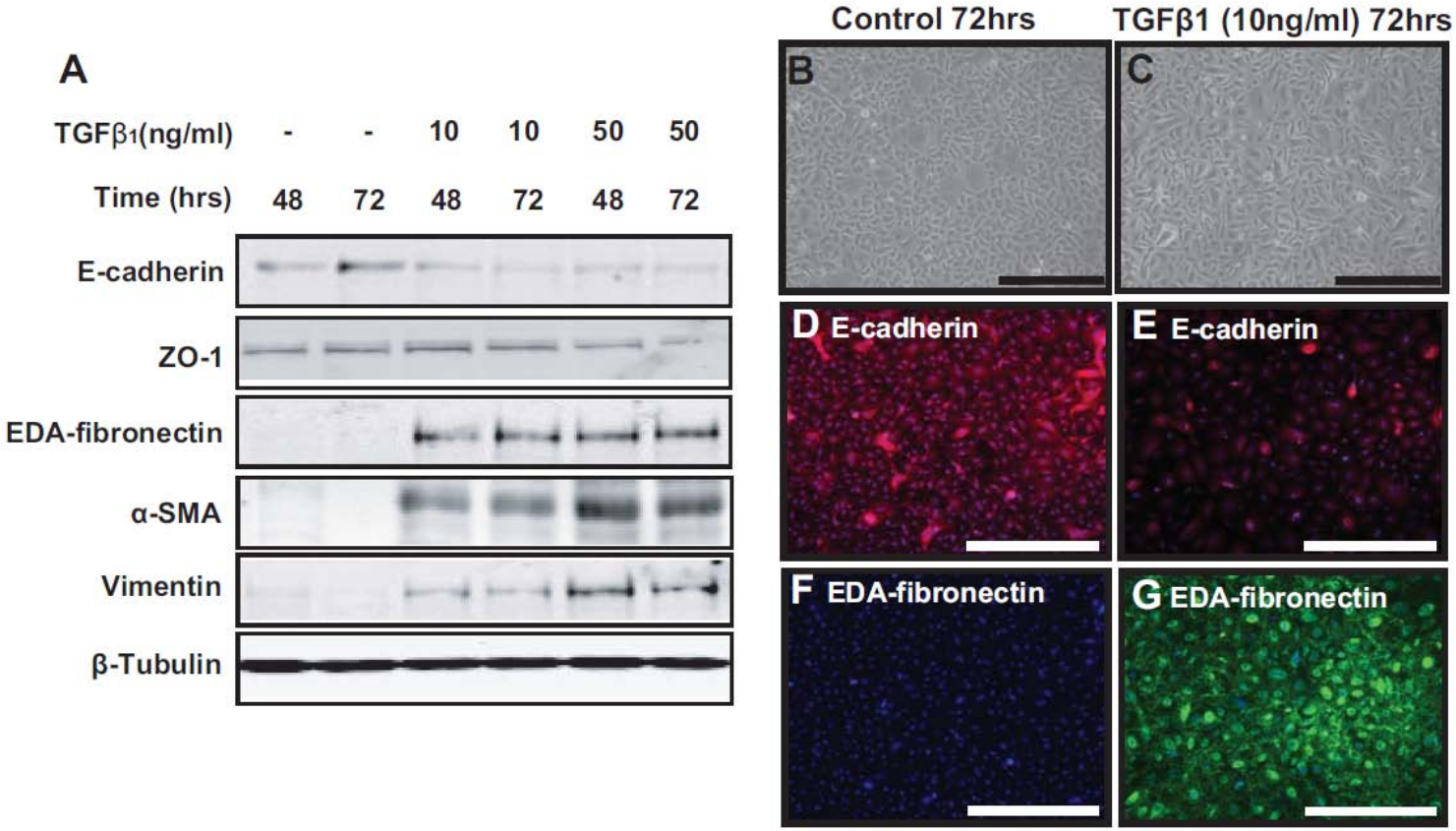


Figure 3.1 TGFβ$_1$-induced epithelial–mesenchymal transition (EMT) occurs in primary human airway epithelial cells (pHAECs). TGFβ$_1$-induced epithelial–mesenchymal transition (EMT) occurs in primary human airway epithelial cells (pHAECs). **(A)** pHAECs were treated with 10 or 50 ng/mL TGFβ$_1$ or vehicle for 48 and 72 hours, total cell lysates were taken for immunoblot or monolayers were fixed for immunofluorescence analysis. Representative immunoblot demonstrates loss of E-cadherin and ZO-1 with concurrent expression of EDA-fibronectin, α-smooth muscle actin, and vimentin in pHAECs after TGFβ$_1$ treatment. Phase contrast images of **(B)** unstimulated cells and **(C)** TGFβ$_1$ (10 ng/mL) treated cells for 72 hours demonstrate characteristic EMT-associated changes in morphology. **(D–G)** Immunofluorescence staining for E-cadherin (red) and EDA-fibronectin (green) with and without TGFβ$_1$ treatment; nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar is equal to 100 μM.

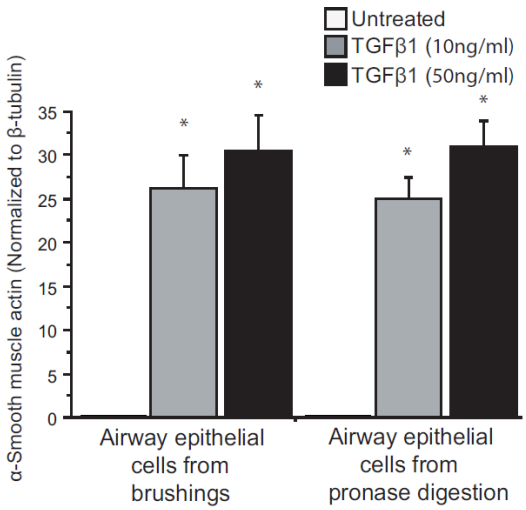


Figure 3.2 TGFβ_1 induces EMT in pHAECs obtained by bronchial brushing or by pronase digestion from donor lungs.
pHAECs obtained from bronchial brushings (n=6) or pronase digestion of donor lungs (n=12) were treated with 10 or 50 ng/mL TGFβ_1 or vehicle control for 72 hours. Densitometry analysis of band intensities for α-smooth muscle actin expression indicate no significant difference in any of the proteins expressed between untreated cells (unfilled bars), TGFβ_1 10 ng/mL treated (light grey bars), or TGFβ_1 50 ng/mL treated (dark grey bars) cells derived from bronchial brushings or pronase digestion of donor lungs. Values given are the mean ± SEM, and all values were normalized to β-tubulin. * Indicates $p<0.05$ compared to untreated control by one-way ANOVA with Dunnett's post-test.

3.3.2 TGFβ_1 induces EMT in airway epithelial cells derived from asthmatic donors

As EMT may play an important role in the sub-epithelial remodeling observed in asthma, epithelial cells from asthmatic patients were examined for their capacity to undergo EMT following exposure to TGFβ_1. Asthmatic epithelial cells exposed to TGFβ_1 underwent identical phenotypic changes associated with EMT as described earlier for non-asthmatic cells (**Figure 3.3**). Densitometric analysis of E-cadherin, ZO-1, EDA-fibronectin, and vimentin expression following exposure to TGFβ_1 at 10 and 50 ng/mL concentrations showed no statistical difference in the magnitude of expression of each marker between asthmatic and normal pHAEC protein lysates (**Figure 3.4A-D**, respectively, n=18).

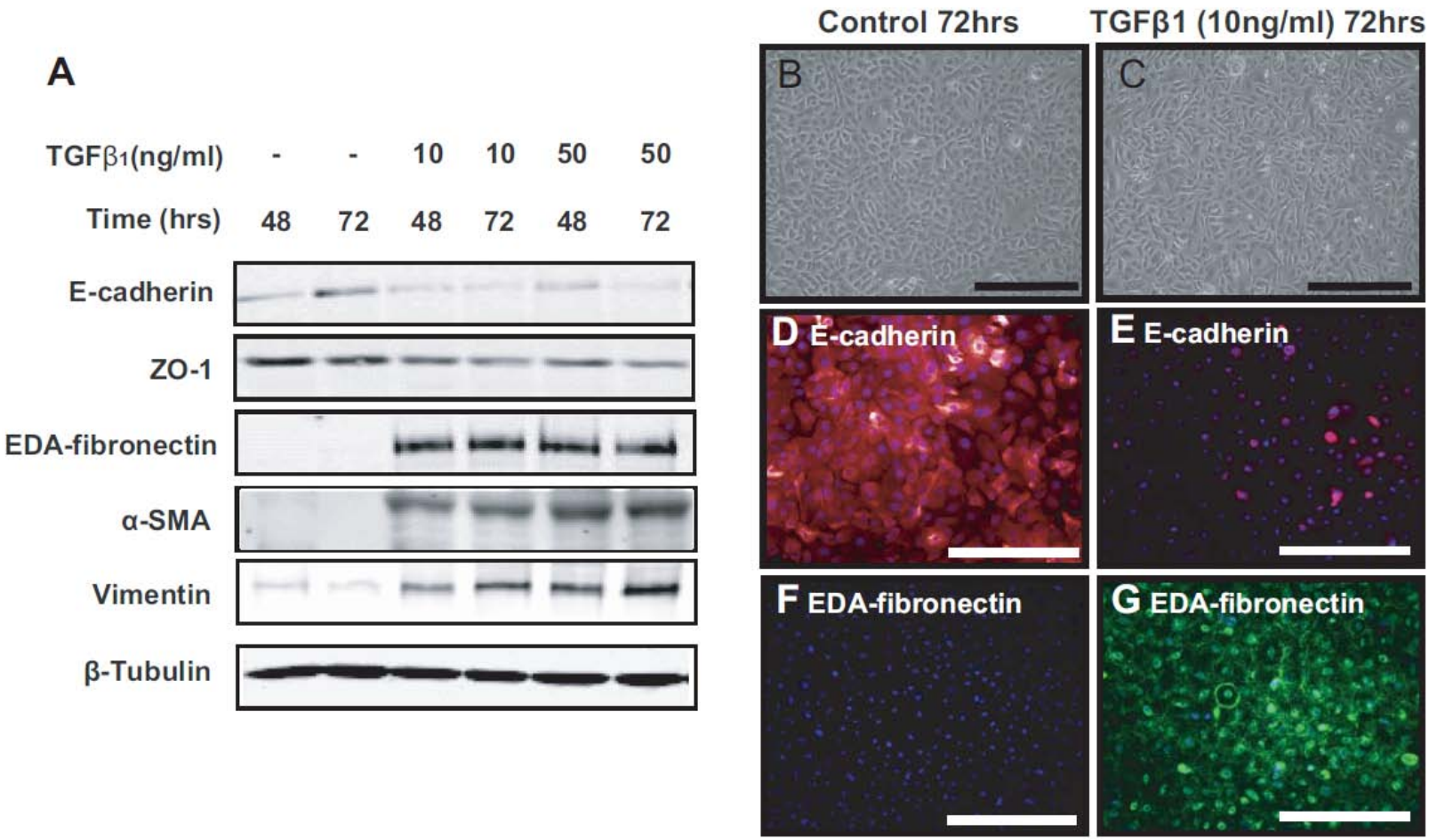


Figure 3.3 TGFβ$_1$ induces epithelial–mesenchymal transition in asthmatic-derived pHAECs.
(A) Representative immunoblot of total cell lysates from asthmatic-derived pHAECs treated with 10 or 50 ng/mL TGFβ$_1$ or vehicle control for 48 and 72 hours and analyzed for E-cadherin, ZO-1, EDA-fibronectin, α-smooth muscle actin, and vimentin expression. β-tubulin is included as a loading control. **(B–G)** Representative phase contrast images and immunofluorescence staining for E-cadherin (*red*) and EDA-fibronectin (*green*) expression in asthmatic-derived pHAEC monolayers treated with or without 10 ng/mL TGFβ$_1$ for 72 hours. *Scale bar* is equal to 100 μM.

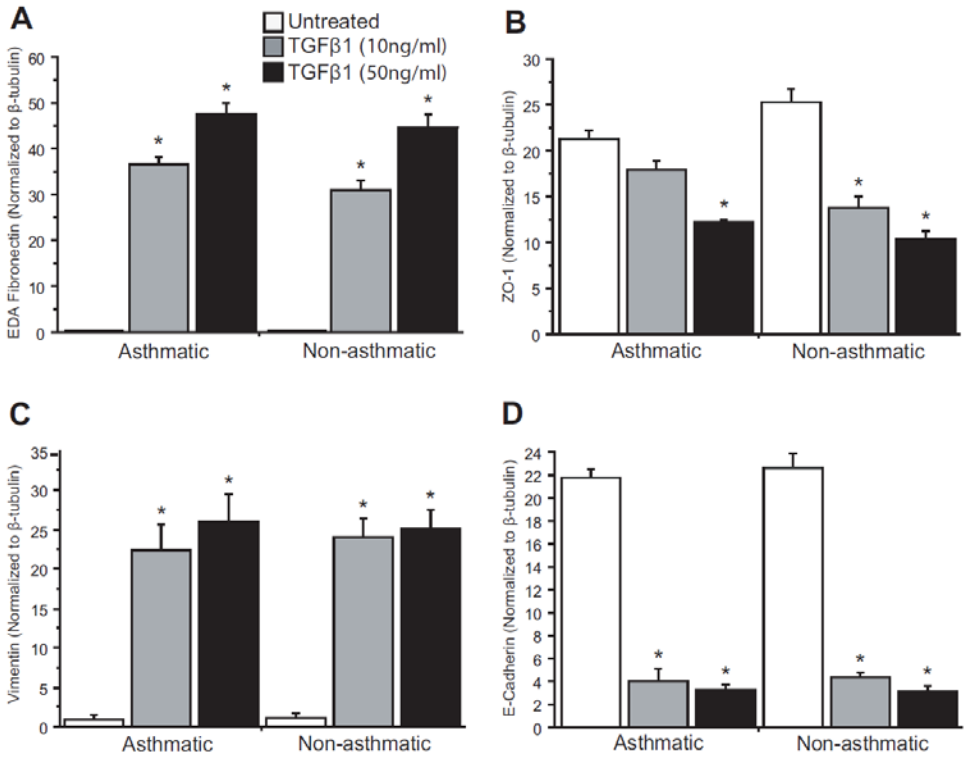


Figure 3.4 Both non-asthmatic and asthmatic pHAECs undergo EMT in monolayer culture.
(**A-D**) Densitometry analyses of band intensities for (**A**) EDA-fibronectin, (**B**) ZO-1, (**C**) vimentin, or (**D**) E-cadherin for each individual in the study (n = 18), which indicated no significant difference in protein expression at 72 hours between untreated cells (*open bars*), 10 ng/mL TGFβ_1 treated (*light grey bars*), or 50 ng/mL TGFβ_1 treated (*dark grey bars*) cells derived from donors with or without asthma. Values given are the mean ± SEM, and all values were normalized to β-tubulin. * Indicates $p<0.05$ compared with untreated control by one-way ANOVA with Dunnett's post-test.

3.3.3 TGFβ_1 induces changes in gene expression during EMT

To determine the profile of mesenchymal and extracellular matrix (ECM) genes expressed during TGFβ_1-induced EMT, mRNA was isolated from pHAECs treated with TGFβ_1 (10 ng/mL) for 24 hours and RNA expression determined using a custom-designed RT-qPCR array for a panel of genes. TGFβ_1 significantly induced RNA expression of multiple mesenchymal markers including vimentin and α-smooth muscle actin (**Figure 3.5**, n=18). TGFβ_1 also up-regulated the

expression of RNAs encoding ECM proteins collagen-1α1, fibrinogen and fibronectin, as well as growth factors connective tissue growth factor (CTGF) and TGFβ$_1$ itself. TGFβ$_1$ also induced expression of its receptor component TGFβRI, but not TGFβRII, and SMAD3-dependent transcription factors Snail 1 and 2. We also observed an increase in expression of the adhesion molecules CD44 and integrin β3 with concurrent loss of E-cadherin. There was no statistical difference in gene expression between asthmatic- or non-asthmatic-derived pHAECs following TGFβ$_1$ treatment.

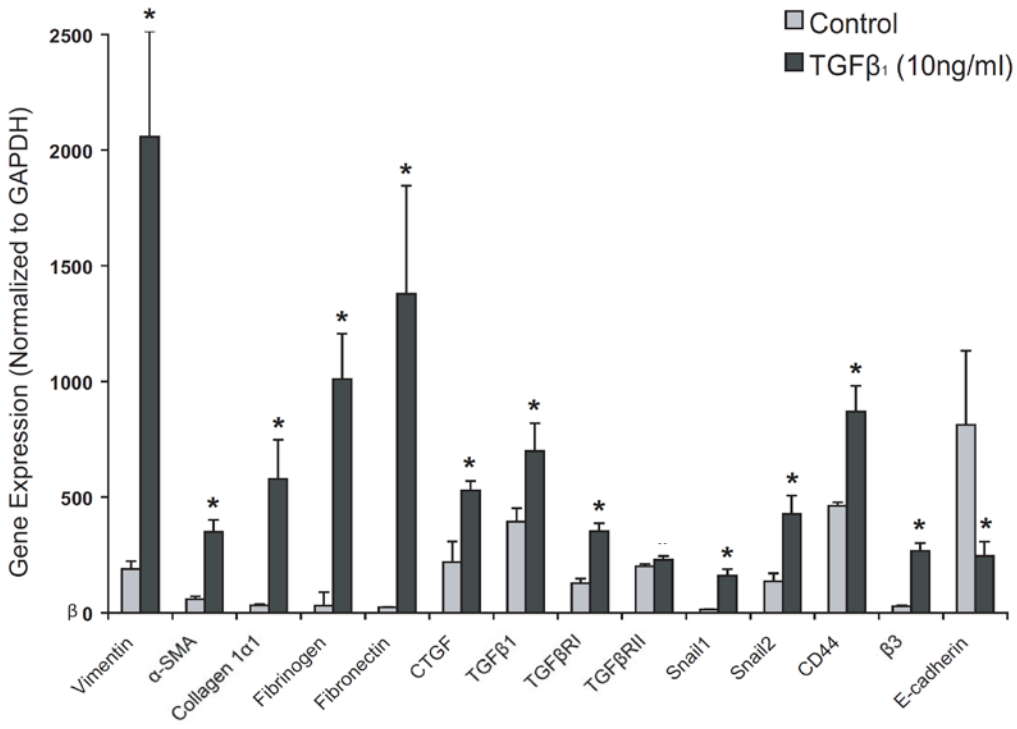


Figure 3.5 TGFβ$_1$ induces expression of mesenchymal and extracellular matrix (ECM) genes during epithelial–mesenchymal transition.
Primary airway epithelial cells (n = 18) were stimulated with 10 ng/mL of TGFβ$_1$ (*solid bars*) or vehicle control (*shaded bars*) for 24 hours. Using a custom-designed RT-qPCR panel, expression of mesenchymal, ECM, growth factors, signaling mechanisms, and cell migration genes previously reported to be induced by TGFβ$_1$ were evaluated. Values given are the mean ± SEM of pooled samples. All values were normalized to GAPDH. * Indicates $p<0.05$ for TGFβ-treated compared to untreated control by Student's unpaired t-test.

In addition to SMAD-mediated transcription, TGFβ_1 activates other signaling pathways, including ERK1/2 and p38 MAP kinase pathways during EMT (Hartsough and Mulder 1995, Zavadil, Bitzer et al. 2001, Bakin, Rinehart et al. 2002). Accordingly, we assessed the relative contribution of SMAD3 or MAPK including p38 and p42/p44 to TGFβ_1-induced EMT in pHAECs. Addition of SMAD3-inhibiting siRNA (10 nM) blocked SMAD3 expression and TGFβ_1-induced EMT as reflected by robust E-cadherin expression and a lack of EDA-fibronectin expression even after 72 hours (**Figure 3.6A**). In contrast, neither the MEK/ERK1/2 inhibitor U0126 (10 μM) nor the p38 inhibitor SB203580 (5 μM) had an effect on TGFβ_1-induced changes in expression of EDA-fibronectin and loss of E-cadherin expression, as shown by the mean densitometry values in **Figure 3.6B** (n=18). Inhibition of MEK/ERK1/2 or p38 alone did not affect the expression of epithelial proteins (**Figure 3.6C**). Taken together, these data demonstrate that SMAD3 signaling is necessary and sufficient for TGFβ_1-induced EMT in pHAECs.

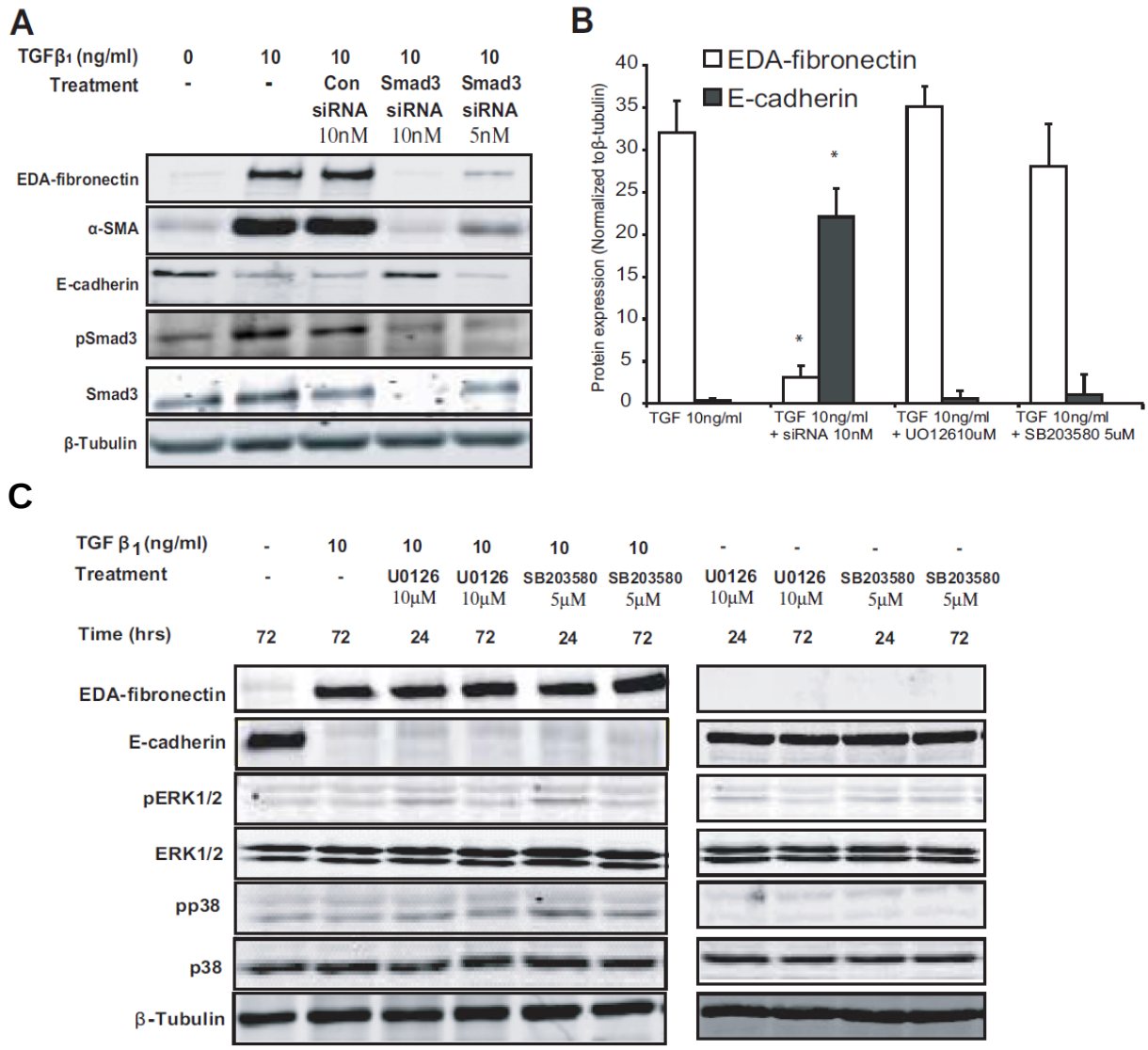


Figure 3.6 TGFβ$_1$-induced phosphorylation of SMAD3 is essential for the expression of mesenchymal and ECM genes during EMT.

(**A**) pHAECs (n=12) were transfected with human SMAD3-inhibiting chimera-RNAi, scrambled siRNA control, or HiPerFect transfection reagent for 24 hours, or the MEK/ERK1/2 inhibitor U0126 (10 μM), the p38 inhibitor SB203580 (5 μM), or media control for 1 hour, and then stimulated with TGFβ$_1$ (10 ng/mL) or vehicle control for a further 72 hours with each relevant inhibitor being added every 24 hours. Representative immunoblot of total cell lysates analyzed for the expression of EDA-fibronectin, α-smooth muscle actin, E-cadherin, pSMAD3, SMAD3, and β-tubulin protein expression. (**B**) Densitometric analysis of EDA-fibronectin (*open bars*) and E-cadherin (*solid bars*) protein expression normalized to β-tubulin after SMAD3 inhibiting siRNA, UO126, or SB203580 treatment. Data are expressed as mean ± SEM. * Indicates $p < 0.05$ compared with TGFβ$_1$ treatment by one-way ANOVA with Dunnet's post-test (n=12). (**C**) Representative immunoblot of total cell lysates analyzed for the expression of EDA-fibronectin, E-cadherin, pERK1/2, pP38 and their un-phosphorylated forms and β-tubulin demonstrates that treatment with UO126 or SB203580 did not inhibit TGFβ$_1$-induced EMT in pHAECs and did not alter the expression of EDA-fibronectin or E-cadherin (n=12).

3.3.4 TGFβ_1 induces EMT in ALI-AEC cultures

Air–liquid interface cultures of airway epithelial cells (ALI-AEC cultures) were used to determine whether specific subtypes of epithelial cells undergo EMT. As demonstrated by the representative hematoxylin and eosin (H&E)-stained section of a non-asthmatic ALI-AEC culture incubated with TGFβ_1 (10 ng/mL), the basal and suprabasal cells lost their cobblestone morphology and gained a spindle-shaped mesenchymal morphology (**Figure 3.7A** and **B**). We demonstrated that the cells that changed morphology were indeed of a basal cell phenotype by staining for the basal cell marker cytokeratin 5 (**Figure 3.7C** and **D**). We further confirmed that the changes in morphology observed following TGFβ_1 stimulation were accompanied by the presence of mesenchymal markers vimentin (**Figure 3.7E** and **F**) and α-smooth muscle actin (**Figure 3.7I** and **J**). Further analysis revealed that the cells expressing these mesenchymal markers and morphological changes were not ciliated or goblet cells since there was no overlap with cytokeratin-18 (**Figure 3.7G** and **H**) or mucin 5AC (**Figure 3.7K** and **L**) staining, respectively. These findings support the concept that basal cells are likely to undergo EMT.

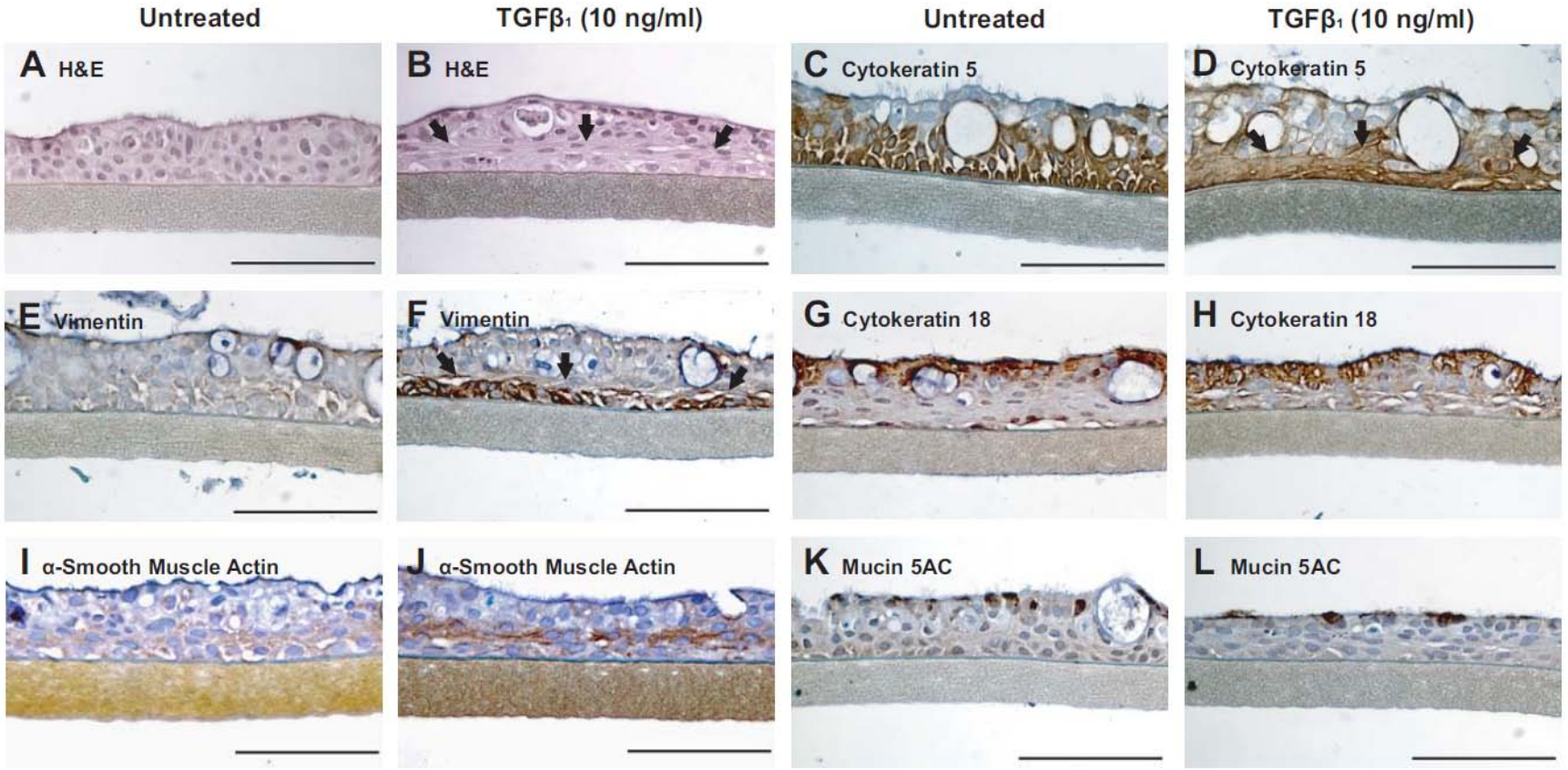


Figure 3.7 TGFβ$_1$ induces EMT in ALI-AEC cultures.
ALI-AEC cultures derived from non-asthmatic pHAECs were incubated with (**B**, **D**, **F**, **J** and **L**) or without (**A**, **C**, **E**, **G**, **I** and **K**) TGFβ$_1$ (10 ng/mL) for 72 hours and then fixed, embedded in paraffin and then sectioned for immunohistochemical analysis. ALI-AEC sections were stained with (**A**, **B**) hematoxylin and eosin (H&E), (**C**, **D**) cytokeratin 5, (**E**, **F**) vimentin, (**G**, **H**) cytokeratin 18, (**I**, **J**) α-smooth muscle actin, and (**K**, **L**) mucin 5AC. Scale bar is equal to 50 μM. Arrows indicate changes in morphology.

ALI-AEC cultures were analyzed by immunoblot to identify changes in protein expression associated with EMT, and to assess the role of SMAD3 in TGFβ$_1$-induced EMT. Robust phosphorylation of SMAD3 was observed in ALI-AEC cultures incubated with TGFβ$_1$ (10 ng/mL), coincident with increased expression of EDA-fibronectin, α-smooth muscle actin and vimentin, and loss of E-cadherin and ZO-1 expression (**Figure 3.8**). The addition of SMAD3-inhibiting siRNA in the basal ALI compartment inhibited TGFβ$_1$-induced mesenchymal markers and maintained E-cadherin expression, supporting the role of SMAD3 in TGFβ$_1$-induced EMT in ALI-AEC cultures. Similar findings were observed in ALI-AEC cultures derived from asthmatic donors, although the magnitude of changes in EDA-fibronectin and E-cadherin were significantly different compared to ALI-AEC cultures derived from non-asthmatic donors (**Figure 3.9**, n=12).

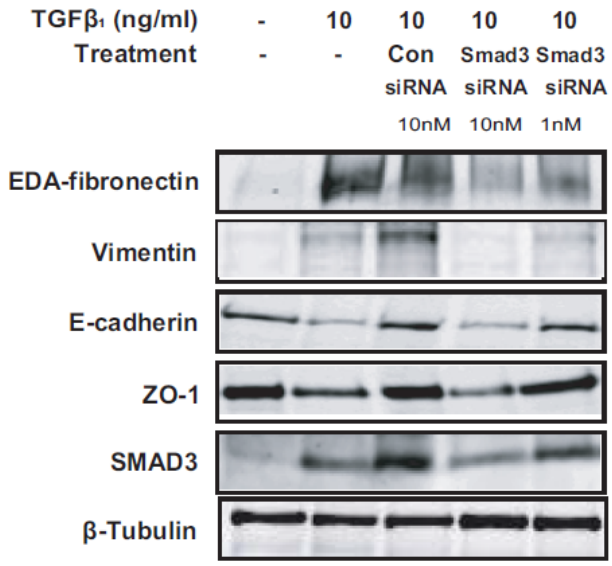


Figure 3.8 SMAD3 mediates TGFβ$_1$-induced EMT in ALI-AEC cultures.
ALI-AEC cultures derived from asthmatic and non-asthmatic pHAECs were transfected with SMAD3-inhibiting siRNA, control scrambled siRNA, or vehicle control for 24 hours, and then incubated with or without TGFβ$_1$ (10 ng/mL) for a further 72 hours. A representative immunoblot of total ALI-AEC culture protein lysates analyzed for EDA-fibronectin, vimentin, E-cadherin, ZO-1, SMAD3, and β-tubulin is shown.

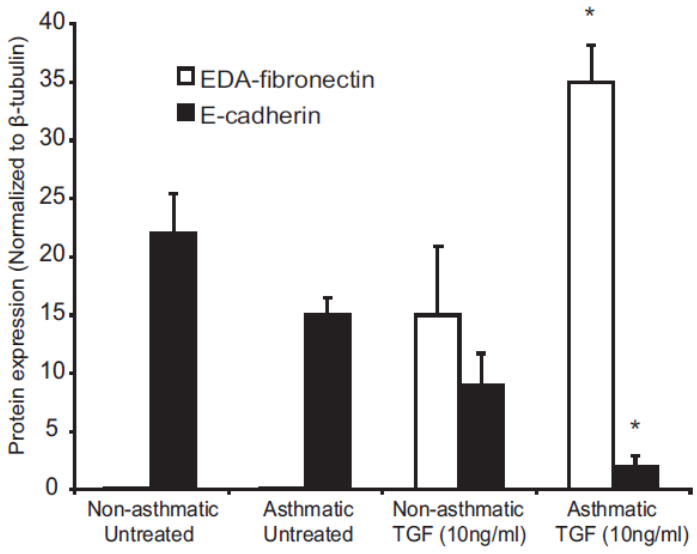


Figure 3.9 TGFβ$_1$ induces pronounced EMT changes in ALI-AEC cultures from asthmatic donors. Densitometry analysis was performed on immunoblots of EDA-fibronectin (*open bars*) and E-cadherin (*solid bars*) expression in ALI-AEC from donors with and without asthma. Data are expressed as mean ± SEM and * indicates $p<0.05$ compared with TGFβ-treated non-asthmatic ALI-AEC cultures (n=12) by Student's unpaired t-test.

3.3.5 Basal epithelial cells in ALI-AEC cultures undergo TGFβ$_1$-induced EMT

To determine which subtypes of pHAECs were able to undergo EMT in asthmatic and non-asthmatic derived ALI-AEC cultures, we used confocal microscopy to analyze entire ALI-AEC cultures. As only one epithelial protein and one mesenchymal protein can be analyzed at the same time with confocal microscopy, we chose to analyze changes in E-cadherin and EDA-fibronectin expression. As shown in the 3-dimensional reconstruction of an untreated ALI-AEC culture (**Figure 3.10A**) and the *en face* sections from the apical and basal surface of the reconstruction (**Figure 3.10B**), all untreated cultures stained for E-cadherin (red), but not EDA-fibronectin (green). Exposure of ALI-AEC cultures derived from non-asthmatic subjects to TGFβ$_1$ (10 ng/mL) for 72 hours resulted in decreased E-cadherin (red) and increased EDA-

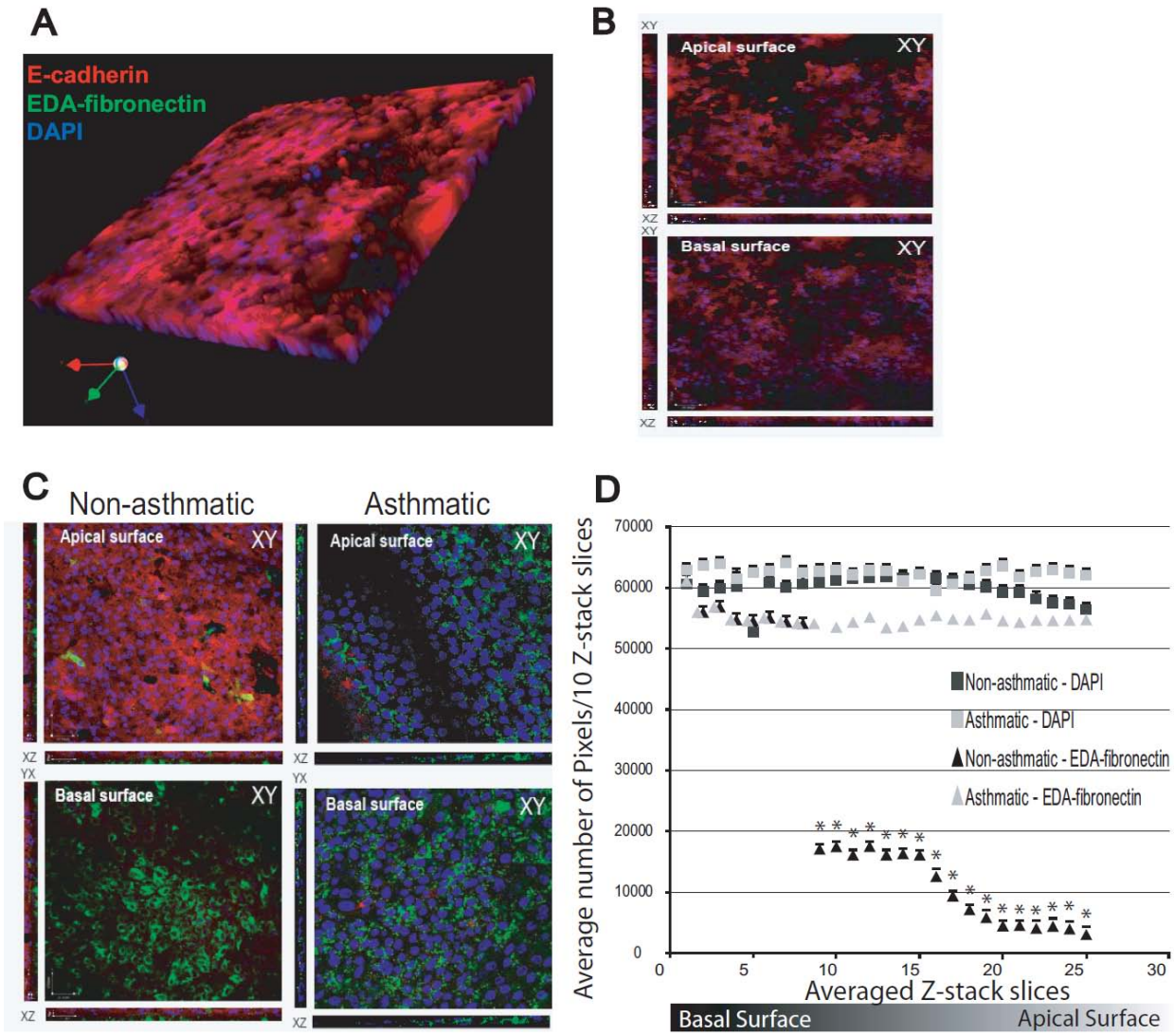


Figure 3.10 TGFβ$_1$-induced EMT occurs in the expanded basal cell population of ALI-AEC cultures from asthmatic donors.

(A) Representative three-dimensional reconstruction of an untreated ALI-AEC culture imaged using confocal microscopy and (**B**) *En face* apical and basal sections from the reconstructed image demonstrate E-cadherin (*red*) staining throughout the entire ALI-AEC but no EDA-fibronectin expression (*green*). (**C**) Confocal images of the *en face* apical and basal surfaces of TGFβ$_1$ (10 ng/mL) treated ALI-AEC cultures derived from donors with and without asthma indicate EDA-fibronectin (*green*), E-cadherin (*red*), and nuclei (*blue*). (**D**) Volocity software classification was used to perform comparative measurements of the number of brightest fluorescent spots for 4′,6-diamidino-2-phenylindole (DAPI) and EDA-fibronectin per every 10 Z-stack slices, showing that EDA-fibronectin is expressed throughout the entire asthmatic-derived ALI-AEC, but restricted to the basal surface in non-asthmatic-derived cultures. Data are expressed as average number of pixels per 10 Z-stack slices ± SEM. * Indicates $p<0.05$ compared with asthmatic-derived ALI-AEC cultures by two-way ANOVA with Bonferroni post-test.

fibronectin (green) staining in cells predominantly localized within the basal cell layer (**Figure 3.10C**, left). In contrast, treatment of asthmatic derived ALI-AEC cultures with TGFβ_1 had profound effects, with large numbers of cells spread diffusely throughout the multilayered tissue showing intense staining for EDA-fibronectin (green) and reduced E-cadherin positivity (red) **(Figure 3.10C**, right). These observations were further validated by image analysis using Volocity classification. As demonstrated in **Figure 3.10D**, the mean number of pixels for EDA-fibronectin (classified for Alexa 488, green) proportional to the number of pixels for DAPI (blue) were constant through the entire asthmatic-derived ALI-cultures compared to non-asthmatic-derived ALI-AEC cultures, wherein staining was restricted to the basal cell layer ($p<0.05$, $n=5$ per group). Finally, we show that the EMT marker EDA-fibronectin and the basal cell markers cytokeratin-5 and p63 were both expressed in pHAECs undergoing EMT (**Figure 3.11A** and **B**).

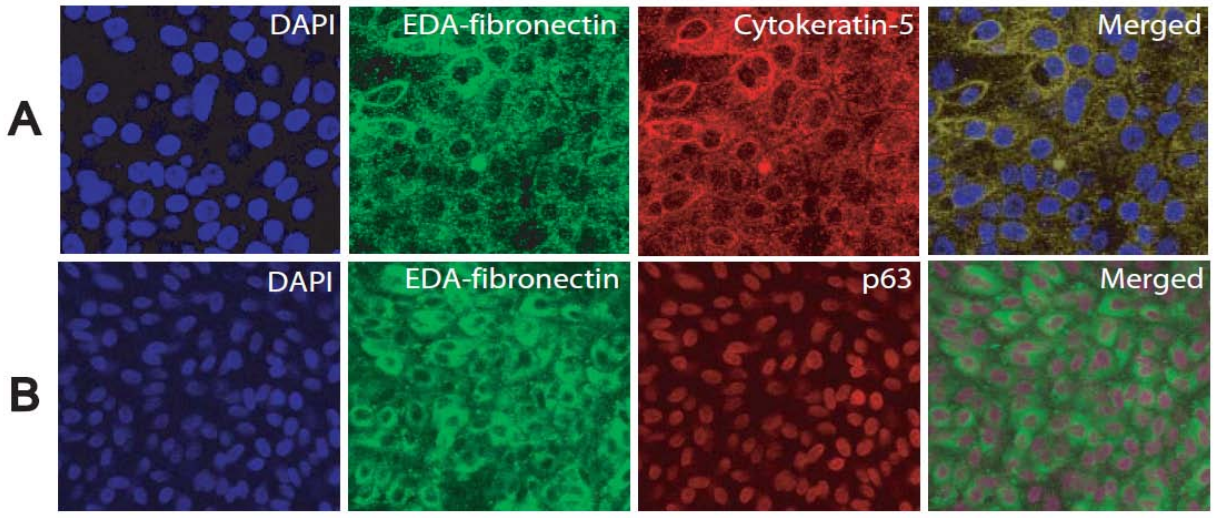


Figure 3.11 Cells expressing basal cell markers CK-5 and p63 undergo TGFβ_1-induced EMT in ALI-AEC cultures.
Representative *en face* confocal images of the basal surface of cells in ALI-AEC cultures treated with TGFβ_1 (10 ng/mL) for 72 hours and stained with **(A)** DAPI (*blue*), EDA-fibronectin (*green*, Alexa 488), and cytokeratin-5 (*red*, Alexa 594); **(B)** DAPI (*blue*), EDA-fibronectin (*green*, Alexa 488), p63 (*red*, Alexa 594); merged sections are shown far right.

3.3.6 Expansion of the basal cell population leads to increased TGFβ$_1$-induced EMT in ALI-AEC cultures from asthmatic donors

The number of basal cells, identified by p63 and cytokeratin-5 expression, was increased in asthmatic compared to non-asthmatic derived ALI-AEC cultures by immunohistochemical staining of transverse ALI-AEC sections (**Figure 3.12A** and **B**). Following exposure to TGFβ$_1$ (10 ng/mL), basally-situated cells in ALI-AEC underwent changes in morphology indicative of EMT, marked by the arrow heads in **Figure 3.12**, and stained for p63 and cytokeratin-5, consistent with a basal cell phenotype.

Using donor-matched airway sections, we demonstrate that airways from asthmatics have increased numbers of cells expressing cytokeratin-5 and p63 *in vivo*, indicating that our *in vitro* ALI-AEC cultures are representative of the asthmatic airway epithelium *in vivo* (**Figure 3.13A** and **B**). Despite the increased numbers of cytokeratin-5 and p63 positive cells, we did not find epithelial cells positive for mesenchymal markers α-smooth muscle actin or vimentin in asthmatic airway epithelium *in vivo* (**Figure 3.13A** and **B**, n=12).

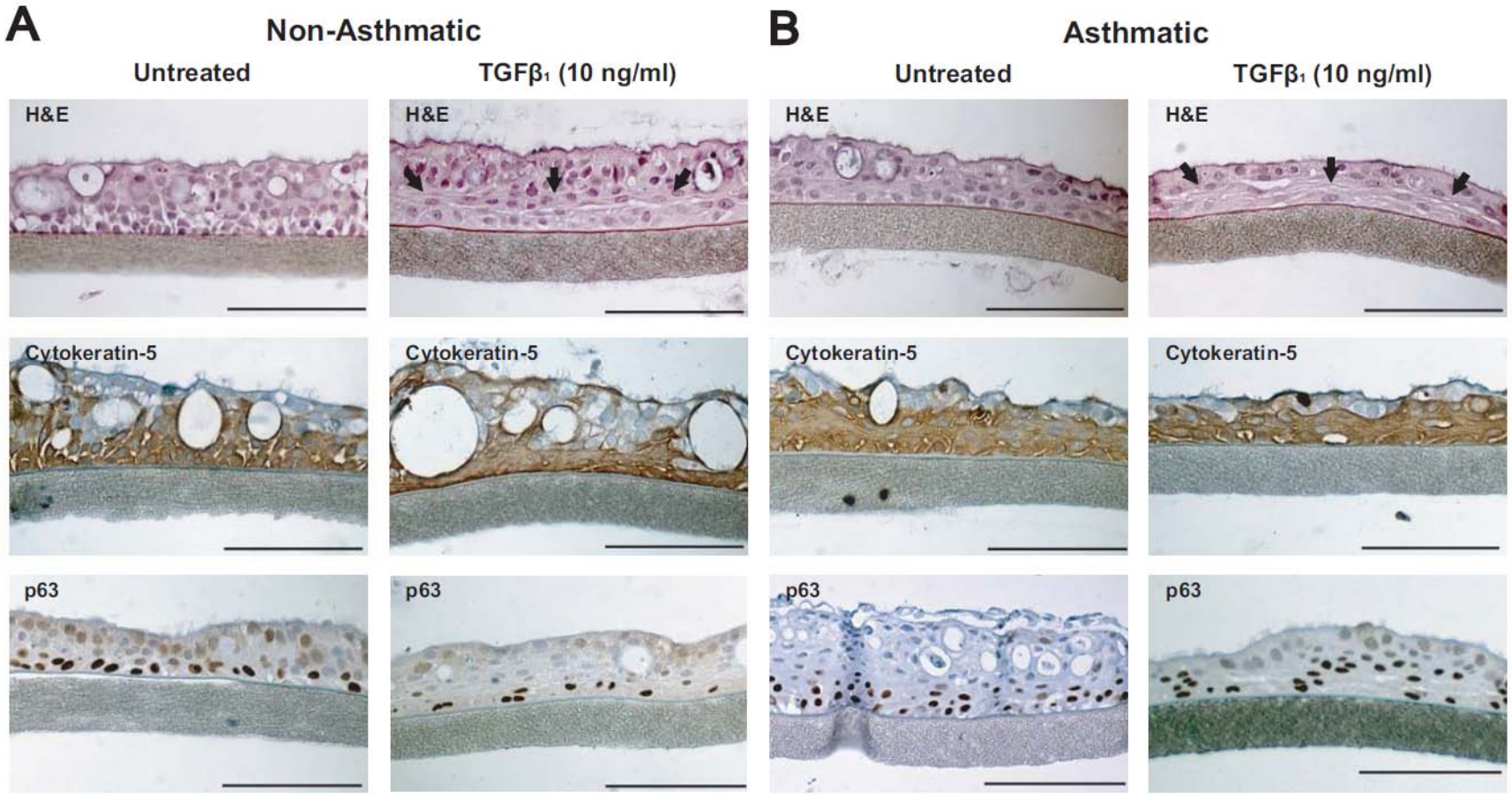


Figure 3.12 TGFβ$_1$-induced EMT occurs to a greater degree in asthmatic-derived ALI-AEC cultures.
ALI-AEC cultures derived from (**A**) non-asthmatic and (**B**) asthmatic donors were incubated with or without TGFβ$_1$ (10 ng/mL) for 72 hours and then fixed, embedded in paraffin, sectioned and stained with H&E, cytokeratin-5 or p63. Arrows indicate changes in morphology. Scale bar is equal to 50 μM.

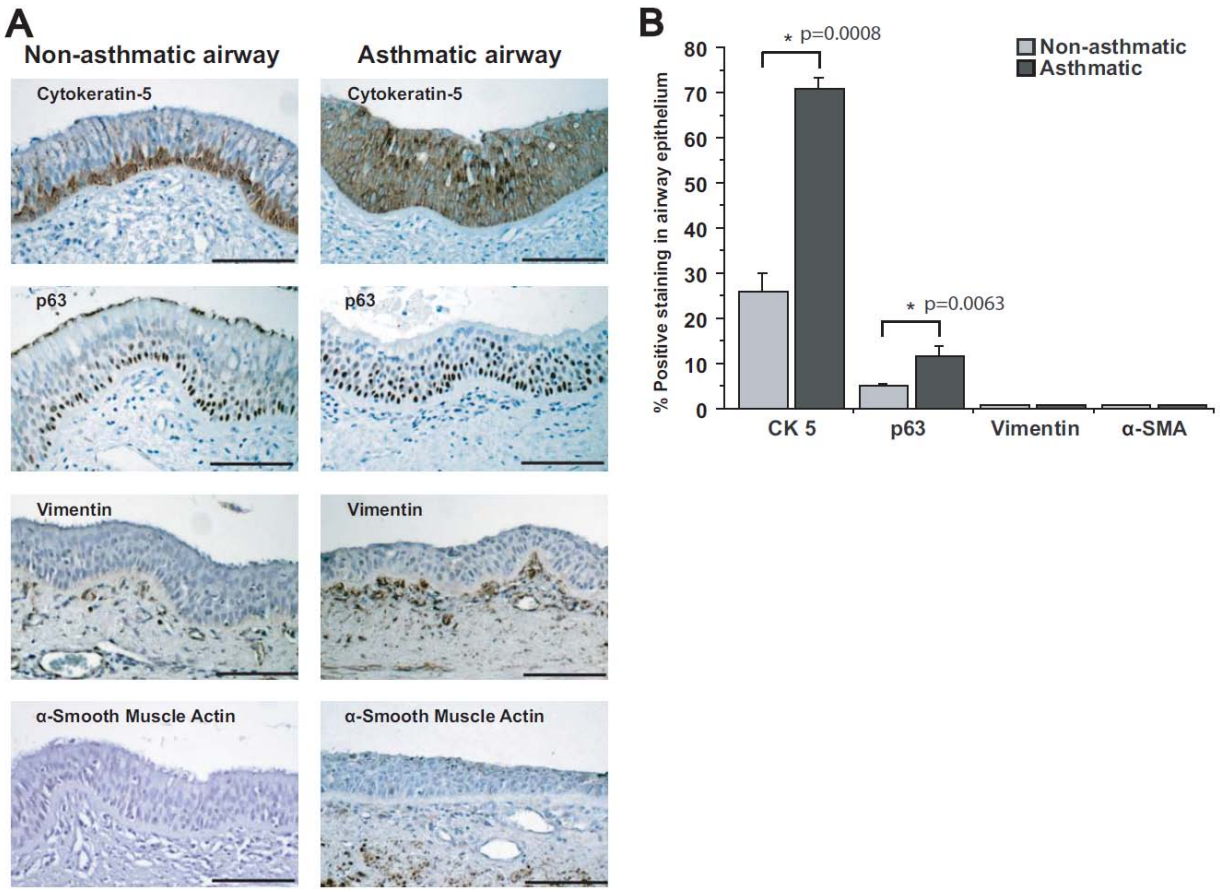


Figure 3.13 Markers of epithelial–mesenchymal transition are not present in asthmatic airways *in vivo*.
(A) Airway sections were immunostained for cytokeratin-5, p63, vimentin, and α-smooth muscle actin staining (*brown*) in airway epithelium of donor-matched nonasthmatic and asthmatic airways. Scale bar is equal to 100 μM. **(B)** The expression of cytokeratin-5, p63, vimentin, and α-smooth muscle actin staining within the epithelium was quantified using Image pro plus software. Data are expressed as % of positive staining in airway epithelium ± SEM. *Light grey bars* indicate normal; *dark grey bars* indicate asthmatic. * Indicates $p<0.01$ compared with non-asthmatic airways by Student's unpaired t-test (n=12).

3.4 Discussion

In this study, we provide evidence that primary human airway epithelial cells in culture undergo EMT in response to $TGF\beta_1$. We demonstrate that $TGF\beta_1$-induced EMT is SMAD3-dependent and characterized by the transition from a typically epithelial cuboidal morphology to a spindle-shaped cell expressing mesenchymal markers EDA-fibronectin, vimentin, and α-SMA, with a

coordinate loss of epithelial proteins E-cadherin and ZO-1. Importantly, we show that in differentiated, multilayered ALI-AEC cultures derived from non-asthmatic donors, $TGF\beta_1$-induced EMT is confined to the basal cell layer. However, in ALI-AEC cultures derived from asthmatic donors there is an increased number of cells that express basal cell markers cytokeratin-5 and p63 and undergo $TGF\beta_1$-induced EMT. We demonstrate that the number of cells expressing basal cell markers cytokeratin-5 and p63 is increased in asthmatic airways *in vivo*. Epithelial cells expressing mesenchymal markers, including vimentin and α-smooth muscle actin, characteristic markers of EMT, were not present *in vivo*.

In asthma, increased deposition of collagen, fibronectin and other ECM proteins in the basement membrane contribute to sub-epithelial fibrosis and airway hyperresponsiveness (Davies, Wicks et al. 2003). The most likely cells responsible for the increased deposition of ECM are fibroblasts and myofibroblasts, with the number of these cells correlating with the magnitude of subepithelial thickening (Gizycki, Adelroth et al. 1997). The origin(s) of these mesenchymal cells remains unknown, although the recruitment of bone marrow-derived fibrocytes and/or the expansion of resident fibroblasts likely play important roles. Epithelial cells undergoing EMT are involved in fibrogenesis in several tissues such as the liver and kidney, contributing up to 36% of the interstitial fibroblast population (Forino, Torregrossa et al. 2006, Kaimori, Potter et al. 2007). With respect to the conducting airway epithelium, few studies of EMT have been reported. Ward and colleagues suggested that EMT occurs in the small airways of clinically stable lung transplant recipients (Ward, Forrest et al. 2005). Molloy et al. showed that EMT-like responses occur in the transformed epithelial cell line BEAS-2B in response to BMP-4 (Molloy, Adams et al. 2008) and in normal airway epithelial cells during repair (McCormack, Molloy et al. 2013). In addition, Wu et al. found evidence of EMT in a small

subset of bronchial epithelial cells following administration of bleomycin in α-smooth muscle actin–Cre reporter transgenic mice (Wu, Yang et al. 2007). These authors also showed an increase in α-smooth muscle actin expression and reduction in E-cadherin expression following exposure to very high concentrations of $TGF\beta_1$ (10 mg/mL) in the SV40-transformed human epithelial cell line 16-HBE^{o-}.

In the present study, we show that primary human airway epithelial cells *in vitro* from both asthmatic and non-asthmatic donors undergo EMT following exposure to 10 ng/mL of $TGF\beta_1$. This was characterized by the hallmark changes associated with EMT, including loss of cobblestone or cuboidal morphology and a marked reduction in E-cadherin and ZO-1 expression, particularly at the cell borders. *In vitro* this would facilitate the disintegration of cell-cell contacts that normally maintain epithelial integrity and barrier function. These more fibroblast-like cells exhibit activated molecular programs allowing the *de novo* synthesis of mesenchymal cytoskeleton proteins α-smooth muscle actin, vimentin and EDA-fibronectin. Our findings are consistent with several studies evaluating EMT in the alveolar type II epithelial carcinoma cell line, A549 (Kasai, Allen et al. 2005, Kim, Kugler et al. 2006, Ando, Otani et al. 2007) and transformed airway epithelial cells (Wu, Yang et al. 2007, Molloy, Adams et al. 2008).

To exclude the possibility of contamination of primary epithelial cultures with fibroblasts, several measures were used. Firstly the method of dissociation of the airway epithelium yields a 98% pure epithelial population by FACS analysis. Epithelial cultures were grown in serum free media which does not support fibroblast growth. Secondly, untreated monolayers exhibited 100% epithelial morphology over passage, with no detectable expression of mesenchymal markers.

To further define the cellular phenotype and processes involved in TGFβ$_1$-induced EMT, a large number of genes were analyzed using a custom-designed RT-qPCR panel (as shown in **Figure 3.5**). Aside from vimentin and α-smooth muscle actin, TGFβ$_1$ induced the expression of the ECM proteins collagen-1α1, fibrinogen and fibronectin. TGFβ$_1$ also upregulated the SMAD3-dependent transcription factors snail1 (SNAI1) and snail2 (SNAI2), which repress E-cadherin transcription (Cano, Perez-Moreno et al. 2000, Carver, Jiang et al. 2001). We also found two genes not previously associated with EMT, integrin β3 and CD44, to be upregulated following TGFβ$_1$ exposure (**Figure 3.5**). The β3 integrin is distributed at the polarized cell front of migrating fibroblasts and is important for cell transmigration on fibronectin (Woods, White et al. 2004). CD44 is a cell adhesion molecule and a receptor for both hyaluronan and fibronectin, which are components of the provisional matrix that facilitate cell migration at wound sites (Knox, Crooks et al. 1986, Turley 1992, Greiling and Clark 1997). CD44 expression is also increased on damaged epithelial cells in asthma (Leir, Baker et al. 2000) and CD44 knockout mice show enhanced inflammation and decreased fibroblast infiltration (Huebener, Abou-Khamis et al. 2008). The upregulation of these pro-migratory factors following TGFβ$_1$ treatment supports the idea that the epithelial cells have gained a mesenchymal phenotype and are highly motile.

We also observed that TGFβ$_1$ increased gene expression of itself, CTGF and TGFβRI, but not TGFβRII (**Figure 3.5**). As TGFβRI is primarily associated with SMAD signaling, we further defined the contribution of this signaling pathway to EMT. Our data show that the TGFβ$_1$-induced expression of mesenchymal genes was accompanied by phosphorylation of SMAD3 in a time- and dose-dependent manner. This finding is consistent with TGFβ$_1$-induced EMT in several cell types including breast, renal and lung epithelial cells (Zavadil, Cermak et al.

2004, Kasai, Allen et al. 2005, Valcourt, Kowanetz et al. 2005). Similarly, several *in vivo* models of EMT and fibrosis have shown that SMAD3 deficiency attenuates the ability of $TGF\beta_1$ to induce key transcriptional regulators of EMT (Sato, Muragaki et al. 2003, Saika, Kono-Saika et al. 2004, Zavadil, Cermak et al. 2004). Our data further demonstrate that knockdown of SMAD3 using SMAD3-inhibiting siRNA in pHAECs maintains E-cadherin expression and prevents expression of EDA-fibronectin. $TGF\beta_1$ signaling through SMAD-independent pathways such as p38 MAPK and ERK1/2 also induce loss of E-cadherin in some models of EMT, particularly in cancer (Ellenrieder, Hendler et al. 2001, Bates and Mercurio 2003). Furthermore, Ross and colleagues reported that activating ERK increases SMAD3 promoter activity in epithelial and smooth muscle cells (Ross, Corey et al. 2007). Here we demonstrate that incubation with the MEK/ERK1/2 inhibitor U0126 or p38 MAPK inhibitor SB203580 did not inhibit $TGF\beta_1$-induced EMT in our model system.

While EMT has been shown to occur in animal lungs *in vivo*, evaluating this process in human lungs *in vivo* is inherently more difficult. In this investigation, we generated ALI-AEC cultures to determine whether EMT occurs in a differentiated mucociliary epithelium. We show that in ALI-AEC cultures derived from non-asthmatic donor airways, a discrete population of cells in the basal layer identified by specific basal cell markers cytokeratin-5 and p63 (Daniely, Liao et al. 2004, Hajj, Lesimple et al. 2007) undergo $TGF\beta_1$-induced EMT characterized by staining for EDA-fibronectin, α-smooth muscle actin and vimentin. To our knowledge, this is the first demonstration of EMT in a multi-layered, differentiated ALI-AEC culture. In support of our findings, Sarrio and colleagues showed that EMT-like changes associated with tumor invasion and metastasis occur preferentially within a basal cell subtype of carcinomas (Sarrio, Rodriguez-Pinilla et al. 2008). Despite our finding of basal cell hyperplasia in asthma, evidence does not

support a causal link between asthma and lung cancer susceptibility (Rosenberger, Bickeboller et al. 2012), and allergic diseases may actually provide a protective effect against cancer (El-Zein, Parent et al. 2014).

In the current study, TGFβ_1 was added to the basal compartment to mimic release of the growth factor from the sub-epithelial fibroblasts or proteolytic release and activation of TGFβ_1 from the ECM. The finding that only basal cells, and not ciliated or goblet cells, undergo EMT could be due to the localized concentration of TGFβ_1 within the basal compartment. However, TGFβ_1 has a molecular weight of 25 kDa and has been shown to diffuse through basal layers via cell junctions (McDowell, Gurdon et al. 2001). Given that basal cells are tissue resident progenitor cells, we hypothesize that EMT occurs within basal cells due to their plasticity and differentiation potential.

We found that ALI-AEC cultures derived from asthmatic donors treated with TGFβ_1 completely lost E-cadherin expression throughout the entire ALI-AEC culture with concurrent gain in expression of EDA-fibronectin, suggesting that susceptibility to EMT is increased in asthmatic epithelia. Following a direct comparison of asthmatic derived ALI-AEC cultures and donor-matched airway sections, we provide evidence that there is a shift toward a less differentiated phenotype characterized by positive staining for basal cell markers cytokeratin-5 and p63 throughout the epithelium both *in vivo* and *in vitro*. This observation is in keeping with our previous finding that epithelial cells obtained via bronchial brushings from asthmatic donors contain a greater percentage of cytokeratin-5 positive epithelial cells compared to non-asthmatics (Kicic, Sutanto et al. 2006). Whether this is the result of a persistent epithelial repair phenotype or a dysregulated differentiation profile is unknown.

Airway remodeling was traditionally considered to be a secondary phenomenon in asthma pathogenesis, developing late in the disease process as a consequence of persistent inflammation (Martinez 2007). However, the importance of remodeling as an early and consistent component of childhood asthma has been emphasized in a number of studies (Fedorov, Wilson et al. 2005, Barbato, Turato et al. 2006). A phenotypic shift in the epithelium of asthmatic airways indicative of stress and injury in both pediatric and adult patients was associated with increased collagen deposition in the lamina reticularis (Fedorov, Wilson et al. 2005). When patient-matched airway sections were stained for markers of EMT, we observed no α-smooth muscle actin or vimentin staining in asthmatic or non-asthmatic airways, suggesting that EMT was not actively occurring *in vivo*.

In conclusion, our data show that TGFβ_1-induced EMT occurs in primary human airway epithelial basal cells *in vitro* via a SMAD3-dependent process. In primary ALI-AEC cultures EMT is confined to basally-situated cells expressing basal cell markers cytokeratin-5 and p63. The number of basal cells is increased in asthmatic ALI-AEC cultures. Staining of patient-matched airway sections demonstrated substantially increased numbers of basal cells expressing cytokeratin-5 and p63 in asthmatics compared to non-asthmatic airway epithelium *in vivo*. We were unable to observe markers of EMT such as α-smooth muscle actin or vimentin in asthmatic airway epithelium. These data support the hypothesis that there is dysregulated differentiation and an increased susceptibility to TGFβ_1-induced EMT in the asthmatic airway epithelium. How these changes in epithelial phenotype affect the progression of asthma and airway remodeling is still unknown.

Chapter 4: Transcription Factor p63 Regulates Key Genes and Wound Repair in Human Airway Epithelial Basal Cells[2]

4.1 Introduction

Goblet cell metaplasia, loss of ciliated epithelial cells, increased susceptibility to oxidant-induced stress, abnormal cytokine and ECM release and mitotic dyssynchrony are associated with asthma (Bucchieri, Puddicombe et al. 2002, Kicic, Sutanto et al. 2006, Freishtat, Watson et al. 2010, Ge, Moir et al. 2010, Kicic, Hallstrand et al. 2010). Our group described a marked expansion of the population of basal cells expressing the transcription factor p63 in the epithelium of asthmatics (Hackett, Shaheen et al. 2008, Hackett, Warner et al. 2009). Importantly, as described in **Chapter 3**, this phenotype was conserved in air-liquid interface (ALI) culture conditions *in vitro* (Hackett, Warner et al. 2009), indicating that this expansion is an intrinsic cellular response and not solely a product of the *in vivo* inflammatory milieu.

The transcription factor p63 is a homologue of the p53 tumor suppressor, and is required for the appropriate development of stratified epithelial tissues. Genetic deletion of p63 in mice results in the absence of stratified epithelia, most notably in the epidermis (Mills, Zheng et al. 1999, Yang, Schweitzer et al. 1999). The subsequent deficiency of barrier function results in perinatal lethality due to dehydration. The airway epithelium is the first structural barrier to the inhaled environment and its ability to repair and regenerate following injury is crucial to

[2]A version of this chapter has been published. Reprinted with permission of the American Thoracic Society. © 2013 American Thoracic Society. Cite: **Warner SM**, Hackett TL, Shaheen F, Hallstrand TS, Kicic A, Stick SM, Knight DA. Transcription Factor p63 Regulates Key Genes and Wound Repair in Human Airway Epithelial Basal Cells. Am J Respir Cell Mol Biol. 2013 Dec;49(6):978-88. Official journal of the American Thoracic Society.

maintain normal tissue function. The basal cells of the airway epithelium exhibit progenitor capacity, and are able to repopulate other cell types, including ciliated and mucus-producing cells, during cellular differentiation (Hackett, Shaheen et al. 2008, Rock, Onaitis et al. 2009) and in response to injury (Musah, Chen et al. 2012). In contrast to the normal pseudostratified airway epithelium consisting of basal, ciliated and mucous cells, the tracheobronchial epithelium of newborn $p63^{-/-}$ mice displays only a single layer of columnar ciliated cells and a complete absence of basal and mucous cells (Daniely, Liao et al. 2004). Thus, the lack of stratified epithelium in p63-/- mice has led to two hypotheses; 1) p63 may play a role in maintaining stem cell populations (Yang, Schweitzer et al. 1999, Pellegrini, Dellambra et al. 2001) or 2) p63 may regulate the commitment to specific epithelial lineages (Mills, Zheng et al. 1999).

Studies of p63 have demonstrated that the gene can be expressed in at least six different mRNA/protein isoforms (Yang, Kaghad et al. 1998). Transcription initiation at the canonical promoter gives rise to isoforms containing the full-length transcriptional activation (TA) domain, whereas initiation from an alternative promoter within intron 3 produces the N-terminally truncated (ΔN) isoforms (**Figure 1A** and **B**). All isoforms share a common DNA-binding domain (DBD) and oligomerization domain (OD), which are required for appropriate function (Natan and Joerger 2012). Alternative splicing at the 3' end produces transcripts of varying length, termed α, β and γ. The α and β splice variants are highly similar; the α variant contains only one additional exon, which encodes a sterile alpha motif (SAM) and a transcription inhibition domain (TID). The γ variant is much shorter and contains a novel 3' end. There have been reports of two additional 3' variants, δ and ε, which function similarly to ΔNp63α (Mangiulli, Valletti et al. 2009), though most current work remains focused on the α, β and γ variants. ΔNp63α is by far the most predominant endogenous isoform in basal cells of stratified epithelial tissues such as the

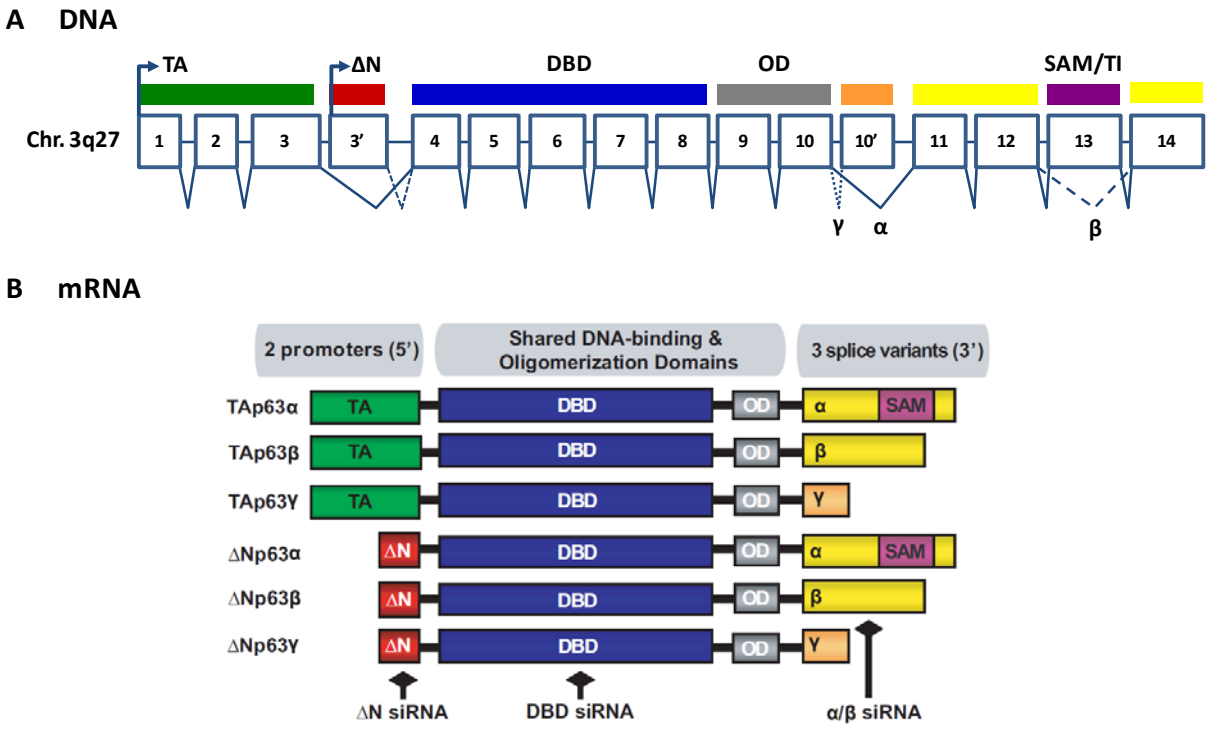


Figure 4.1 Schematic of p63 isoforms arising from the TP63 gene locus.
Two separate promoter variants (TA vs. ΔN), a shared DNA-binding (DBD) and oligomerization (OD) domain, and at least three 3' splice variants (α, β and γ) lead to at least 6 possible p63 isoforms. The α isoforms contain a sterile alpha motif (SAM). Approximate binding sites of siRNA to the ΔN, DBD and α/β domains are indicated. Figure modified from Kawasaki et al. (Kawasaki, Tanioka et al. 2006).

skin (Yang, Kaghad et al. 1998), cornea (Kawasaki, Tanioka et al. 2006) and prostate (Signoretti, Waltregny et al. 2000), and thus has been the most frequent subject of functional studies. To date, the expression and role of specific p63 isoforms in the maintenance and repair of the human airway epithelium has not been explored.

In this study, we hypothesized that p63 regulates basal cell functions such as epithelial repair and expression of repair-related genes in pHAECs. Our aims were to identify which isoforms of p63 are expressed in pHAECs, and to determine the effect of altered p63 expression on 1) the expression of putative target genes and 2) the process of epithelial repair.

4.2 Methods and materials

4.2.1 Cell culture conditions

This study was approved by the Research Ethics Board of the University of British Columbia. Primary human airway epithelial cells (pHAECs) were isolated from non-asthmatic and asthmatic donor lungs deemed unsuitable for transplant, as described in **Chapter 2.1**. In addition, pHAECs were obtained by bronchial brushing of non-asthmatic and asthmatic subjects (Hallstrand, Wurfel et al. 2010) or from commercial sources (Normal Human Bronchial Epithelial cells (NHBE; Lonza, Basel, Switzerland)). PHAECs were maintained in bronchial epithelial growth media (BEGM; Lonza) and supplemented with antibiotic/antimycotic (Gibco, Burlington, ON, Canada). Experiments were conducted on pHAECs at passage 2 or 3. Patient demographics of pHAECs used for immunoblot of total p63 in monolayer culture are found in **Table 4.1**, while those used for the epithelial gene expression array are shown in **Table 4.2**.

A549 human alveolar adenocarcinoma cells were cultured in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and 1x penicillin/streptomycin (50 U and 50 µg per mL, respectively; Gibco).

Minimally-immortalized HBEC6-KT bronchial epithelial cells were generously provided by Dr. John Minna (Ramirez, Sheridan et al. 2004). HBEC6-KT cells were maintained in keratinocyte serum-free media (KSFM; Invitrogen, Burlington, ON, Canada) supplemented with EGF (0.4 ng/mL), bovine pituitary extract (BPE; 50 µg/mL) and 1x penicillin/streptomycin (50U/50µg per mL, respectively; Gibco). Experiments were conducted on cells at passages 12 and 13.

Donor†	Sex	Age°	Asthma Status*	Sample Type	Cause of Death
N1	F	26	None; PC_{20} > 8.0	Bronchial brushing	
N2	M	20	Absent	Donor Lung	Head trauma
N3	F	23	None; PC_{20} > 8.0	Bronchial brushing	
N4	F	47	Absent	Donor Lung	Head trauma
N5	M	29	None; PC_{20} > 8.0	Bronchial brushing	
A1	F	23	PC_{20} = 0.757	Bronchial brushing	
A2	F	23	PC_{20} = 0.25	Bronchial brushing	
A3	M	11	Doctor-diagnosed; age 2	Donor Lung	Anoxia; asthma
A4	F	29	PC_{20} = 0.42	Bronchial brushing	
A5	M	25	Doctor-diagnosed; age 11	Donor Lung	Anoxia; asthma

Table 4.1 Donor demographics of pHAEC cultures used for immunoblot of p63 isoforms.
† N = Non-asthmatic; A = Asthmatic.
° Unpaired, two-tailed t-test confirmed no significant difference in mean age between disease groups (p = 0.267).
* For cells obtained by bronchial brushing, methacholine challenge data either confirmed (provocative concentration $(PC)_{20}$ < 1.0 mg/mL) or excluded (PC_{20} > 8.0 mg/mL) asthma diagnosis.

Donor	Sex	Age	Sample Type	Other info
1	M	20	Donor Lung	Head trauma
2	F	20	Donor Lung	Head trauma
3	M	21	NHBE (Lonza)	
4	F	23	Bronchial Brushing	
5	M	47	Donor Lung	Head trauma

Table 4.2 Donor demographics of non-asthmatic pHAEC cultures used for epithelial gene expression array.
†Airway epithelial cells were obtained from upper airways of human lungs deemed unsuitable for transplant, from bronchial brushing or commercially available NHBEs. All donors lacked doctor-diagnosed asthma.

4.2.2 Modulation of p63 expression

Small interfering RNA (siRNA) constructs with a stability-enhancing modification (Stealth RNAi, Invitrogen) were designed using Primer 3 (http://frodo.wi.mit.edu/) to target specific domains of p63, as shown in **Figure 4.1.** Constructs with the forward sequences shown in **Table 4.3** were obtained. For experiments, pHAECs at approximately 60% confluence were transfected with 50nM siRNA using 0.5% (v/v) HiPerFect transfection reagent as per manufacturer's instructions (Qiagen).

Adenoviruses encoding ΔNp63α (King, Ponnamperuma et al. 2003), TAp63α, TAp63γ and LacZ (Sasaki, Morimoto et al. 2001) were kindly provided by Dr. Wendy Weinberg. pHAECs were infected at 70% confluency at a multiplicity of infection (MOI) of 0.15 plaque-forming units (pfu) per cell. A549 cells were infected at 30% confluence with an MOI of 1.0 or 3.0 pfu/cell. Samples were collected after 48 hours (RNA) or 72 hours (protein).

siRNA	Forward Sequence	Targets
ΔN	UGCCCAGACUCAAUUUAGUGAGCCA	ΔNp63α, ΔNp63β and ΔNp63γ
DBD	CAGCCAUGCCCAGUAUGUAGAAGAU	All p63 isoforms
α/β	GGGUGAGCGUGUUAUUGAUGCUGUG	ΔNp63α, ΔNp63β, TAp63α, TAp63β
Scrambled	CUACAGACCAGCAGUACAUGGUGAU	No specific target

Table 4.3 Sequences of siRNA constructs for targeting of p63 isoforms.

4.2.3 Gene expression analysis

RNA was extracted according to manufacturer's instructions using the RNeasy Mini Kit (Qiagen, Valencia, CA). 1 μg total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Ontario, CA) using the random primer method. Gene expression was

determined by real-time polymerase chain reaction (PCR) using the Taqman Universal PCR Master Mix (Applied Biosystems) and pre-developed Taqman gene expression assays (shown in **Table 4.4**) as per manufacturer's instructions (Applied Biosystems). Gene expression levels were calculated as normalized to GAPDH using the equation: expression = 10000 x $2^{CtGAPDH - CtGene}$.

For semi-quantitative PCR of the p63 isoforms, primers were designed to specific domains of the gene to enable identification of the full-length transcripts. Primer sequences can be found in **Table 4.5**. PCR was carried out for 40 cycles, with an extension time of 90 seconds per cycle. Resulting amplicons were separated by electrophoresis on 1% agarose gel and visualized using GelRed Nucleic Acid Gel Stain (Biotium, Hayward, USA).

Target	Taqman Assay ID
TAp63	Hs00978349_m1
ΔNp63	Hs00978339_m1
β-Catenin	Hs0355049_m1
EGFR	Hs00914223_m1
Jagged1	Hs1070032_m1
GAPDH	Hs9999905_m1

Table 4.4 Taqman assays used in Chapter 4.

Primer name	Symbol	Primer Sequence
TA forward	TA-f	GAAGATGGTGCGACAAACAA
ΔN-forward	ΔN-f	CTGGAAAACAATGCCCAGAC
α/β reverse	α/β-r	GTCTCACTGGAGCCCACACT
γ reverse	γ-r	TTTTGGAGTTTCTCTCCGGG

Table 4.5 Sequences of primers used for semi-quantitative PCR of p63 isoforms.

4.2.4 Focused epithelial gene expression array

Reverse transcription (RT^2) kits (SA Biosciences, a QIAGEN company) were utilized to synthesize cDNA from 500 ng total RNA. Pre-loaded RT^2 Profiler Arrays (SA Biosciences) containing primers for 21 different epithelial genes were custom designed to contain putative p63-regulated genes as well as the reference gene GAPDH. Gene expression was normalized to GAPDH as described above and values expressed as fold change vs. control conditions. Data are expressed as mean fold change ± SEM between all experiments (n=5 donors, **Table 4.2**).

4.2.5 Immunoblot

Please refer to **Chapter 2.1** for detailed description of immunoblot protocol. Primary antibodies used in **Chapter 4** can be found in **Table 4.6**.

Epitope	Host	Catalog #	Company	Uses
ΔNp63	Rabbit	619002	BioLegend, San Diego, USA	IB
p63(4A4)	Mouse	sc-8341	Santa Cruz Biotechnology, Santa Cruz, USA	IB
β-tubulin	Mouse	05-661	EMD Millipore Corporation, MA, USA	IB
E-cadherin	Mouse	sc-8426	Santa Cruz Biotechnology, Santa Cruz, USA	IB
EDA-Fibronectin	Mouse	MAB1940	Chemicon International, CA, USA	IB

Table 4.6 List of all primary antibodies used in Chapter 4.

4.2.6 Wound repair kinetics

HBEC6-KT cells were seeded in 4-well chamber slides and grown to 70% confluence and then transfected with 25nM siRNA and 0.3% (v/v) hiperfect reagent. 24 hours later, cells were scratch-wounded in a cross pattern, rinsed once with basal media and then re-transfected. Wounds were photographed using a digital camera mounted to a phase-contrast microscope in

the centre of the cross area immediately after wounding (time 0) and after 4, 8 and 24 hours. ImagePro Plus software (Media Cybernetics, Rockville, MD, USA) was used to quantify the percentage area of each image that was denuded. Wound repair is reported as a percentage of the denuded area compared to that observed at time 0, where 100% indicates a fully reconstituted epithelium.

4.2.7 Ki-67 immunocytochemistry

Monolayer cultures of HBEC6-KT were fixed for 20 minutes in 4% paraformaldehyde in PBS and then stored in PBS at 4°C. Slides were blocked with normal goat serum. Primary antibody was applied in PBS containing 0.1% saponin overnight at 4°C (rabbit anti-ki67, Abcam ab15580). Goat anti-rabbit IgG-biotin (1/100; Vector Labs) was applied for 2 hours at room temperature in PBS/0.1% saponin, followed by streptavidin/HRP (Dako Cytomation) and DAB chromogen (Dako Cytomation). All washes were carried out in PBS with 0.1% saponin. Nuclei were counterstained using Harris-modified hematoxylin (Sigma-Aldrich, St. Louis, USA). Slides were dehydrated through graded ethanol and allowed to dry before applying coverslips with non-aqueous mounting media (Cytoseal 60, Thermo Fisher Scientific, Waltham, USA). Slides were photographed on a Nikon Eclipse microscope using the SPOT Advanced software. Five images for each treatment condition, in three replicate experiments, were manually tagged and quantified for immunoreactivity using ImagePro Plus software.

4.2.8 Statistics

One-way ANOVA with Dunnett's post-test was used to assess statistical significance of treatment conditions vs. control (GraphPad Prism 5.0) when multiple conditions were present.

When only two categories were present, a two-tailed, unpaired t-test was used. In all cases, probability values of less than 0.05 ($p<0.05$) were considered statistically significant.

4.3 Results

4.3.1 ΔNp63α is the most abundant p63 isoform in human airway epithelial basal cells

When submerged in culture, airway epithelial cells form a relatively homogenous cuboidal monolayer that is representative of basal cells within the airway epithelium. We have shown that p63 is only expressed in basal cells identified by positive staining for cytokeratin-5, CD151, and tissue factor (Hackett, Shaheen et al. 2008). Given the multiple possible isoforms of p63 (**Figure 4.1**), we first set out to determine the relative contribution of the ΔN and TA isoforms in pHAECs. Using quantitative PCR, we observed abundant expression of ΔNp63 in pHAECs whereas TAp63 expression was virtually undetectable (**Figure 4.2A**).

We then used semi-quantitative PCR to detect the presence of full-length mRNA isoforms of p63 in pHAECs (**Figure 4.2C**). As shown in **Figure 4.1**, it is not possible to design primers that are specific for the p63β isoform, so we used primers that were specific for both the p63α/β isoforms and γ isoforms. Using this strategy, we determined that the most predominant isoforms expressed were ΔNp63α/β, followed by ΔNp63γ. Although TAp63α/β was detected, it was at a very low level even after 40 cycles of amplification, which is in agreement with our quantitative PCR data for the TA isoform. However, endogenous TAp63γ mRNA was not detected. As TAp63 isoforms were expressed at such low levels, we used adenovirus-mediated overexpression of TAp63α or TAp63γ in pHAECs as positive controls and detected robust expression, indicating that endogenous levels of TAp63 isoforms are indeed extremely low in pHAECs.

We used immunoblot to confirm our mRNA findings and demonstrate that the ΔNp63α/β isoform is the most abundantly expressed protein isoform in basal pHAECs (**Figure 4.2C**, left panel). Adenovirus-mediated overexpression of ΔNp63α, TAp63α and TAp63γ in HEK cells are included as positive controls and for molecular weight comparison (**Figure 4.2C**, right panel), indicating that although we detected exogenous TAp63α and TAp63γ in HEK cells, the endogenous levels were too low to detect in pHAECs.

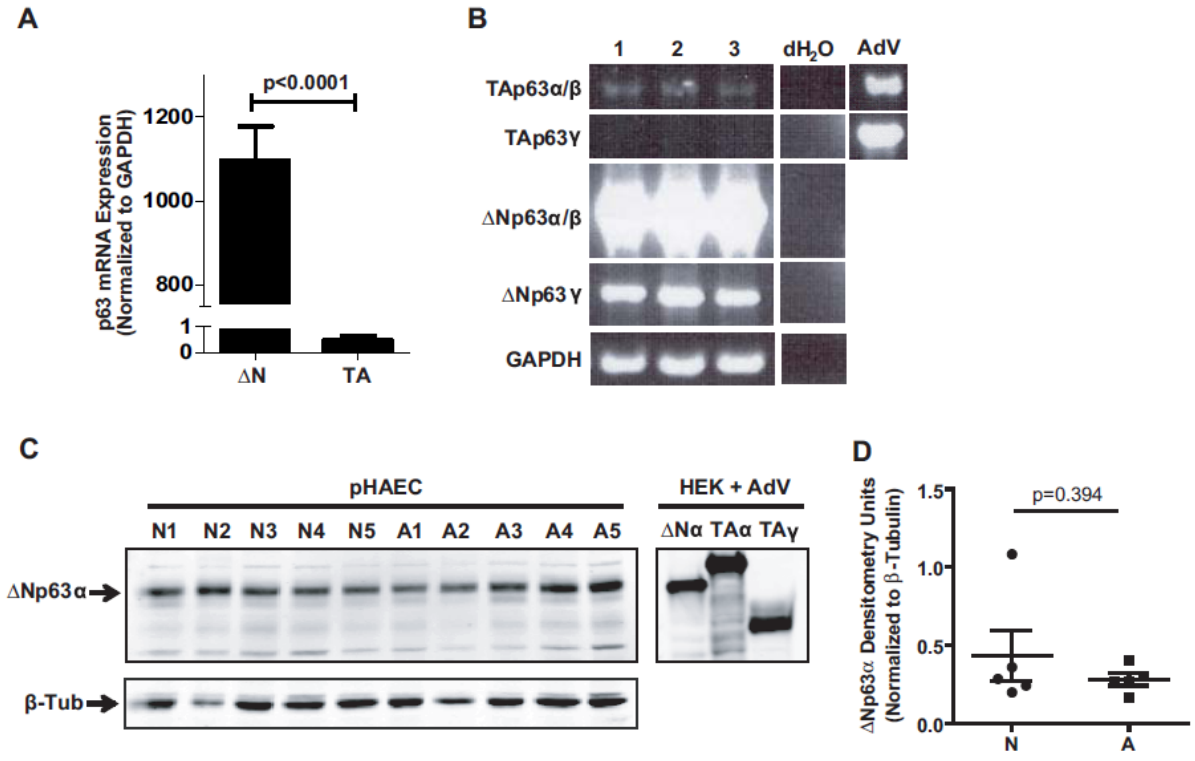


Figure 4.2 ΔNp63α is the most abundant isoform in human airway epithelial cells *in vitro*.
(A) RT-qPCR for ΔN vs. TAp63 mRNA in cultured pHAECs indicates robust expression of the ΔN but not the TA isoforms (n=7). (B) Semi-quantitative PCR for full-length p63 isoforms in cultured pHAECs from n=3 donors indicates that ΔNp63α/β are the predominant mRNA isoforms. Adenoviral (AdV) overexpression provided a positive control for TAp63α and TAp63γ, which were not endogenously detected. (C) Immunoblot for total p63 in monolayer pHAEC cultures from non-asthmatic (N) and asthmatic (A) donors (left panel; n=5 each). Adenoviral overexpression of ΔNp63α, TAp63α and TAp63γ in HEK cells (right panel) included as positive controls. β-Tubulin is included as a loading control. (D) Densitometry analysis of ΔNp63α protein followed by two-tailed unpaired t-test indicates that ΔNp63α expression is not significantly different between disease groups (p=0.394).

The number of epithelial cells expressing p63 is increased in asthmatic compared to non-asthmatic donors in the airway and in air-liquid interface cultures (Hackett, Warner et al. 2009). To determine whether the expression of p63 was altered in a homogenous basal culture system, we compared the expression of ΔNp63α protein in monolayer cultures of pHAECs derived from non-asthmatic (N) and asthmatic (A) donors (**Figure 4.3D**; see **Table 4.1** for patient data). In contrast to our findings in differentiated airway epithelium, the expression of ΔNp63α protein did not differ between basal pHAECs derived from asthmatic and non-asthmatic donors as measured by densitometry (**Figure 4.2E**). These data suggest an expansion of the p63-expressing basal cell population in asthma rather than increased p63 expression in individual cells.

4.3.2 Inhibition of ΔNp63α decreases expression of genes involved in epithelial repair and differentiation

To understand the effects of loss of function of ΔNp63α in pHAECs, we designed small interfering RNA (siRNA) constructs to target different regions of this mRNA, including the 5' (ΔN), DNA-binding (DBD) and 3' α/β domains of this isoform. Knockdown using ΔN, DBD or α/β siRNA each significantly decreased total ΔNp63 mRNA in pHAECs ($p<0.001$, **Figure 4.3A**) but did not significantly affect TAp63 mRNA (**Figure 4.3B**). We next determined the impact of ΔNp63 mRNA knockdown on ΔNp63α protein expression by immunoblot (**Figure 4.3C**). Densitometry analysis of three independent experiments showed a significant reduction in ΔNp63α protein expression following transfection with each of the siRNAs ($p<0.01$, **Figure 4.3D**). Given that ΔN and α/β are most specific for the ΔNp63α isoform, we used these siRNA constructs for our analysis of candidate target genes regulated by ΔNp63α.

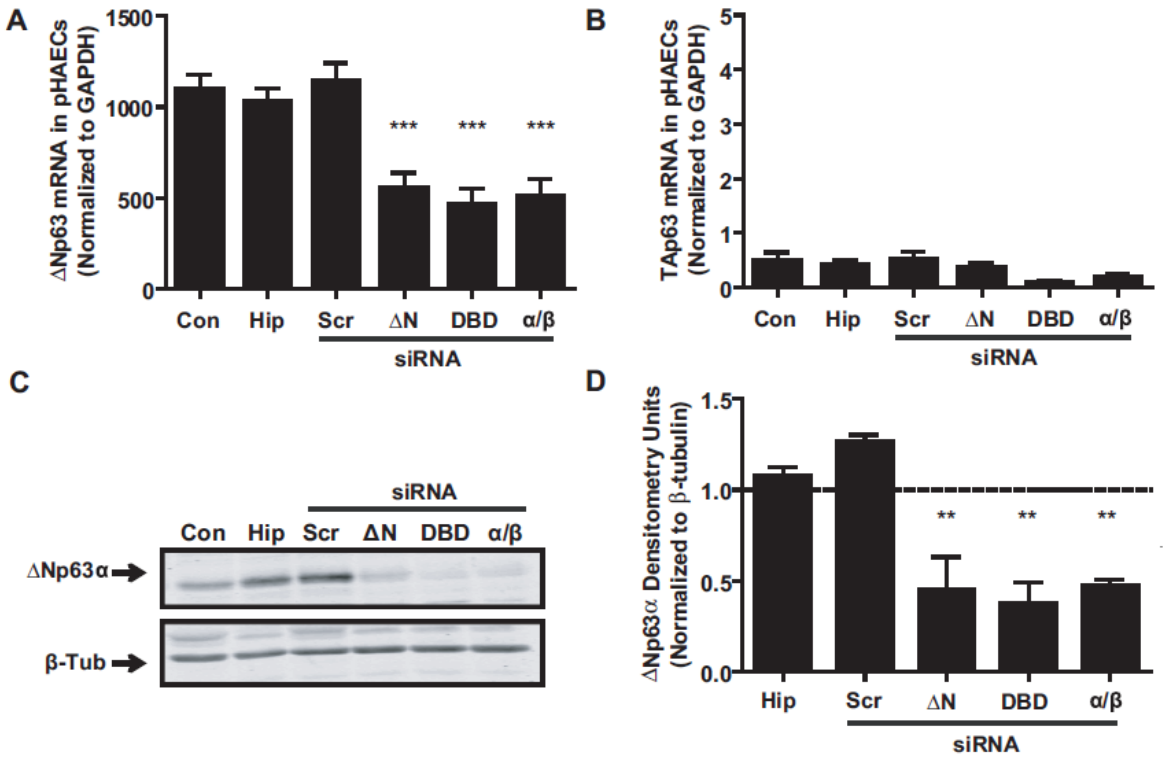


Figure 4.3 p63 siRNA significantly downregulates ΔNp63α mRNA and protein.
RT-qPCR analysis of pHAECs 48 hours after transfection with siRNA to ΔN, DNA Binding (DBD) or α/β domains of p63 indicated **(A)** significantly decreased expression of ΔNp63 with each of the p63-specific siRNAs but no change with HiPerFect transfection reagent (Hip) or scrambled siRNA construct (Scr) and **(B)** no change in TAp63 mRNA with any siRNA construct. **(C)** Representative immunoblot of ΔNp63α protein 72 hours after transfection with 50nM siRNA to ΔN, DBD or α/β domains of p63. **(D)** Densitometry analysis of ΔNp63α protein expression in pHAECs (n=3) showed decreased ΔNp63α protein expression after treatment with all three p63-specific siRNAs. Values were normalized to β-tubulin as a loading control and are shown as fold change over untreated control (denoted with dashed line).

To determine the impact of ΔNp63 knockdown on genes involved in key epithelial differentiation and repair functions, we designed an array of PCR primers for 21 genes (**Table 4.7**) that are regulated by p63 in other epithelial tissues and/or that contain a p63 response element in their promoter region (according to the p53 FamTaG database (Sbisa, Catalano et al.

2007)). We found that 11 of 21 candidate target genes were significantly inhibited by siRNAs to ΔNp63α with either the ΔN or α/β siRNA construct as compared to the scrambled control. We found no RNAs that were increased following inhibition of ΔNp63α.

Gene	ΔN siRNA		α/β siRNA	
	Fold Change ± SEM†	**p-value°**	**Fold Change ± SEM**	**p-value**
CTNNB1	0.71 ± 0.04	p<0.001	0.64 ± 0.06	p<0.001
LAMC2	0.46 ± 0.07	p<0.001	0.68 ± 0.05	p<0.01
TJP1	0.62 ± 0.02	p<0.001	0.62 ± 0.07	p<0.001
CD44	0.68 ± 0.05	p<0.01	0.64 ± 0.08	p<0.001
EGFR	0.40 ± 0.15	p<0.01	0.50 ± 0.06	p<0.05
EP300	0.66 ± 0.06	p<0.01	0.54 ± 0.07	p<0.001
F11R/JAM1	0.57 ± 0.07	p<0.01	0.56 ± 0.07	p<0.001
GSK3B	0.74 ± 0.05	p<0.01	0.81 ± 0.06	p<0.01
PLEC1	0.50 ± 0.08	p<0.05	0.50 ± 0.10	p<0.05
CCND1	0.84 ± 0.06	p<0.05	0.77 ± 0.11	p<0.01
JAG1	0.48 ± 0.10	ns	0.39 ± 0.09	p<0.05
CDKN1A	0.86 ± 0.07	ns	0.91 ± 0.08	ns
CEBP	1.14 ± 0.18	ns	0.92 ± 0.09	ns
KRT18	0.68 ± 0.07	ns	0.69 ± 0.10	ns
KRT5	0.57 ± 0.07	ns	0.52 ± 0.13	ns
CLDN1*	0.95 ± 0.05	p<0.001	0.66 ± 0.08	p<0.001
NOTCH2*	0.83 ± 0.04	p<0.001	0.75 ± 0.06	p<0.001
BMP7	low expression		low expression	
EGF	low expression		low expression	
FGFR2	low expression		low expression	
WNT1	low expression		low expression	

Table 4.7 Epithelial gene expression array overview.
† Values are expressed as fold change ± SEM of gene expression in siRNA-treated cultures relative to untreated control (n=5 donors).
° One-way ANOVA followed by Dunnett's post-test was used to assess statistical significance vs. scrambled siRNA construct.
* Indicates genes which were significantly altered by scrambled siRNA treatment vs. untreated control.

As demonstrated by three of the p63-regulated genes, β-catenin (CTNNB1; **Figure 4.4A**), epidermal growth factor receptor (EGFR; **Figure 4.4B**), and jagged1 (JAG1; **Figure 4.4C**), both the ΔN and α/β siRNA constructs resulted in decreased target gene expression, indicating that ΔNp63α/β is the predominant isoform of p63 regulating these genes. These three genes were selected for further study based on their potential contribution to epithelial differentiation and repair in asthma. β-Catenin is a subunit of the adherens junction that anchors the actin cytoskeleton to regulate cell growth and adhesion between cells, and can also function as a transcriptional co-activator (Drewelus, Gopfert et al. 2010, Talos, Schulz et al. 2010). EGFR regulates responses to the epithelial mitogen epidermal growth factor (EGF) and regulates the process of repair in the epithelium (Puddicombe, Polosa et al. 2000, Xu, Ding et al. 2004). Jagged1 is a ligand for the Notch family of proteins that is involved in cell fate determination in tissue-resident progenitor cells (Wu, Rollin et al. 2012).

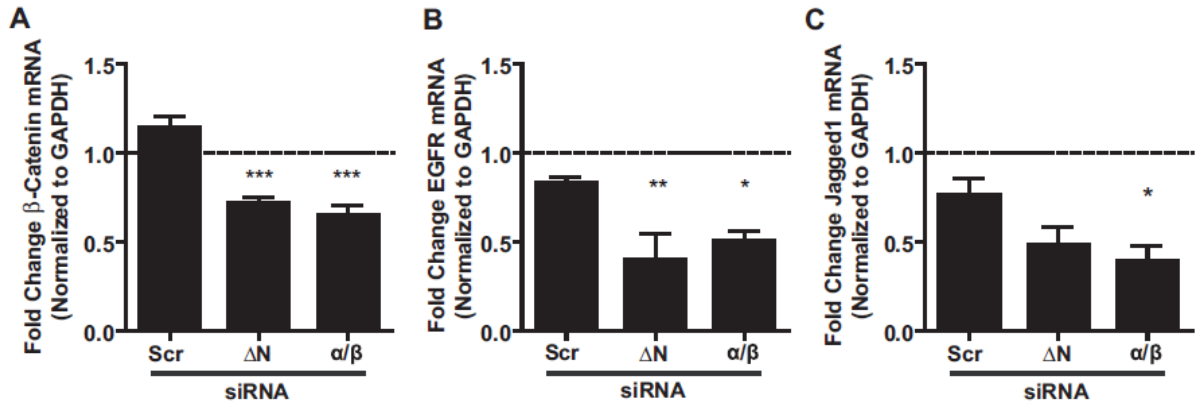


Figure 4.4 Loss of p63 inhibits genes involved in epithelial function.
(A)-(C): RT-qPCR of candidate p63 target genes by epithelial gene expression array 48 hours following knockdown of ΔN or α/β isoforms of p63 in n=5 pHAECs; **(A)** β-Catenin, **(B)** Epidermal Growth Factor Receptor and **(C)** Jagged1. Gene expression (relative to GAPDH) is shown as fold change compared to controls (dashed line). Statistical significance was assessed by one-way ANOVA with Dunnett's post-test, with *p<0.05, **p<0.01, ***p<0.001 compared to scrambled siRNA.

4.3.3 ΔNp63α alters expression of genes involved in epithelial function

Having shown that inhibition of ΔNp63α/β reduced mRNA expression of β-catenin, EGFR and jagged1, we next investigated the effects of expressing ΔNp63α using adenoviral vectors (King, Ponnamperuma et al. 2003). Infection with adenovirus encoding ΔNp63α but not LacZ (MOI = 0.15 pfu/cell) increased ΔNp63 mRNA after 48 hours (**Figure 4.5A**, $p<0.01$). Increased ΔNp63α protein was also observed 72 hours after infection with ΔNp63α-encoding adenovirus (**Figure 4.5B** and **C**). Messenger RNA for any of the three candidate genes, β-catenin, EGFR or jagged1, was not significantly changed by ΔNp63α overexpression (**Figure 4.5D-F**). As endogenous ΔNp63α is highly expressed within basal pHAECs, we hypothesized that the lack of effect on target gene expression was due to saturation of ΔNp63α signaling despite further exogenous ΔNp63α expression. To test this hypothesis, we infected an alveolar type II cell line (A549) that lacks endogenous ΔNp63α with ΔNp63α-encoding adenovirus. ΔNp63α protein (**Figure 4.6B**) was induced by ΔNp63α adenovirus in A549s. Adenovirus-mediated expression of ΔNp63α (MOI 3.0 pfu/cell) in A549s significantly increased expression of β-catenin (**Figure 4.6C**, $p<0.01$), EGFR (**Figure 4.5D**, $p<0.001$) and jagged1 (**Figure 4.5E**, $p<0.001$) mRNA, supporting our hypothesis that ΔNp63α induces these three target genes in human pulmonary epithelial cells.

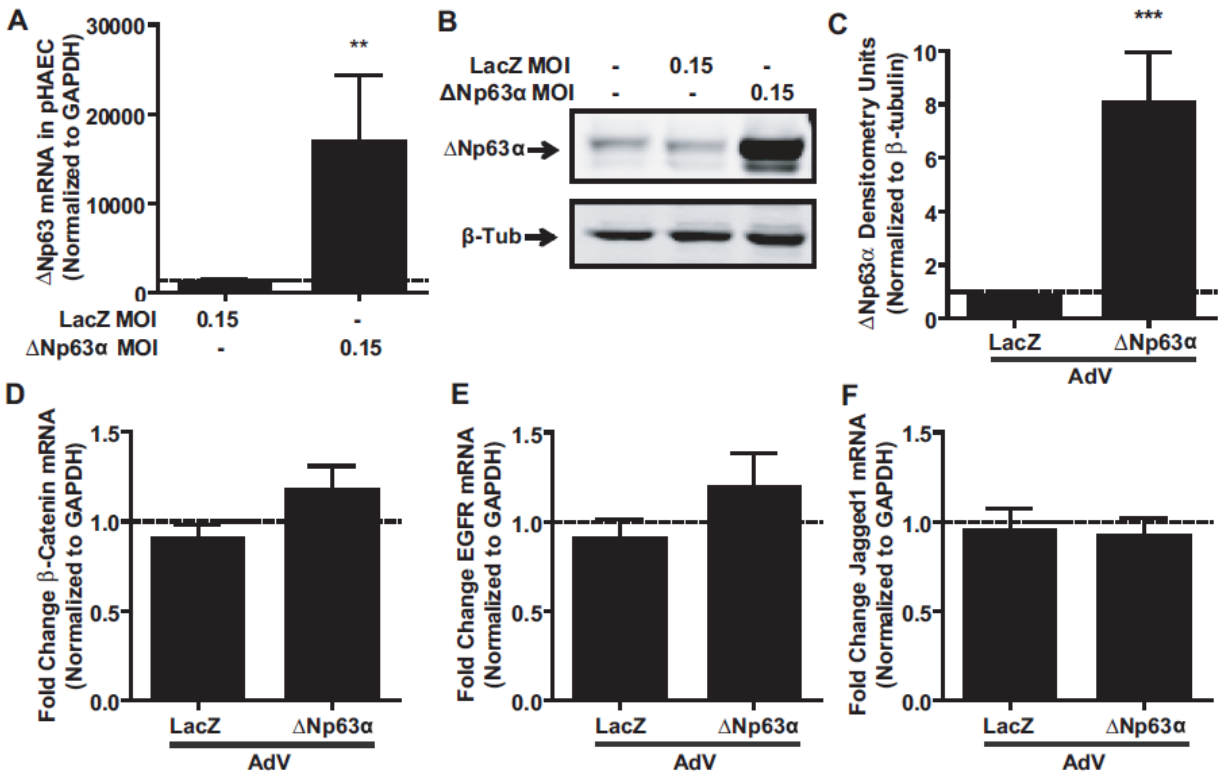


Figure 4.5 Exogenous ΔNp63α does not induce target gene expression in pHAECs.
(A) RT-qPCR analysis indicated robust induction of ΔNp63 mRNA expression 48 hours following transduction with adenovirus expressing ΔNp63α but not LacZ (MOI = 0.15 pfu/cell). **(B)** Representative immunoblot of ΔNp63α protein 72 hours following transduction with adenovirus and **(C)** densitometry analysis of ΔNp63α protein expression indicated significant upregulation of ΔNp63α protein (n=3). Values were normalized to β-tubulin as a loading control and are shown as fold change over untreated control (dashed line). **(D)-(F):** RT-qPCR of candidate downstream target genes 48 hours following adenoviral transduction in pHAECs (n = 4) showed no significant change in expression of **(D)** β-Catenin, **(E)** EGFR or **(F)** jagged1. Dashed line represents values in untreated control samples. Statistical significance was assessed using unpaired two-tailed t-test between LacZ and ΔNp63α adenovirus treatment and ** indicates $p<0.01$, ***$p<0.001$.

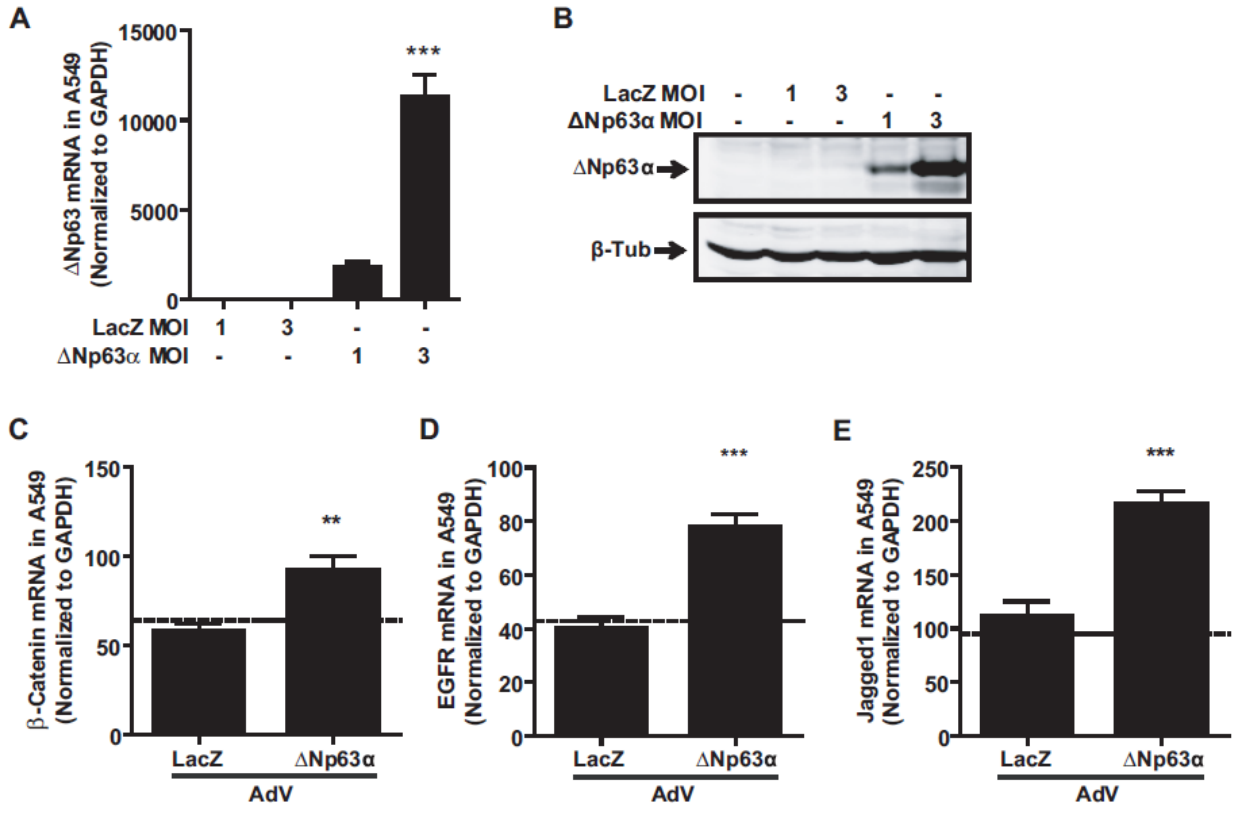


Figure 4.6 Exogenous ΔNp63α increases candidate gene expression in p63-null A549 cells.
(A) RT-qPCR analysis demonstrated negligible endogenous ΔNp63 mRNA in A549 cells but significant upregulation following treatment with ΔNp63α adenovirus for 48 hours at MOI of 1.0 or 3.0 pfu/cell, as assessed by one-way ANOVA with Dunnett's post-test (n=6). **(B)** Representative immunoblot of total p63 protein demonstrated expression of ΔNp63α protein only in the presence of ΔNp63α adenovirus. β-Tubulin is included as loading control. RT-qPCR analysis of **(C)** β-catenin, **(D)** EGFR and **(E)** jagged1 demonstrated significant upregulation of target gene mRNA 48 hours following treatment of A549s with ΔNp63α but not LacZ adenovirus (AdV; MOI = 3.0 pfu/cell; n = 6). Expression data were normalized to GAPDH. Dashed line represents values in untreated control samples. Statistical significance was assessed using unpaired two-tailed t-test between LacZ and ΔNp63α adenovirus treatment and ** indicates $p<0.01$, ***$p<0.001$.

4.3.4 Loss of ΔNp63 impairs epithelial cell wound repair

Having shown that ΔNp63α regulates several genes known to affect epithelial function, we next wanted to determine if knockdown of ΔNp63α/β isoforms impacts epithelial wound repair. For these studies we used minimally-immortalized HBEC6-KT cells that, like pHAECs, express predominantly ΔNp63α that is responsive to knockdown of protein expression using either the ΔN or α/β siRNA constructs (**Figure 4.7A**). We used a scratch wound repair model and as shown by the representative light microscopy images of HBEC6-KT cultures at time 0 and 24 hours following wounding, ΔNp63 siRNA impaired wound repair (**Figure 4.7B**). Further, by quantifying repair over time, we demonstrate that while control or scrambled siRNA-treated cultures were equally able to reconstitute over 90% of the wound area within 24 hours, knockdown of total ΔNp63 inhibited wound closure to only 71±5% (**Figure 4.7C**, $p<0.01$). In contrast, specific knockdown with α/β siRNA construct did not alter wound repair. We demonstrate that inhibition of ΔNp63α using either the ΔNp63 or α/β siRNA construct maintained downregulation of ΔNp63 (**Figure 4.8A**), β-catenin (**Figure 4.8B**), and jagged1 (**Figure 4.8D**) mRNA in HBEC6-KT cells 24 hours after wounding, but EGFR mRNA was not significantly decreased (**Figure 4.8C**).

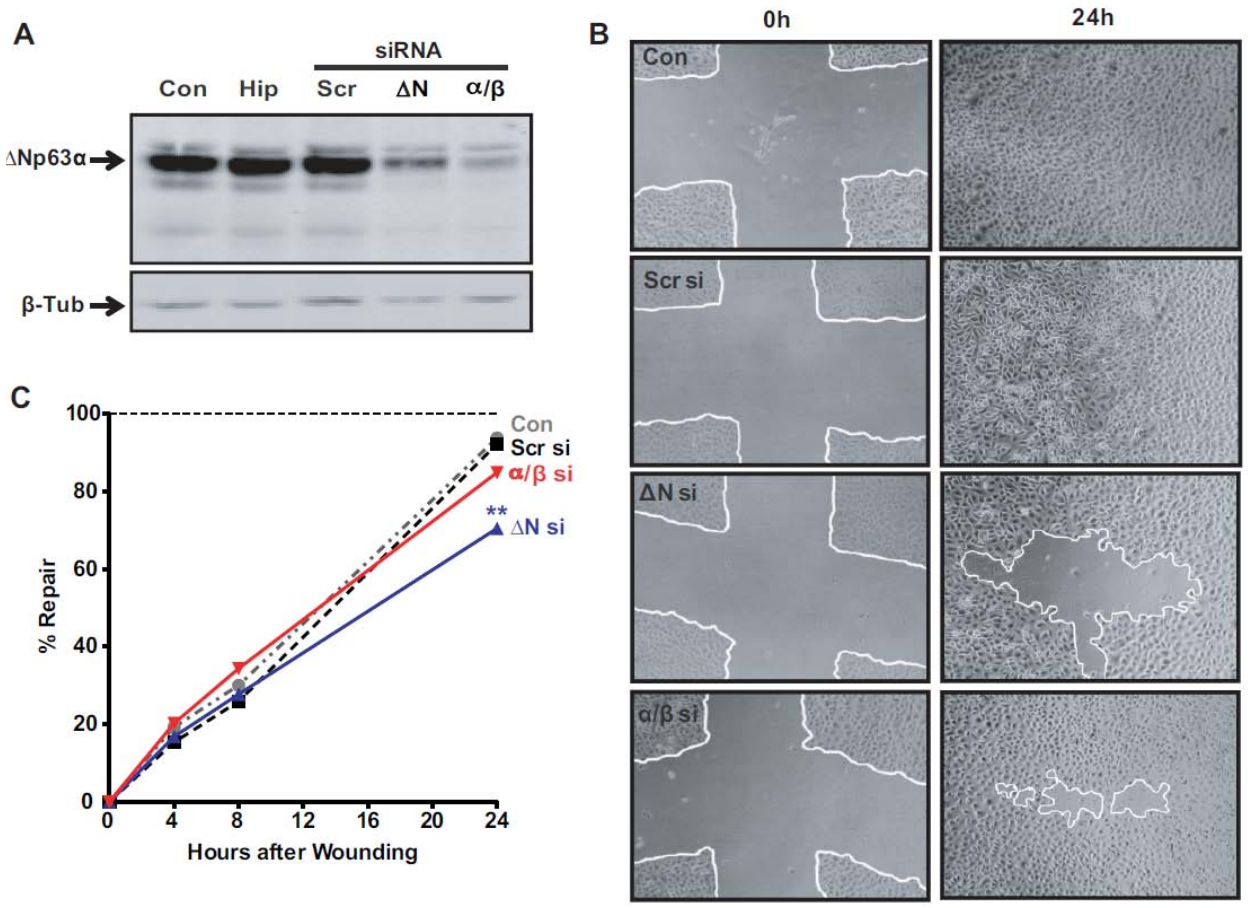


Figure 4.7 Loss of ΔNp63 significantly impairs repair in scratch-wounded monolayer cultures of human bronchial epithelial cells.

(A) Representative immunoblot of monolayer cultures of HBEC6-KT cells 72 hours after transfection with 25nM p63-specific siRNA, indicating decreased ΔNp63α protein expression. **(B)** Representative phase-contrast images of confluent monolayer cultures of HBEC6-KTs at time of scratch wounding (0h) and after 24 hours (24h) indicate repair progress in the presence of scrambled, ΔN or α/β siRNA constructs. White lines denote the edge of the wound area. **(C)** Analysis of wound repair at 0, 4, 8 and 24 hours following scratch wounding showed significantly impaired repair with ΔN siRNA only (n = 6). Wound repair was calculated as percentage area reconstituted compared to the initial wound area (time 0), where horizontal dashed line indicates 100% closure. Statistical significance was assessed at each time point using one-way ANOVA with Dunnett's post-test, and ** indicates $p<0.01$ compared to scrambled siRNA conditions.

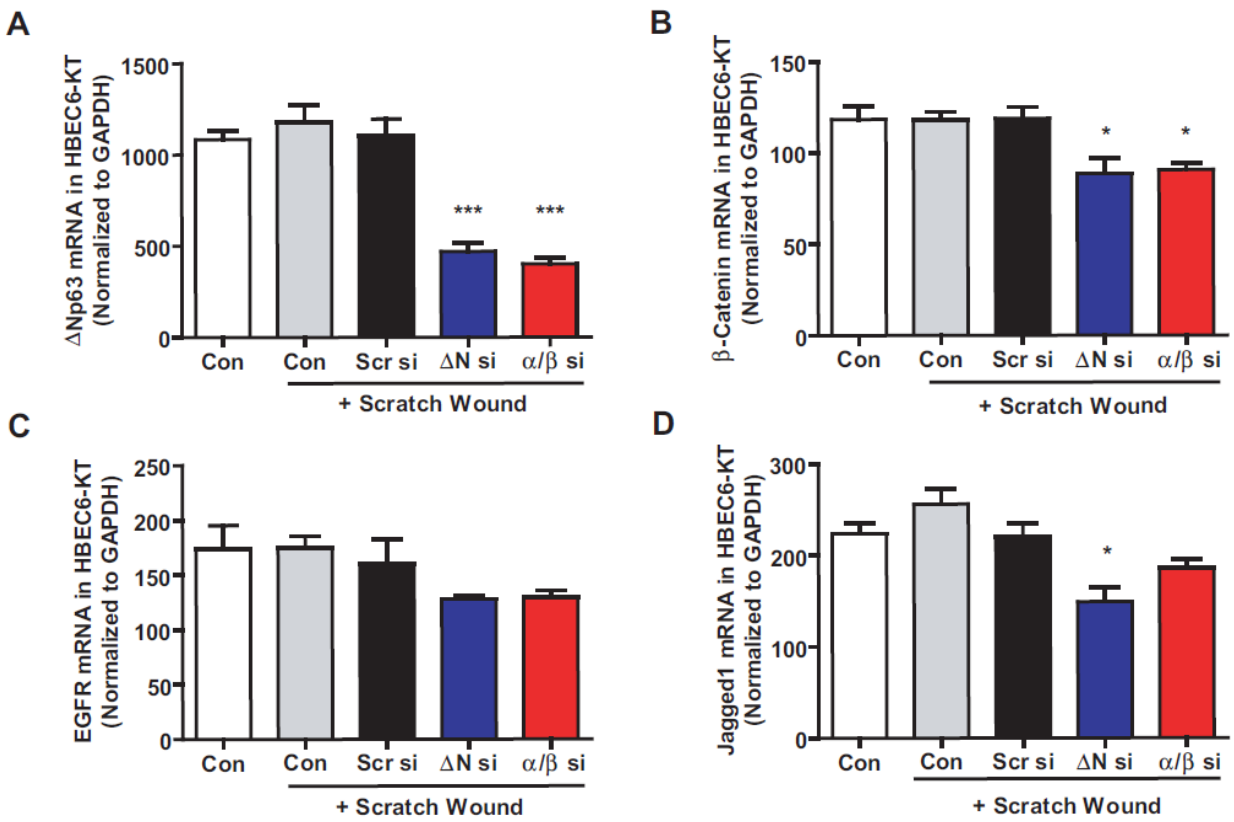


Figure 4.8 Suppression of ΔNp63, β-catenin and jagged1 was maintained during wound repair. **(D-G)** RT-qPCR of target gene mRNA 24 hours after media change or scratch wounding in the presence of Scrambled, ΔN or α/β siRNA indicates sustained downregulation of **(A)** ΔNp63, **(B)** β-catenin, **(C)** EGFR and **(D)** jagged1 mRNA expression. Statistical significance was assessed using one-way ANOVA with Dunnett's post-test with * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to scrambled siRNA treatment.

To determine whether the impaired repair was due to effects on proliferation, we stained cultures for the proliferation marker Ki-67 (**Figure 4.9A**) 24 hours after wounding. ΔNp63 siRNA reduced the number of Ki-67 positive cells (**Figure 4.9B**, $P<0.05$), but use of the α/β siRNA construct did not, suggesting that p63 may exert isoform-specific effects on epithelial proliferation. Although Ki-67 expression is generally thought to be restricted to the nucleus (grey arrow), we observed abundant cytoplasmic staining (red arrow). We separately quantified expression of nuclear (**Figure 4.9B**, grey bars) and cytoplasmic (red bars) Ki-67 expression and

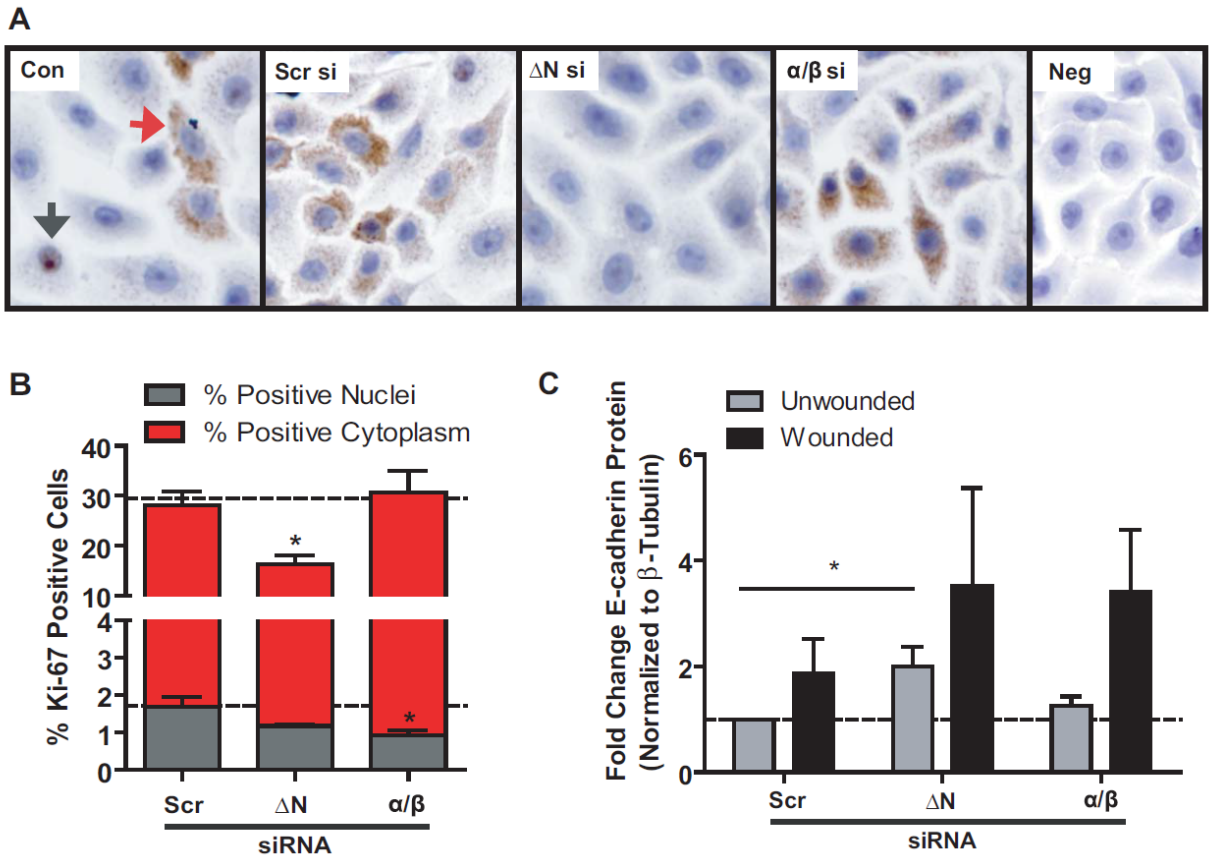


Figure 4.9 Loss of ΔNp63 significantly impairs epithelial proliferation in scratch wounded monolayers of human bronchial epithelial cells.
(A) Immunocytochemistry for proliferation marker Ki-67 (brown) indicated both nuclear (grey arrow) and cytoplasmic (red arrow) staining. Nuclei were counterstained with hematoxylin (blue staining). Negative staining control demonstrated antibody specificity. **(B)** Quantification by manual point-counting indicated significantly decreased Ki-67 cytoplasmic expression with ΔNp63 siRNA treatment (red bars) but decreased nuclear expression (grey bars) following α/β siRNA treatment. Staining was performed on five images from each of N=3 independent experiments 24 hours after wounding. Dashed lines represent untreated control conditions. **(C)** Densitometry quantification of n=3 immunoblots for E-caderin protein 24 hours following media change or scratch wounding of confluent HBEC6-KT monolayers pre-treated with ΔN, DBD or α/β siRNA constructs indicates significant upregulation of E-cadherin protein with the ΔNp63 siRNA construct. Statistical significance was assessed using one-way ANOVA with Dunnett's post-test, where * indicates $p<0.05$, **$p<0.01$ compared to treatment with scrambled siRNA.

demonstrate that knockdown with the ΔNp63 siRNA construct primarily reduced cytoplasmic staining compared to control ($p<0.05$), and this accounted for the significant loss of Ki-67. As both proliferation and migration occur during wound repair, and are linked to cell adhesion, we

quantified expression of the adherens junction protein E-cadherin by immunoblot (**Figure 4.9C**) and found that ΔNp63 siRNA significantly increased E-cadherin content compared to scrambled control ($p<0.05$), suggesting that ΔNp63 is a negative regulator of E-cadherin. We propose that, as E-cadherin loss and redistribution are essential for cytokinesis during epithelial cell proliferation (Guillot and Lecuit 2013) and for dissolution of adherens junctions during migration, loss of ΔNp63 leads to increased expression of E-cadherin, and as a consequence a less migratory and proliferative cell phenotype.

4.4 Discussion

Expression of the transcription factor p63 is confined to the basal cells of airway epithelium (Daniely, Liao et al. 2004, Hackett, Shaheen et al. 2008, Rock, Onaitis et al. 2009). Here we demonstrate that ΔN is the exclusive promoter used and that the α splice variant is the most abundant, leading to expression of the ΔNp63α isoform in human airway epithelial basal cells. We show that loss of function of ΔNp63α using targeted siRNA decreases the expression of a number of genes involved in epithelial differentiation and repair. Further, we demonstrate a role for ΔNp63 in epithelial repair through regulation of epithelial proliferation and E-cadherin expression during wound closure *in vitro*. These data support our hypothesis that ΔNp63α is an important regulatory protein in the human airway epithelial basal cell.

This finding of the predominance of ΔNp63α in airway epithelial basal cells is supported by previous studies demonstrating expression of ΔNp63α in basal cells in non-pulmonary epithelial tissues, including skin, prostate, cornea, and breast (Yang, Schweitzer et al. 1999, Signoretti, Waltregny et al. 2000, Kawasaki, Tanioka et al. 2006, Yalcin-Ozuysal, Fiche et al. 2010). The role of ΔNp63 in maintaining the basal cell population within epithelial tissues is

supported by studies that demonstrated a striking lack of epithelial tissues and in particular the epidermis in p63-null mice (Mills, Zheng et al. 1999, Yang, Schweitzer et al. 1999). Further, Candi et al. found that complementation of the p63 null background with ΔNp63 rescued the basal cell layer of the epidermis (Candi, Rufini et al. 2006). Finally, the recent study from Romano et al. found that $\Delta Np63^{-/-}$ mice phenocopied the epidermal abnormalities of p63-null mice, indicating the crucial role of ΔNp63 in development and maintenance of epithelial tissues (Romano, Smalley et al. 2012). Interestingly, we demonstrated that the number of basal cells expressing ΔNp63 is significantly greater in the epithelium of asthmatics *in vivo* (Hackett, Warner et al. 2009), indicating a defect in the normal mechanism of epithelial restitution and/or differentiation. We therefore wanted to determine the expression levels of each p63 isoform in pHAECs from asthmatic and non-asthmatic donors. When cultured as a submerged monolayer culture, pHAECs form a cuboidal epithelium consisting of relatively undifferentiated basal cells (Hackett, Shaykhiev et al. 2011). When we measured the expression of ΔNp63 isoforms we found that ΔNp63α is the predominant isoform in basal pHAECs and there were no differences in expression between asthmatic and non-asthmatic subjects. These data indicate that although the number of basal cells expressing ΔNp63α is increased in asthma compared to non-asthmatics, the ΔNp63α protein content in individual basal cells does not differ. Our finding that TAp63 mRNA expression is exceptionally low (approximately 2000-fold less than the ΔN promoter variant, while protein expression was not detectable) is consistent with previous studies that demonstrate that TAp63 is not expressed in non-cancerous adult epithelial basal cells (Koster, Kim et al. 2004, Kurita, Cunha et al. 2005). Thus, ΔNp63α is the most abundant isoform expressed in human airway epithelial basal cells.

As we wanted to understand the role of p63 in airway epithelial basal cell function, we investigated the role of ΔNp63α in epithelial repair by modulating its expression in pHAECs from non-asthmatic donors. Epithelial repair is a highly integrated series of processes that includes migration, proliferation, and differentiation. We selected 21 candidate target genes involved in these processes based on the knowledge that they are regulated by p63 in other epithelial tissues (King, Ponnamperuma et al. 2003, Koster, Kim et al. 2004, Carroll, Carroll et al. 2006, King, Ponnamperuma et al. 2006, Birkaya, Ortt et al. 2007, Carroll, Brugge et al. 2007) and/or contain p63-response elements (Sbisa, Catalano et al. 2007). We found that 11 of 21 candidate genes were significantly decreased by modulation of ΔN or α/β p63 isoforms (Table 3; CTNNB1, LAMC2, TJP1, CD44, EGFR, EP300, F11R/JAM1, GSK3B, PLEC1, CCND1 and JAG1), in pHAECs. Four genes were unchanged by p63 modulation (CDKN1A, CEBPA, KRT18 and KRT5). Of these, CDKN1A ($p21^{WAF1}$) is repressed by ΔNp63α in keratinocytes (Westfall, Mays et al. 2003) and thus we would have expected increased expression with loss of ΔNp63α. It is possible that this mechanism of regulation is not predominant in airway epithelial cells, or may be a consequence of the pro-proliferative growth conditions. Our model is also one of undifferentiated cells, which may partially explain why we found that four genes involved in development (BMP7, EGF, FGFR2 and WNT1) were expressed at very low levels.

Since abnormalities in differentiation and repair are associated with asthma, we subsequently focused our efforts on the study of three candidate genes involved in these processes: CTNNB1 (β-catenin), EGFR and JAG1 (jagged1). Although β-catenin is dispensable for repair of mouse airway epithelium following ablation of club cells (Zemke, Teisanu et al. 2009), inhibition of β-catenin in cytokeratin 14-expressing basal cells significantly delayed normal epithelial repair processes following chemical injury, and activation of β-catenin in these

cells increased proliferation (Giangreco, Lu et al. 2012). Expression of EGFR is increased in the airway epithelium of asthmatics (Puddicombe, Polosa et al. 2000), suggesting an important role for this receptor in epithelial repair. ΔNp63α is a direct transcriptional activator of EGFR expression in pancreatic cancer cell lines (Danilov, Neupane et al. 2011) and keratinocytes (Testoni, Borrelli et al. 2006). Reciprocally, p63 may also be regulated by EGFR (Matheny, Barbieri et al. 2003), providing further rationale for its inclusion in this analysis. Jagged ligands are involved in epithelial differentiation and repair through signaling via Notch family receptors. Jagged1 is transcriptionally regulated by p63 in keratinocytes (Testoni, Borrelli et al. 2006).

Adenoviral-mediated expression of ΔNp63α in pHAECs had no effect on target gene expression, despite a significant increase in expression of ΔNp63α itself. This surprising finding led us to postulate that endogenous levels of ΔNp63α were constitutively high, perhaps saturating the response elements of target genes. Infection of the alveolar type II cell line (A549), which lacks p63 expression, induced a significant increase in all target genes, verifying that p63 indeed regulates these targets. In support of this, King et al. demonstrated that ΔNp63α maintained primary murine keratinocytes in a basal phenotype (King, Ponnamperuma et al. 2006), and the ΔN domain was sufficient to maintain cells in a proliferative state. These findings highlight that endogenous levels of ΔNp63α in pHAECs in monolayer culture may in fact be sufficient for target gene induction, but further increases in expression may have no additive effect. Loss of p63 expression in pHAECs did not completely abrogate the expression of β-catenin, EGFR and jagged1, indicating either incomplete knockdown of ΔNp63α or the existence of other transcriptional regulators of these genes. β-catenin is regulated by the Wnt ligands at the protein level and at the transcription level by the TCF4 transcription factor and by β-catenin protein itself, as has been shown during migration of cancer cells (Bandapalli, Dihlmann et al.

2009). EGFR is regulated by the mitogen EGF, as well as multiple other growth factors. Jagged1 expression is regulated by Notch signaling, and interestingly is also significantly regulated by Wnt/β-catenin/TCF signaling (Rodilla, Villanueva et al. 2009). Chromatin immunoprecipitation (ChIP) experiments in human keratinocytes have previously demonstrated that p63 can directly bind to the EGFR and jagged1 promoters (Testoni, Borrelli et al. 2006). In contrast, the mechanism of action of p63 on β-catenin expression is somewhat less clear; p63 may form protein complexes with β-catenin, suggesting that ΔNp63 acts as a regulator of β-catenin signaling through non promoter binding properties (Patturajan, Nomoto et al. 2002). Alternatively, p63 may complex with the TCF/LEF family of Wnt-related transcription factors, to recruit transcriptional modifiers to Wnt-responsive genes, such as β-catenin, as shown in urinary bladder carcinoma cells (Drewelus, Gopfert et al. 2010). Many factors can contribute to the ability of a transcription factor to bind a given promoter, including epigenetic mechanisms such as promoter methylation, histone modifications, or steric hindrance due to the presence of other transcription factors. While the elucidation of the specific regulatory mechanisms by which ΔNp63α functions in primary human airway epithelial cells is of interest, these experiments are beyond the scope of the current study.

We found that inhibition of ΔNp63 significantly compromised the capacity of airway epithelial cells to repair. In contrast, in a scratch wound model of immortalized keratinocytes, inhibition of ΔNp63α by siRNA treatment increased migration, independently of proliferation (Ichikawa, Suenaga et al. 2008), a finding that has also been reported to occur in primary keratinocytes (Barbieri, Tang et al. 2006). However, as p63 has been shown to have distinct cell-type specificity, we believe that ΔNp63α is in fact necessary for repair in airway epithelial basal cells, primarily by regulating cellular proliferation. In contrast to the inhibition with ΔNp63

siRNA, specific targeting of the α/β isoforms did not significantly affect wound repair. There are two potential explanations for this: Firstly, TAp63α may be induced upon wounding, and may have an inhibitory effect on repair; as has been shown in a murine deep skin wound model (Bamberger, Hafner et al. 2005). We did not observe any induction of TAp63 mRNA following wounding (data not shown). The second possibility is that ΔNp63γ may play a role in wound repair. In mammary epithelial cells, loss of ΔNp63α/β allowed ΔNp63γ to confer a more migratory phenotype, leading to epithelial-mesenchymal transition (EMT) (Lindsay, McDade et al. 2011). However, in human epidermal keratinocytes, over-expression of ΔNp63α resulted in enhanced TGFβ_1-induced EMT (Oh, Kim et al. 2011). We have previously shown that airway epithelial basal cells are highly susceptible to TGFβ_1-induced EMT (Hackett, Warner et al. 2009). It is possible that when p63α isoforms are inhibited, expression of ΔNp63γ may confer a degree of cell plasticity, enabling repair via epithelial migration or EMT-like effects. Since we did not observe enhanced repair beyond or even to the same degree as that of the control conditions, it is unlikely that ΔNp63γ alone is able to compensate for the ΔNp63α-dependent loss of proliferation.

We tested the effect of ΔNp63 knockdown on wound repair by examining expression of Ki-67, which has been shown to be a specific marker of proliferation. Interestingly, we saw the vast majority of Ki-67 staining within the cytoplasm, with lesser amounts in the nucleus of cells. Cytoplasmic expression of Ki-67 has been documented previously, although the functional cause or effect of this remains unclear. For example, cytoplasmic Ki-67 in breast cancer biopsies correlated with worse prognosis (Faratian, Munro et al. 2009), but was a disease-independent phenomenon in rat atrial cells undergoing postnatal remodeling (Ciulla, Acquistapace et al. 2009). Whether our finding is physiological or pathological in nature is unclear at this time, but it

is noteworthy that we observed dramatically decreased cytoplasmic Ki-67 expression in response to loss of ΔNp63, indicating that wound closure was inhibited due to decreased proliferation.

The process of wound repair requires epithelial cells to both proliferate and migrate. E-cadherin is known to be involved in the maintenance of adherens junctions that tether the actin cytoskeleton between adjacent cells. Loss of E-cadherin expression is required for cytokinesis during proliferation (Bauer, Motosugi et al. 2008) and to permit cell migration (Masterson, Molloy et al. 2011). We found that ΔNp63 siRNA significantly upregulated E-cadherin, but α/β siRNA did not. The present data support our finding that knockdown of ΔNp63 was able to significantly prevent wound repair in pHAECs, which we propose could be due to enhanced E-cadherin expression. In concordance with this idea, forced expression of E-cadherin blocked cell migration in a lung metastatic cell melanoma cell line (Molina-Ortiz, Bartolome et al. 2009) and inhibited both migration and proliferation in intestinal epithelial cells (Hermiston, Wong et al. 1996). Also in agreement with our present finding, we have previously shown both increased ΔNp63 (Hackett, Warner et al. 2009) and decreased E-cadherin (Hackett, Singhera et al. 2011) in the asthmatic airway epithelium *in vivo* and in ALI culture *in vitro*, indicating the potential for negative regulation of E-cadherin by ΔNp63α.

In the present study, we have attempted to elucidate the roles of ΔNp63α in primary human airway epithelial cells. We have focused here on the use of epithelial cells in monolayer culture, as they represent a homogenous model of basal cells expressing p63 and cytokeratin-5 (Hackett, Shaykhiev et al. 2011). The high degree of shared domains between various p63 isoforms presents a technical challenge; targeting a specific region of the p63 molecule with siRNA will impact a subset of isoforms rather than one specific isoform. Here we targeted either the ΔNp63 domain, targeting all ΔNp63 isoforms, or a region shared by both the α and β

isoforms, which will leave only the γ isoform expressed. We chose this as a two-pronged approach to target ΔNp63α, based on the abundance of this isoform in our study conditions. To minimize ambiguity from this approach, we have described reciprocal regulatory effects using an adenovirus expressing ΔNp63α, further showing that ΔNp63α is the primary isoform responsible for target gene regulation.

In conclusion, we demonstrated that ΔNp63α is the predominant isoform of p63 expressed in pHAECs. This isoform regulates several epithelial genes, and its loss impairs epithelial repair in basal cells in monolayer culture. Further elucidation of the role and upstream regulation of ΔNp63α will have important implications for our understanding of epithelial differentiation and regeneration and how this pertains to the pathophysiology of epithelial injury in asthma.

Chapter 5: Impaired Ciliary Differentiation of Airway Epithelial Cells from Asthmatics With and Without Exercise-Induced Bronchoconstriction

5.1 Introduction

The airway epithelium, both *in vivo* and when cultured at air-liquid interface *in vitro*, is pseudostratified, containing basal cells, columnar ciliated and mucus-producing goblet cells. In submerged monolayer conditions, the epithelial phenotype is reminiscent of basal cells *in vivo*, including expression of basal cell markers CK-5 and the transcription factor p63, and the absence of markers of ciliated and secretory cells (Hackett, Shaykhiev et al. 2011). Basal cells have high proliferative potential, and can act as progenitor cells to repopulate basal, ciliated, and goblet cells through the process of mucociliary differentiation (Hackett, Shaheen et al. 2008, Rock, Onaitis et al. 2009, Dvorak, Tilley et al. 2011).

Several *in vivo* and *in vitro* studies have demonstrated abnormal differentiation in the epithelium of asthmatic subjects. Epithelial damage and fragility in asthmatic individuals is evidenced by loss of columnar ciliated epithelial cells and decreased epithelial junction proteins, such as ZO-1 and E-cadherin (de Boer, Sharma et al. 2008, Hackett, Singhera et al. 2011, Xiao, Puddicombe et al. 2011, Hackett, de Bruin et al. 2013). An undifferentiated, immature phenotype has also been suggested due to the increase in the proportion of p63- and CK-5-expressing basal cells within the asthmatic epithelium (Kicic, Sutanto et al. 2006, Hackett, Warner et al. 2009, Hackett, Singhera et al. 2011). Importantly, we and others have demonstrated that many of the features of epithelial remodeling are maintained when cells are cultured in ALI *in vitro* (Hackett,

Warner et al. 2009, Parker, Sarlang et al. 2010, Hackett, Singhera et al. 2011), although whether these reported findings occur in sub-groups of asthmatic subjects is unknown.

There is increasing interest in obtaining a more precise understanding of the clinical and pathological phenotypes of asthma, as complex and costly clinical trials have failed in the general asthma population, despite their efficacy in specific sub-groups (Wenzel 2012). One such clinical phenotype is exercise-induced bronchoconstriction (EIB), which 30 to 50% of asthmatics experience (Cabral, Conceicao et al. 1999, Crapo, Casaburi et al. 2000, Hallstrand, Moody et al. 2005). In individuals with EIB, exercise challenge leads to a decrease in the forced expiratory volume in one second (FEV_1) of 15% or greater (Haby, Anderson et al. 1994, Miller, Hankinson et al. 2005). Although the mechanisms underlying EIB remain elusive, the epithelium is postulated to have a central role. Epithelial water transport is thought to be defective in EIB patients during exercise-related hyperpnea, resulting in sustained hyperosmolality of the airway surface liquid, and the increased production of inflammatory mediators (Anderson and Daviskas 2000). Moreover, induced sputum from EIB(+) asthmatics contains significantly more shed columnar epithelial cells compared to EIB(-) asthmatics, providing further evidence that an altered epithelial barrier contributes to disease pathogenesis (Hallstrand, Moody et al. 2005).

We hypothesized that in addition to an altered epithelial phenotype in asthmatic-derived cultures, the airway epithelium from EIB(+) asthmatic donors would show distinct differences in ciliated cells, basal cells and adherens junctions as compared to both EIB(-) and control donors. Our aim was to comprehensively phenotype epithelial cells from EIB(+) and EIB(-) asthmatic and healthy control donors *in vivo* as well as throughout the course of differentiation *in vitro*. This study also forms the foundation for the RNA sequencing analysis that will be described in

Chapter 6, the aim of which is to understand what miRNAs and mRNAs regulate both mucociliary differentiation and the differences between EIB(+) and EIB(-) asthmatics.

5.2 Methods and materials

5.2.1 Endobronchial biopsies and brushing

Samples were collected by endobronchial biopsy and bronchial brushing of healthy control donors and asthmatic patients with (+) and without (-) EIB as described previously (Hallstrand, Moody et al. 2005, Hallstrand, Chi et al. 2007, Hallstrand, Debley et al. 2007, Hallstrand, Lai et al. 2013). Control donors had no asthma history, a baseline FEV_1 greater than 80% predicted and methacholine PC_{20} less than 8 mg/mL. Asthmatic patients had been diagnosed by a physician at least one year prior to study enrollment, and had mild to moderate disease severity. EIB(+) status was classified as at least 15% decrease in FEV_1 after exercise challenge, while a change of less than 7% was considered EIB(-). All patients were never-smokers and gave informed consent for the study as required by the human protocol accepted by the University of Washington ethics committee. Donor demographics for ALI cultures can be found in **Table 5.1** and biopsy samples in **Table 5.2**.

Subject		Age	Gender	Ethnicity	FEV_1/ FVC	Meth PC_{20}	Max EIB[$]	AUC_{30}[#]	Therapy[&]
Control	**1**	29	m	cauc	0.88	8	-1.0	-57.3	
	2	58	f	cauc	0.85	8	1.9	2.1	
	3	23	f	cauc	0.91	8	4.8	104.3	
	4	22	f	asian	0.92	8	-1.2	-65.7	
	5	25	f	cauc	0.98	8	1.9	52.1	
	Mean	**31.4**			**0.91**	**8**	**1.3**	**7.1**	
	SD	**15.1**			**0.0**	**0.0**	**2.5**	**72.3**	
	Median	**25**							
EIB(-)	**1**	23	f	cauc	0.70	0.25	2.8	7.5	
	2	23	f	cauc	0.77	0.76	1.6	-45.0	Advair
	3	38	f	cauc	0.83	2.64	5.3	99.8	
	4	25	m	cauc	0.71	0.84	2.6	-83.3	
	5	26	m	cauc	0.63	0.93	-0.5	-109.5	
	Mean	**27**			**0.73**	**1.08**	**2.4**	**-26.1**	
	SD	**6.3**			**0.1**	**0.9**	**2.1**	**83.0**	
	Median	**25**							
EIB(+)	**1**	44	m	cauc	0.61	0.06	25.8	621.4	
	2	24	f	cauc	0.86	0.06	24.5	429.3	
	3	22	f	cauc	0.78	0.78	20.3	490.8	Serevent
	4	37	f	cauc	0.77	0.12	24.7	499.5	
	5	21	f	cauc	0.72	0.19	34.5	826.7	
	Mean	**29.6**			**0.75**	**0.24**	**25.9**	**573.5**	
	SD	**10.3**			**0.1**	**0.3**	**5.2**	**157.7**	
	Median	**24**							
Statistics†									
Con vs. EIB(-)		ns	ns	ns	**	****	ns	ns	
Con vs. EIB(+)		ns	ns	ns	*	****	***	***	
EIB(-) vs. EIB(+)		ns	ns	ns	ns	ns	***	***	

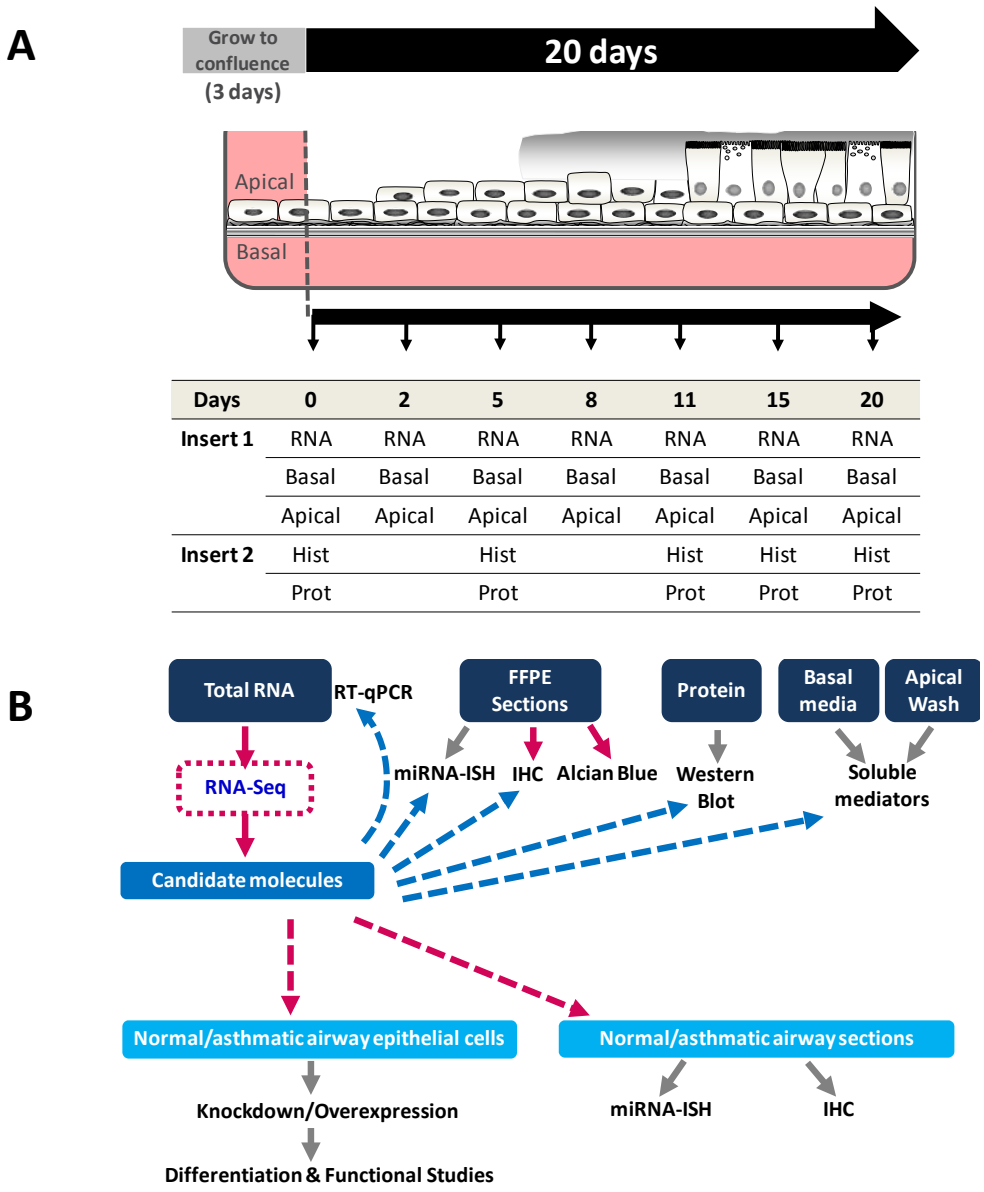
Table 5.1 Patient demographics of epithelial cells used in air-liquid interface cultures.
$ Maximum EIB: Maximum % fall in FEV_1 after exercise challenge
# AUC30: Area under FEV_1-time curve, to quantify severity of EIB over a 30-minute period after exercise.
& Controller therapy was discontinued for 30 days prior to bronchoscopy.
† One-way ANOVA with Tukey post-test; * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Subject		Age	Gender	Ethnicity	FEV_1/ FVC	Meth. PC_{20}	Max. EIB$	AUC_{30}#	Therapy&
Control	**1**	22	f	asian	0.92	8.00	-1.2	-65.7	
	2	24	f	cauc	0.78	8.00	3.4	27.7	
	3	25	f	cauc	0.98	8.00	1.9	52.1	
	4	50	f	cauc	0.83	8.00	2.3	-26.1	
	Mean	**30.3**			**0.88**	**8.0**	**1.6**	**-3.0**	
	SD	**6.6**			**0.04**	**0.0**	**1.0**	**26.5**	
	Median	**25**							
EIB(-)	**1**	23	f	cauc	0.77	0.76	1.6	-45.0	Advair
	2	24	f	cauc	0.85	2.45	-1.1	-77.2	
	3	23	f	cauc	0.91	0.48	7.4	36.7	
	4	25	m	cauc	0.71	0.84	2.6	-83.3	
	5	26	m	cauc	0.63	0.93	-0.5	-109.5	
	Mean	**24.5**			**0.78**	**1.2**	**2.1**	**-58.3**	
	SD	**0.6**			**0.06**	**0.4**	**1.9**	**32.4**	
	Median	**25**							
EIB(+)	**1**	44	m	cauc	0.61	0.06	25.8	621.4	
	2	24	f	cauc	0.86	0.06	24.5	429.3	
	3	21	f	cauc	0.84	0.71	18.7	488.5	
	4	21	f	cauc	0.72	0.19	34.5	826.7	
	Mean	**27.5**			**0.76**	**0.3**	**25.8**	**591.5**	
	SD	**5.5**			**0.06**	**0.2**	**3.3**	**88.1**	
	Median	**23**							
Statistics†									
Con vs. EIB(-)		ns	ns	ns	ns	***	ns	ns	
Con vs. EIB(+)		ns	ns	ns	ns	***	***	***	
EIB(-) vs. EIB(+)		ns	ns	ns	ns	ns	***	***	

Table 5.2 Patient demographics of endobronchial biopsy samples.
$ Maximum EIB: Maximum % fall in FEV_1 after exercise challenge
# AUC30: Area under FEV_1-time curve, to quantify severity of EIB over a 30-minute period after exercise.
& Controller therapy was discontinued for 30 days prior to bronchoscopy.
† One-way ANOVA with Tukey post- test; * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

5.2.2 Air-liquid interface (ALI) culture

PHAECs obtained by bronchial brushing were cultured in monolayer in Bronchial Epithelial Growth Media (BEGM) (Lonza Inc., Basel, Switzerland). At 80% confluence, pHAECs were trypsinized and 2 x 10^5 cells were seeded in 12 mm diameter/0.4 µm pore size transwell inserts (Corning, New York), and submerged in ALI growth media supplemented with 10 ng/mL EGF (VWR) and 30 ng/mL retinoic acid (Sigma) (Fulcher, Gabriel et al. 2005). ALI growth media consisted of a 1:1 DMEM:BEBM mix supplemented with hydrocortisone, insulin, epinephrine, bovine pituitary extract and transferrin (as specified by the manufacturer, Lonza), 80 uM ethanolamine, 0.3 mM magnesium chloride, 0.4 mM magnesium sulphate, 25 µg/mL fluconazole, 0.5 mg/mL bovine serum albumin (all Sigma) and 1x penicillin/streptomycin (Invitrogen). Initially, 1.5 mL of ALI media was added to the basal compartment and 0.5 mL to the apical compartment. After 24 hours, media in the basal compartment was changed. Forty-eight hours after this (72 hours after seeding), the apical media was removed, and media in the basal compartment was replaced with ALI growth media containing 0.5 ng/mL EGF and 30 ng/mL retinoic acid, to induce differentiation. Basal media was changed every 48 hours, and from day 5, apical surfaces were washed twice per week with PBS to remove mucus build-up. At days 0, 5, 11, 15 and 20, total RNA, protein, basal media, and apical washes were collected, and cultures were formalin fixed and paraffin embedded (FFPE) for histological examination (**Figure 5.1**). Additional RNA, basal media and apical wash samples were collected at days 2 and 8.



Days	0	2	5	8	11	15	20
Insert 1	RNA	RNA	RNA	RNA	RNA	RNA	RNA
	Basal	Basal	Basal	Basal	Basal	Basal	Basal
	Apical	Apical	Apical	Apical	Apical	Apical	Apical
Insert 2	Hist		Hist		Hist	Hist	Hist
	Prot		Prot		Prot	Prot	Prot

Figure 5.1 Schematic of sample collection and experimental plan for pHAEC ALI cultures.
(A) At each timepoint, the apical surface of one ALI insert was washed with PBS to collect secretions (Apical) and conditioned media was collected from the basal compartment (Basal). The culture was then lysed to collect total RNA (RNA). At days 0, 5, 11, 15 and 20, a second insert was also collected. One half was formalin-fixed and paraffin-embedded for histology (Hist) and the second half was lysed to collect protein in the presence of phosphatase and protease inhibitors (Prot). **(B)** RNA collected on days 0, 5, 11 and 20 will be sequenced to identify candidate mRNAs and miRNAs, as will be described in **Chapter 6**. In future studies, candidate molecules will be confirmed by RT-qPCR, miRNA *in situ* hybridization (miR-ISH), immunohistochemistry (IHC), Western Blot, or various methods to assess soluble mediators in basal media and apical wash. Knockdown or overexpression studies will be carried out to assess the functional role of candidate molecules in ALI cultures of pHAECs from non-asthmatic or asthmatic donors. Expression of candidate molecules will also be assessed in an independent cohort using FFPE sections of human donor airways from non-asthmatic and asthmatic individuals.

5.2.3 Histology

FFPE sections were stained with either Alcian blue to visualize mucins (counterstained with 1% neutral red), or for specific proteins by immunohistochemistry (IHC). For IHC, antigens were retrieved by autoclaving in citrate buffer (Dako Cyomation, Burlington, ON), endogenous peroxidase blocked in 3% H_2O_2, and non-specific binding blocked with appropriate sera. Expression of cytokeratin-5, p63 or E-cadherin (see **Table 5.3**) was visualized using DAB (Dako Cytomation). Nuclei were counterstained with Harris hematoxylin (Sigma-Aldrich, St Louis, MO).

Epitope	Host	Catalog #	Company	Dilution
Cytokeratin 5 (D5/16 B4)	Mouse	M7237	Dako, Burlington, Canada	1/50
E-cadherin	Mouse	sc-8426	Santa Cruz Biotechnology, Santa Cruz, USA	1/50
p63(4A4)	Mouse	sc-8341	Santa Cruz Biotechnology, USA	1/250

Table 5.3 Antibodies used in chapter 5.

5.2.4 Image analysis

All ALI samples were blinded by random coding to ensure no observer bias was introduced to the image analysis. For each donor, five images were captured by phase-contrast digital microscopy (Spot Imaging Solutions, Sterling Heights, MI) and analyzed either by point counting or by colour segmentation using Image-Pro Plus (Media Cybernetics, Rockville MD). In Alcian blue-stained sections, cilia length was measured on up to 20 ciliated cells from each donor.

5.2.5 Enyzme-linked immunosorbent assay (ELISA)

Inflammatory mediators interleukin-6 (IL-6) and IL-8 were assessed by ELISA (R&D Systems, Minneapolis, MI) in the conditioned basal media of the ALI cultures at each time point as described in **Chapter 2.5**. Samples were analyzed in duplicate and expressed as pg/mL of media per 24 hours in culture.

5.2.6 Statistics

Data are presented as median with interquartile range. Paired comparisons were analyzed with Mann-Whitney U-test and Kruskal-Wallis with Dunn's post-test was used for comparison between three or more groups. All statistical analyses were done using Prism Version 5.04 (GraphPad Inc., San Diego, CA), with *P* values less than 0.05 considered statistically significant.

5.3 Results

5.3.1 Airway epithelial remodeling occurs *in vivo*

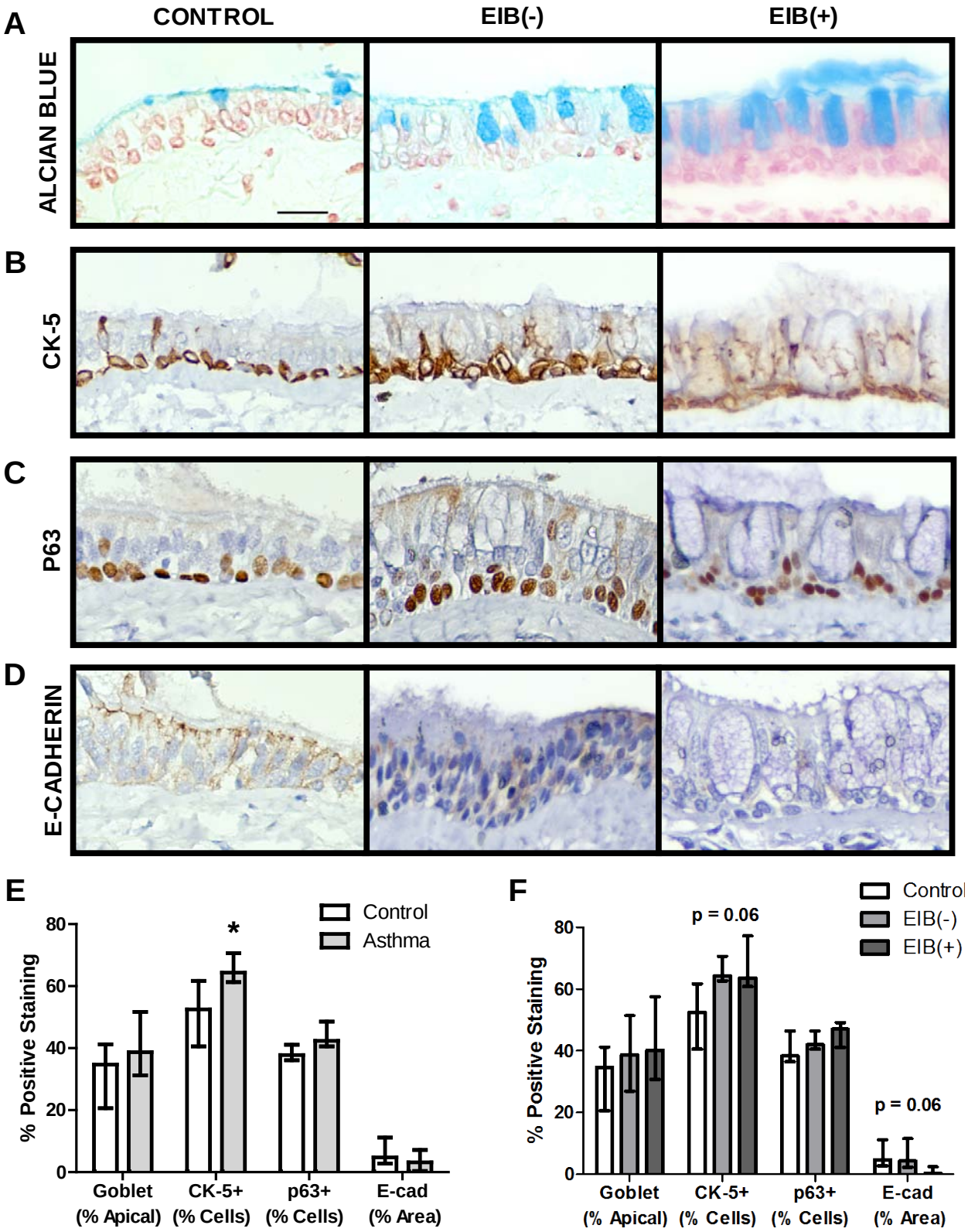
We first set out to determine whether the airway epithelium *in vivo* lacks features of differentiation in EIB(+) as compared to EIB(-) asthmatic and control donors. To do this, we obtained serial sections of endobronchial biopsies and stained sections with Alcian blue to visualize mucins (**Figure 5.2A**) and performed immunohistochemistry for the basal cell markers cytokeratin (CK)-5 (**Figure 5.2B**) and p63 (**Figure 5.2C**), and the adherens junction protein E-cadherin (**Figure 5.2D**). Quantification of the various cell types in control as compared to asthmatic donors demonstrated that there was no significant difference in goblet cell numbers in the asthmatic epithelium (**Figure 5.2E**), or in individual EIB phenotypes (**Figure 5.2F**). However, quantification of CK-5 demonstrated significantly more basal cells in the asthmatic

epithelium ($p<0.05$; **Figure 5.2E**), with approximately equal increases in EIB(+) and EIB(-), though neither was significantly different from control cultures ($p=0.06$; **Figure 5.2F**). Interestingly, another basal cell marker, p63, was not significantly different between disease states (**Figure 5.2E**). Finally, expression of E-cadherin was not significantly different between control and asthmatic donors by percentage positive area (**Figure 5.2E**) or between disease phenotypes ($p=0.06$ overall; **Figure 5.2F**).

(Next Page)

Figure 5.2 Asthmatic airways contain more CK-5$^+$ basal cells *in vivo*.

(A-D) Endobronchial biopsies from control (n=4), EIB(-) asthmatic (n=5) and EIB(+) asthmatic (n=4) donors were stained with **(A)** Alcian blue or immunostained for **(B)** cytokeratin (CK)-5, **(C)** p63 or **(D)** E-cadherin. Scale bar is 20 μm. **(E)** Quantification by point counting of positive cells (CK-5 and p63) or by colour segmentation of positive area (E-cadherin) was used to determine phenotypic differences between control (white bars) and asthmatic (grey bars). Data are presented as median with interquartile range and significance was assessed with Mann-Whitney U-test (* indicates $p<0.05$). For E-cadherin, one outlier was excluded from the EIB(+) group (n=3). **(F)** Asthmatic groups were further separated to assess EIB(-) (light grey bars) and EIB(+) (dark grey bars). Data are presented as median with interquartile range, and significance was assessed by Kruskal-Wallis test. P-values are provided.


A
CONTROL
EIB(-)
EIB(+)
ALCIAN BLUE
B
CK-5
C
P63
D
E-CADHERIN
E
Control
Asthma
*
% Positive Staining
80
60
40
20
0
Goblet (% Apical)
CK-5+ (% Cells)
p63+ (% Cells)
E-cad (% Area)
F
Control
EIB(-)
EIB(+)
p = 0.06
p = 0.06
% Positive Staining
Goblet (% Apical)
CK-5+ (% Cells)
p63+ (% Cells)
E-cad (% Area)

5.3.2 Ciliated cell differentiation is altered in asthmatic-derived pHAECs *in vitro*

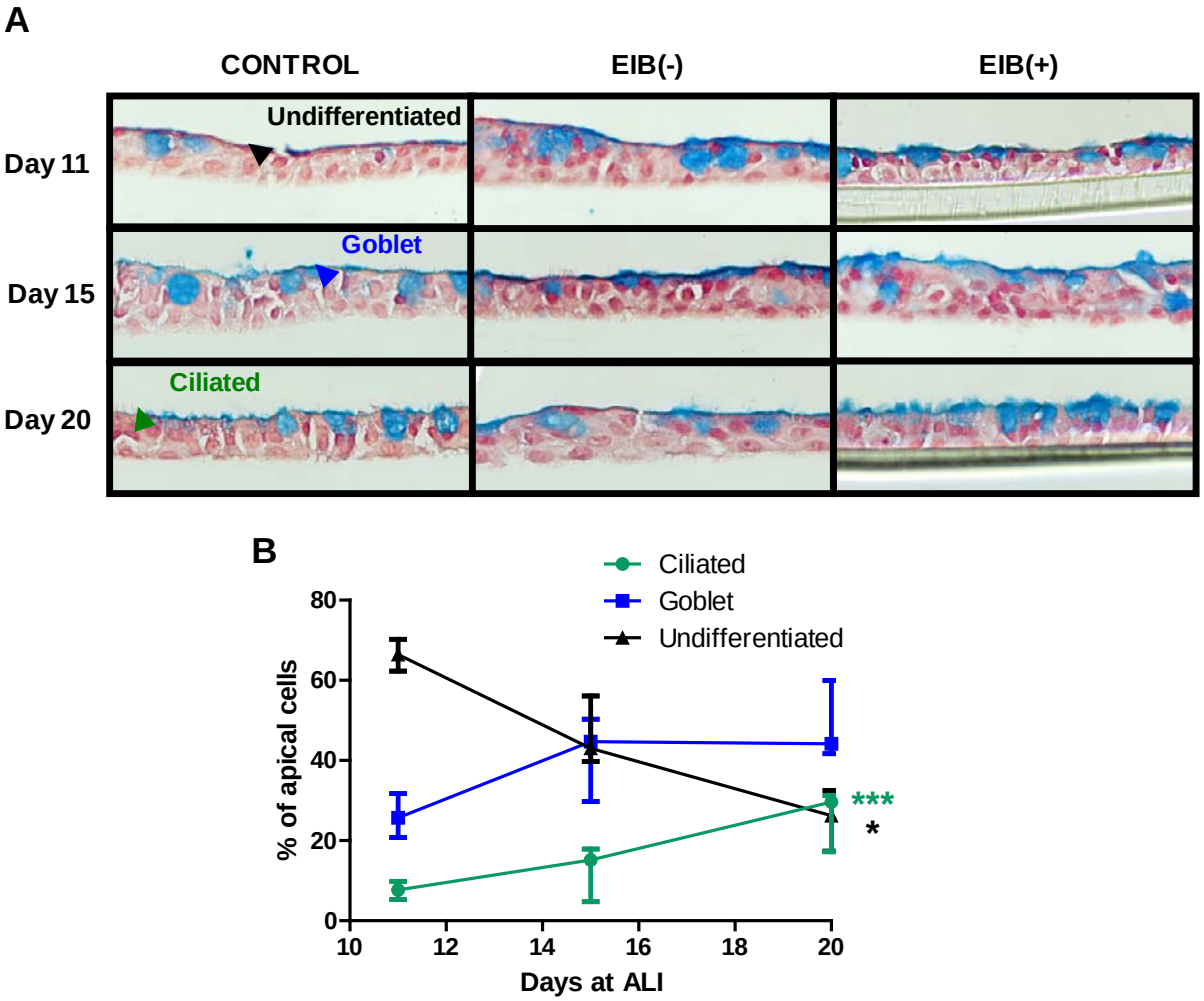
We next wanted to determine whether pHAECs from EIB(+) and EIB(-) asthmatic donors maintained an intrinsically altered phenotype when differentiated in ALI culture *in vitro*. To this end, we grew ALI cultures of pHAECs from control donors (n=5), EIB(-) asthmatic (n=5) and EIB(+) asthmatic (n=5) donors. To explore the differentiation profile of the ALI cultures, we assessed markers of mucociliary differentiation over 20 days. Alcian blue stained sections were used to visually distinguish cells at the apical surface as goblet cells (blue arrowhead), ciliated cells (green arrowhead), or cells that lacked characteristics of either goblet or ciliated cells, which we termed undifferentiated cells (black arrowhead) (**Figure 5.3A**). In ALI cultures generated from non-asthmatic donors, we observed a significant increase in the percentage of ciliated cells at day 20 compared to day 11 and 15 ($p<0.001$ and $p<0.01$, respectively; **Figure 5.3B**, green line). In contrast to the increase in ciliated cells, we observed a significant decrease in undifferentiated apical cells in non-asthmatic cultures from day 11 to day 20 ($p<0.05$, black line). While the proportion of goblet cells also increased over time, the difference between days 11 and 20 was not statistically significant (blue line). This indicates that undifferentiated cells are able to differentiate into either goblet or ciliated cells in ALI cultures of pHAECs from non-asthmatic donors.

In contrast, when we compared the cellular composition of the apical surface of differentiated ALIs at day 20 between disease phenotypes, we found that there was a striking decrease in the proportion of ciliated cells in asthmatic-derived ALIs ($p<0.001$; **Figure 5.3C**). We observed slightly more undifferentiated apical cells in asthmatic-derived ALIs at day 20, although this did not reach statistical significance. There was no difference in the proportion of goblet cells between asthmatic and control cultures at day 20, although there was a trend toward

an increase in asthma. When we further separated the asthmatic donors by EIB status, we found that both EIB(-) and EIB(+) displayed this defect in ciliated cells compared to controls ($p<0.05$ for both; **Figure 5.3D**). There were no significant differences in the percentage of goblet or undifferentiated cells at the apical surface in either EIB(+)or EIB(-) cultures compared to control at day 20 (**Figure 5.3D**). Overall, we found that ciliated cell differentiation was similarly defective in ALI cultures of pHAECs from both EIB(+) and EIB(-) donors.

(Next page)

Figure 5.3 Differentiation of ciliated cells is impaired in ALI cultures from asthmatic donors.
(A) pHAECs from control, EIB(+) asthmatic and EIB(-) asthmatic donors (n=5 each) were assessed for mucus production by Alcian Blue at days 11, 15 and 20 of ALI culture. **(B)** Analysis of mucociliary differentiation was assessed by point counting of ciliated (green arrowhead in (A), goblet (blue arrowhead) and undifferentiated cells (black arrowhead) at the apical surface of control cultures (n=5). Signifcance was assessed by two-way ANOVA with Bonferroni post-test to compare day 20 with day 11. **(C)** Analysis of the percentage positive cells of each type in control (white bars) and asthmatic-derived (grey bars) cultures, significance assessed by Mann-Whitney U-test. (D) Asthmatic groups were further separated to assess percentage of goblet, ciliated and undifferentiated cells in control (white bars), EIB(-) (light grey bars) and EIB(+) (dark grey bars) ALIs and significance was assessed by Kruskal-Wallis with Dunn's post-test. All data are presented as median with interquartile range, with * $p<0.05$, *** $p<0.001$.


A
CONTROL
EIB(-)
EIB(+)
Day 11
Day 15
Day 20
Undifferentiated
Goblet
Ciliated
B
Ciliated
Goblet
Undifferentiated
% of apical cells
Days at ALI
***
*
C
Control
Asthma
% of apical cells
***
Goblet
Ciliated
Undifferentiated
D
Control
EIB(-)
EIB(+)
% of apical cells
* *
Goblet
Ciliated
Undifferentiated

Following our finding of a decreased number of ciliated cells at the apical surface of asthmatic-derived ALI cultures, we were interested to determine whether the cilia of these cells were comparable in length to those from non-asthmatic donors. At day 20, we observed ciliated cells at the apical surface of 5 of 5 control cultures, 4 of 5 EIB(-) cultures, and 4 of 5 EIB(+) cultures. Of those ALI cultures that had ciliated cells, we queried whether the cilia differed in length (**Figure 5.4A**). Cilia were significantly shorter in asthmatic (median 4.58 µm) compared to control cultures (median 5.25 µm; **Figure 5.4B**). By Mann-Whitney U-test, the EIB(-) group had shorter cilia than control cultures, but the EIB(+) cultures did not due to the large range in cilia length from these donors (2.36 – 6.20 µm) (**Figure 5.4C**). This may indicate that in addition to a defect in commitment to the ciliated cell lineage in asthma, there is also an intrinsic defect in cilia formation in those cells that do commit to this lineage, particularly in EIB(-) asthma.

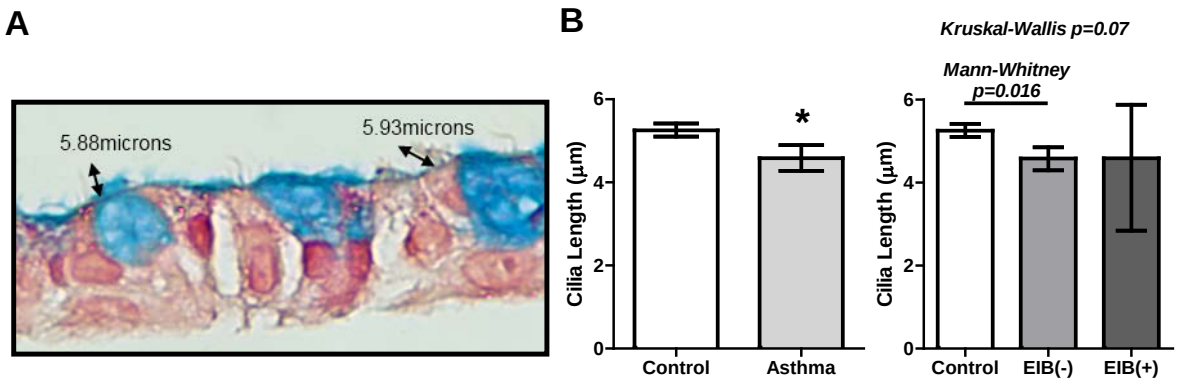


Figure 5.4 Cilia are significantly shorter in primary human airway epithelial cells from EIB(-) asthmatic donors.
(A) Representative image of ALI day 20 culture from control donor stained with Alcian blue to visualize mucin. Cilia were measured as demonstrated. **(B)** Cilia length measurements in control (n=5) and asthmatic (n=8) at day 20. Statistical significance was assessed by Mann-Whitney U-test. **(C)** Comparison of cilia length between control (n=5), EIB(-) (n=4), and EIB(+)-derived (n=4) ALI cultures at day 20. Statistical significance between all three groups was assessed using Kruskal-Wallis test with p-value provided. Mann-Whitney U-test was used to assess statistical significance between control and EIB(-) groups, with p-value provided. All data expressed as median with interquartile range.

5.3.3 Basal cell populations are expanded in asthmatic-derived ALI cultures *in vitro*

We next wanted to determine if molecular markers of epithelial remodeling were still altered in ALI culture, and whether these differed with donor disease status. ALI cultures at day 20 were stained for basal epithelial markers CK-5 (**Figure 5.5A**) and p63 (**Figure 5.5B**), and the adherens junction protein E-cadherin (**Figure 5.5C**). The percentage of CK-5+ cells was significantly higher in cultures from asthmatic compared to non-asthmatic donors ($p<0.05$; **Figure 5.5D**). When we separated the asthmatics by EIB status, we found that EIB(-) asthmatic cultures alone displayed significantly more CK-5-positive cells than control cultures ($p<0.05$; **Figure 5.5E)**, whereas EIB(+) cultures did not. As with the *in vivo* biopsies, we found that the proportion of p63-positive cells was not significantly higher in asthma (Figure 5.5D) or for either asthma phenotype alone (**Figure 5.5E**). In contrast to the trend toward downregulation *in vivo*, the percentage area of the epithelium that stained for E-cadherin was increased in asthmatic-derived ALIs compared to controls ($p<0.05$; **Figure 5.5D**). When separated by disease phenotype, the EIB(-) cultures had significantly greater E-cadherin immunoreactivity ($p<0.05$), while EIB(+) cultures did not (**Figure 5.5E**). Overall, in addition to an inability to differentiate at the apical surface, we found that there were increased numbers of CK-5-expressing basal cells in asthmatic-derived pHAECs, particularly in EIB(-) asthma.

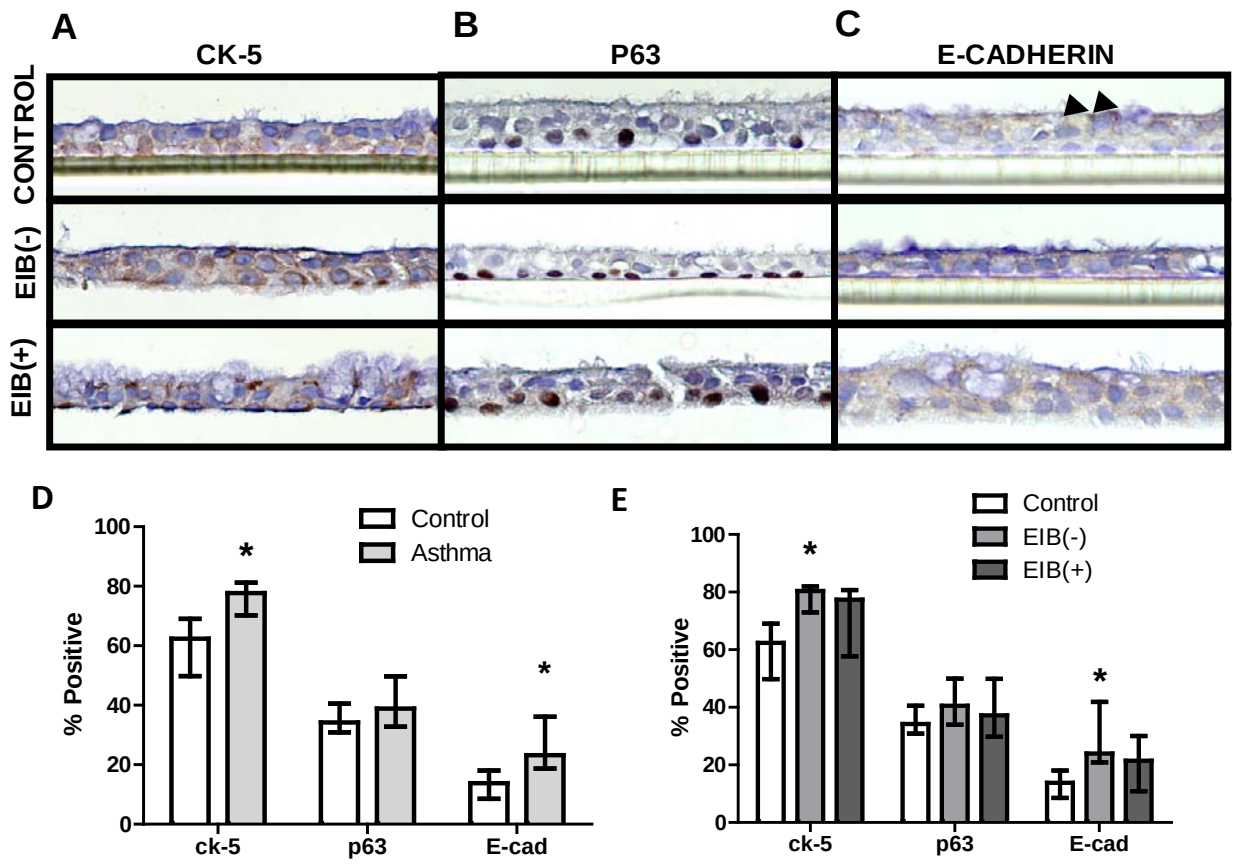


Figure 5.5 ALI cultures from asthmatic donors contain more CK-5$^+$ basal cells.
(A-C) Formalin-fixed paraffin-embedded ALI cultures (20 days) of pHAECs from control, EIB(+) asthmatic and EIB(-) asthmatic donors (n=5 each) were immunostained for **(A)** cytokeratin (CK)-5, **(B)** p63 and **(C)** E-cadherin (all brown) and hematoxylin counterstain (blue). **(D)** Quantification by point counting of positive cells (CK-5 and p63) or by colour segmentation of positive area (E-cadherin) was used to determine phenotypic differences between control (white bars) and asthmatic (grey bars) by Mann-Whitney U-test. **(E)** Asthmatic groups were further separated to assess EIB(-) (light grey bars) and EIB(+) (dark grey bars) and significance was assessed by Kruskal-Wallis with Dunn's post-test. * Indicates $p<0.05$ compared to control cultures.

5.3.4 Release of inflammatory mediators differs between disease states and during differentiation

Inflammatory mediator responses are increased in pHAECs from asthmatic patients, potentially mediated in part by phosphorylation of p38 (Hackett, Singhera et al. 2011). We assessed the release of innate pro-inflammatory mediators IL-6 and IL-8 during the 20 days of ALI differentiation. IL-8 decreased between day 0, which mimics a wound repair state with only undifferentiated cells, and day 20, in which cultures are differentiated ($p<0.0001$; **Figure 5.6A**). When the asthmatic subgroups were combined, IL-8 secretion was significantly different from control cultures ($p<0.0001$; **Figure 5.6A**). This was particularly evident at day 2, when ALIs from asthmatic donors secreted less IL-8 than control cultures ($p<0.05$). IL-8 release was distinct among the three disease phenotypes (**Figure 5.6B**), wherein EIB(-) cultures released the least IL-8 and control donors released the most. At day 2, EIB(-) cultures released significantly less IL-8 than control cultures ($p<0.05$).

Asthmatic subgroups together produced more IL-6 than control cultures throughout differentiation ($p<0.01$ by two-way ANOVA; **Figure 5.6C**). IL-6 release was increased in the asthmatic subgroups ($p<0.01$; **Figure 5.6D**). Production of IL-6 varied throughout differentiation in EIB(+) asthmatic cultures. IL-6 release was increased at day 11 in EIB(+) cultures compared to control ($p<0.05$).

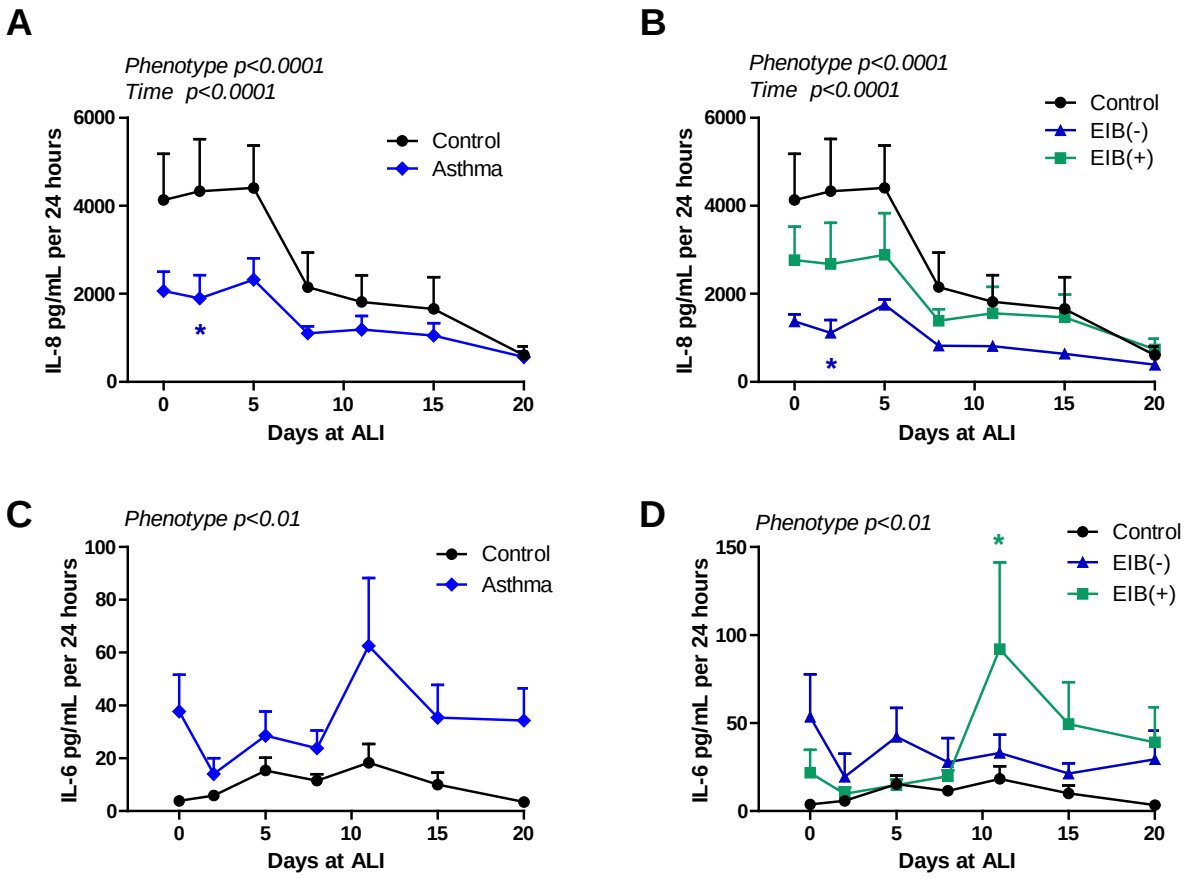


Figure 5.6 Release of inflammatory mediators differs in asthma and during differentiation.
Media from the basal compartment collected throughout differentiation was used to measure release of inflammatory mediators **(A)** IL-8 and **(C)** IL-6 from ALI cultures from both asthmatic sub-groups combined (blue) compared to control (black). Release of **(B)** IL-8 and **(D)** IL-6 from control (black), EIB(-) (blue) and EIB(+) (green) donors was also compared. Two-way ANOVA was used to assess statistical significance between donor phenotypes and over time (p-values are shown in top left corner of each graph). Data are presented as mean ± SEM and * $p<0.05$ at individual timepoints by Bonferroni post-test.

5.4 Discussion

Airway remodeling is a defining feature of asthma, and is associated with numerous epithelial alterations (Davies 2009). Close to half of all asthmatic patients experience EIB, but the structure and function of the airway epithelium in this clinical subtype are largely unknown. Here we have examined in detail the phenotype of the airway epithelium from non-asthmatic and asthmatic donors with and without EIB, both *in vivo* and over the course of differentiation *in vitro*. We demonstrate that the airway epithelium from EIB(+) and EIB(-) asthmatic donors exhibits defective ciliary differentiation *in vitro* and that asthmatic donors had an expanded CK-5-expressing basal cell population *in vivo* and *in vitro*. However, we did not find any differences in mucociliary differention between EIB(+) and EIB(-) asthmatic donors.

Loss of ciliated epithelial cells in asthmatic patients has been described since 1943 (Hilding 1943). Sloughing of columnar cells, termed Creola bodies in BAL fluid, has also been described early in the disease progression (Barbato, Turato et al. 2006). Few *in vitro* studies have explored the ability of airway epithelial cells from asthmatic donors to undergo ciliary differentiation, and to our knowledge no studies have focused on this process in EIB(+) asthma. We found that in ALI cultures from EIB(-) and EIB(+) donors, the percentage of ciliated cells at day 20 was approximately five-fold lower than non-asthmatic cultures, indicating a striking defect in ciliated cell differentiation. This is in keeping with previous work showing that ALI cultures from pediatric asthmatic donors had fewer ciliated cells at day 28 (Parker, Sarlang et al. 2010). Mucociliary differentiation is crucial for normal development of the pseudostratified airway epithelium, as well as for epithelial restitution following injury. Studies of mucociliary differentiation in normal airway epithelial cells *in vitro* have reported the appearance of ciliated cells as early as 14 days in ALI culture (de Jong, van Sterkenburg et al. 1994, Bernacki, Nelson

et al. 1999, Parker, Sarlang et al. 2010), others by 18 days (Gray, Guzman et al. 1996), and some as late as 24 days (Pohl, Hermanns et al. 2009). The number of ciliated cells has been shown to increase drastically between days 21 and 31 of ALI culture (de Jong, van Sterkenburg et al. 1994). In line with these reported studies, we observed ciliated cells in non-asthmatic ALI cultures by day 11, and by day 20 ciliated cells comprised approximately 25% of the apical cells present. Though we cannot rule out the possibility that complete mucociliary differentiation would occur at a time point after 20 days in our ALI model, it is clear that early commitment to the ciliated cell lineage is aberrant in epithelial cells from asthmatic donors, regardless of EIB status.

Mucus-producing goblet cells have been shown to be present in ALI cultures prior to ciliated cells. Specifically, goblet cells exceeded ciliated cells in number at days 7 and 14 in ALI cultures (de Jong, van Sterkenburg et al. 1994, Gray, Guzman et al. 1996, Bernacki, Nelson et al. 1999) but the two cell types were equivalent by day 21 (de Jong, van Sterkenburg et al. 1994). In agreement with these studies, we found that goblet cells were abundant from day 11 onward. However, in our study we found that the number of goblet cells continued to be greater than ciliated cells up to day 20 of the ALI model. This difference in goblet cell number may have been due to differences in coating of the ALI membrane. In our study methodology we did not coat the ALI membrane with ECM proteins, and instead the seeded epithelial cells synthesized and secreted their own ECM. In the de Jong et al. study (de Jong, van Sterkenburg et al. 1994), the ALI insert was coated with collagen type I, which has been shown to promote ciliogenesis in rat tracheal epithelial cells (Davenport and Nettesheim 1996). While these methodological differences may have contributed to the differences in the ratios of goblet and ciliated cells

observed in these two studies, it does not account for the reported decrease in the number of ciliated cells in asthmatic compared to non-asthmatic ALI cultures.

Mucus hypersecretion and goblet cell hyperplasia are well-described features of airway remodeling in asthma (Ordonez, Khashayar et al. 2001). However, we found no differences in the numbers of goblet cells in either EIB(-) or EIB(+) compared to non-asthmatic epithelium, either *in vivo* or *in vitro.* In contrast, previous studies found that ALI cultures derived from patients with asthma produced more mucins (Gras, Bourdin et al. 2012) and had increased populations of goblet cells (Parker, Sarlang et al. 2010, Blume, Swindle et al. 2013). In comparison with our cohort of mild to moderate asthmatics, who were free of controller medications for at least 30 days prior to bronchoscopy, the asthmatic patients in the studies described above required ongoing controller medications, suggestive of more severe disease (Parker, Sarlang et al. 2010 and Gras, Bourdin et al. 2012). Further, there is mounting evidence that goblet cell hyperplasia is a response to the allergic T_H2-predominant inflammatory milieu *in vivo,* which was not recapitulated in our ALI culture system (Tyner, Kim et al. 2006, Park, Korfhagen et al. 2007). In support of these data, we have previously shown that the number of goblet cells was not increased in asthmatic-derived ALIs, although in matched airway sections from the same donors we did observe mucus cell metaplasia (Hackett, Singhera et al. 2011). Finally, few donors in our cohort of mild to moderate asthmatic EIB(+) and EIB(-) donors required controller therapy prior to study enrollment, suggesting that there was minimal persistent T_H2 airway inflammation. This may indicate why we did not observe goblet cell hyperplasia *in vivo* at the time of biopsy collection (Tyner, Kim et al. 2006, Park, Korfhagen et al. 2007).

Ciliated cells of the respiratory epithelium contain 200-300 motile cilia per cell, which move in a concerted fashion to transport mucus and trapped particles toward the pharynx. Defects in ciliary function can lead to mucus congestion in the airways due to impaired mucociliary clearance, resulting in conditions such as bronchiectasis and chronic sinusitis (Afzelius 2004). We found that, in addition to decreased numbers of ciliated cells, the median length of cilia that were present was significantly shorter in ALI cultures derived from EIB(-) asthmatic patients than in non-asthmatic-derived cultures. Thomas and colleagues demonstrated that although cilia length did not differ significantly in bronchial brushings from mild, moderate, and severe asthmatic donors compared to control subjects, ciliary function and ultrastructure was compromised in severe asthma (Thomas, Rutman et al. 2010). Several factors, including environmental toxicants, can affect ciliary length and function. For example, cigarette smoke exposure leads to shortened airway cilia through histone deacetylase (HDAC)6-mediated autophagy of cilia components (Lam, Cloonan et al. 2013). In physiological settings, free of harmful stimuli, control of cilia structure and length is a complex and dynamic process. Multiciliated epithelial cells require that hundreds of centrioles, termed basal bodies, are formed and transported to the apical cell surface. Out of these basal bodies, the cilium is extended through a process of intraflagellar trafficking (IFT) of proteins from the cytoplasm (Ishikawa and Marshall 2011), reaching a final length of 6-7 μm for human respiratory cilia. As microtubule subunits are continually being recycled through a process involving kinesin family members, maintenance of cilia length requires ongoing molecular trafficking and cilia elongation (Stephens 1997, Marshall and Rosenbaum 2001, Song and Dentler 2001, Blaineau, Tessier et al. 2007). In keeping with this concept, the kinesin gene KIF3A was recently reported as a novel candidate asthma-associated gene, with decreased expression in uncontrolled asthma (Kovacic, Myers et al.

2011). Although cilia are not evident until approximately two weeks in ALI culture, the transcriptional activation and formation of ciliary components begins much earlier, between four and ten days in culture (Ross, Dailey et al. 2007, Hoh, Stowe et al. 2012). Therefore, defects in regulatory genes of ciliogenesis and cilia function will be assessed further as candidate genes in the RNA sequencing analysis of these samples described in **Chapter 6**.

As we have identified differences in EIB(+) and EIB(-) mucociliary differentiation, it was important to assess the progenitor basal cells present within the epithelium. In the present study we observed an increased proportion of CK-5-expressing basal cells in the epithelium of asthmatic donors *in vivo* and *in vitro* compared to non-asthmatic donors, and in the EIB(-) subgroup specifically. These findings are consistent with our previous studies demonstrating increased CK-5-expressing basal cells in the airway epithelium of asthmatics (Hackett, Shaheen et al. 2008, Hackett, Warner et al. 2009, Hackett, Singhera et al. 2011). Interestingly, we did not see a concurrent elevation in numbers of cells expressing p63, another basal cell marker that is crucial for maintenance and formation of stratified epithelial tissues (Yang, Schwitzer et al. 199; Mills, Zheng et al. 1999), and which we have previously shown to be elevated in asthmatic subjects (Hackett, Shaheen et al. 2008, Hackett, Warner et al. 2009, Hackett, Singhera et al. 2011). As basal cells differentiate into a polarized mucociliary tissue, formation of adhesion junctions by the transmembrane protein E-cadherin is crucial for formation of tight junctions, and thus for an effective mucociliary barrier (Tunggal, Helfrich et al. 2005). We and others have previously demonstrated decreased E-cadherin expression in the epithelium of asthmatic donors (de Boer, Sharma et al. 2008, Hackett, Singhera et al. 2011, Xiao, Puddicombe et al. 2011, Hackett, de Bruin et al. 2013). However, in the current study we did not find E-cadherin immunoreactivity to be decreased in asthmatic biopsies or ALI cultures. Our analysis enabled

quantification of the total percentage area of E-cadherin, but future analyses will incorporate a measure of staining intensity to determine whether there may be aberrant E-cadherin localization as opposed to decreased protein expression. Our observation of increased numbers of basal cells therefore support our hypothesis of a defect in mucociliary differentiation in asthmatic subjects, however the formation of adhesion junctions did not appear to be impaired in this mild asthmatic cohort.

In addition to structural alterations, we also assessed the production of inflammatory cytokines IL-6 and IL-8. IL-8 is increased in airway secretions in asthma, although this is most evident in severe, or neutrophilic, asthma (Nocker, Schoonbrood et al. 1996, Folkard, Westwick et al. 1997, Ordonez, Khashayar et al. 2001). The role of IL-8 in EIB is unclear, but hyperosmolality of airway surface liquid is postulated to contribute to EIB, and exposure of bronchial epithelial cells to hyperosmolar media induces IL-8 production (Hashimoto, Matsumoto et al. 1999). However, we did not find that IL-8 release was specifically altered in the EIB(+) group at any time point. Interestingly, we found that in the early stages of differentiation, IL-8 release was attenuated in asthmatic-derived ALIs, particularly in the EIB(-) subgroup. This is in contrast to Kicic et al, who found no difference in IL-8 release in asthmatic monolayers of pediatric epithelial cells (Kicic, Sutanto et al. 2006). This may be due to differences in storage versus release of IL-8 in our unstimulated cultures, as we only measured spontaneous release, but not IL-8 RNA or protein within the cells. In line with this, our unstimulated ALI cultures at day 20 released similar amounts of IL-8 regardless of donor disease status, which is in agreement with previous studies of well-differentiated ALI cultures (Parker, Sarlang et al. 2010, Hackett, Singhera et al. 2011). This may reflect the milder disease severity of our cultures, as Gras et al. report that ALI cultures derived from patients with severe asthma

released more IL-8 than patients with mild asthma (Gras, Bourdin et al. 2012). Although beyond the scope of the present study, it would be of interest to assess IL-8 release following damage of EIB(-) and EIB(+) cultures, as asthmatic-derived ALIs released more IL-8 upon damage by viral infection, particulate matter, and mechanical wounding than control-derived cultures (Hackett, Singhera et al. 2011).

IL-6 induces mucus hypersecretion, and inhibits T_H1 polarization while favouring the T_H2 and T_H17 pathways, indicating a clear role for this cytokine in affecting airway function and asthma pathogenesis (Neveu, Allard et al. 2009). Our finding that IL-6 release was consistently higher in asthmatic-derived cultures, regardless of timepoint, is in agreement with a previous report from Kicic et al, in which pHAECs from pediatric donors with mild asthma released significantly more IL-6 in monolayer culture (Kicic, Sutanto et al. 2006). In a previous study we also demonstrated enhanced IL-6 release in response to environmental exposures such as respiratory syncytial virus (RSV), particulate matter and mechanical wounding in asthmatic compared to non-asthmatic derived ALI cultures (Hackett, Singhera et al. 2011). Taken together, these findings indicate that release of inflammatory cytokines is altered in the asthmatic airway epithelium *in vitro*, even in unstimulated conditions.

There are some limitations to our study. Firstly, the sample size in this study was limited by the availability of endobronchial biopsy tissue and epithelial brushings from well-phenotyped asthmatic donors with and without EIB. Secondly, we utilized biopsies and epithelial cells collected by bronchoscopy, whereas our previous studies took advantage of the availability of intact airways from unusable transplant lung tissue (Hackett, Warner et al. 2009, Hackett, Singhera et al. 2011). This may have influenced the cell populations we were able to observe, as enzymatic digestion of entire airways yields abundant progenitor and basal cells (Hackett,

Shaheen et al. 2008). Lastly, our previous studies on whole lung tissue provided intact whole airways for histological examination of entire cross-sections of multiple airway generations rather than single biopsy samples, which have been well discussed within the literature to have artifact limitations (Labonte, Laviolette et al. 2008).

In summary, we provide here the first detailed phenotypic analysis of the airway epithelium from both EIB(-) and EIB(+) asthmatic donors during the kinetics of differentiation *in vitro*. Epithelial cells from asthmatic patients as a whole had aberrant ciliary differentiation, increased numbers of CK-5-expressing basal cells and altered release of inflammatory mediators IL-8 and IL-6. When separated by disease subgroup, all of the aforementioned features were present in EIB(-) compared to control cultures, but only the defect in ciliary differentiation was common to both EIB(-) and EIB(+) cultures, indicating a central, asthma-related mechanism. Cell fate decisions during mucociliary differentiation are highly regulated and temporally governed by transcription factors (Notch, Foxj1), microRNAs (miR-449a), and can be modified by exogenous growth factors (EGF, $TGF\beta_1$) and cytokines (IL-6). A detailed assessment of the transcriptional events occurring during mucociliary differentiation will be the focus of **Chapter 6**, and will allow us to further elucidate the underlying cause of the epithelial abnormalities found in EIB(-) and EIB(+) asthma.

Chapter 6: Distinct Epithelial mRNA and miRNA Expression Profiles During Differentiation and Between EIB(+) and EIB(-) Asthma Phenotypes

6.1 Introduction

In **Chapter 5**, we demonstrated that airway epithelial cell plasticity is abnormal in both EIB(+) and EIB(-) asthma, with defective differentiation of ciliated cells and greater numbers of CK-5-expressing cells in air-liquid interface (ALI) culture compared to non-asthmatic donors. We have also previously reported increased numbers of basal and progenitor cells in the epithelium of fatal asthmatics (Hackett, Warner et al. 2009, Hackett, Shaheen et al. 2008). Together, these findings suggest a defect in mucociliary differentiation in asthma. However, the mechanisms underlying this defect are thus far unknown.

Cell fate decisions in the epithelium are governed by numerous pathways and transcription factors and within the lung several key pathways have been described. For example, the Notch signaling pathway is centrally involved in mucociliary differentiation and is required for basal cell differentiation to a secretory cell fate (Guseh, Bores et al. 2009, Rock, Gao et al. 2011) through the inhibition of the ciliated cell lineage (Tsao, Vasconcelos et al. 2009). Developmental pathways such as the sox (SRY (sex determining region Y)-box) (Que, Luo et al. 2009) and alternate forkhead box (fox) transcription factors such as foxa2 are also involved in cell fate specification during prenatal development and postnatal epithelial differentiation (Mucenski, Nation et al. 2005). T_H2 cytokines, acting through transcription factors SPDEF and STAT-6, are known to induce goblet cell differentiation (Tyner, Kim et al. 2006, Park,

Korfhagen et al. 2007). Despite these candidate molecule analyses, very few studies have carried out comprehensive analyses of the transcriptional events that occur throughout mucociliary differentiation of airway epithelial cells *in vitro*. Studies have previously compared the transcriptome of monolayer and differentiated epithelia from healthy donors *in vitro* (Hackett, Shaykhiev et al. 2011, Martinez-Anton, Sokolowska et al. 2013), or tested the validity of differentiated *in vitro* cultures by comparing ALI cultures to bronchial brushings (Dvorak, Tilley et al. 2011, Pezzulo, Starner et al. 2011). One compelling study used microarray technology to assess the epithelial transcriptome at 11 timepoints between days 0 and 28 in ALI culture of primary human airway epithelial cells obtained by bronchial brushing from three healthy donors, to provide a transcriptional atlas of mucociliary differentiation (Ross, Dailey et al. 2007). The main pathways that were shown to be differentially expressed during mucociliary differentiation were the TGFβ, EGFR, and wnt/β-catenin pathways, in addition to genes regulating ciliogenesis (Ross, Dailey et al. 2007). Although Ross et al. did not observe morphological evidence of ciliary and mucus cell formation until day 21, the associated transcriptional events were shown to occur prior to day 10, highlighting the importance of assessing multiple timepoints during mucociliary differentiation.

One of the most important mechanisms of regulating mRNA expression is through the action of endogenous short RNAs known as microRNAs (miRNAs), that can either suppress mRNAs by targeted degradation or via translation repression, or may alternatively enhance protein expression (Vasudevan, Tong et al. 2007). Production of mature miRNA molecules is a coordinated, multi-step process as shown in **Figure 6.1** (Filipowicz, Bhattacharyya et al. 2008), that usually begins with *de novo* transcription of long primary transcripts (pri-miRNA), although

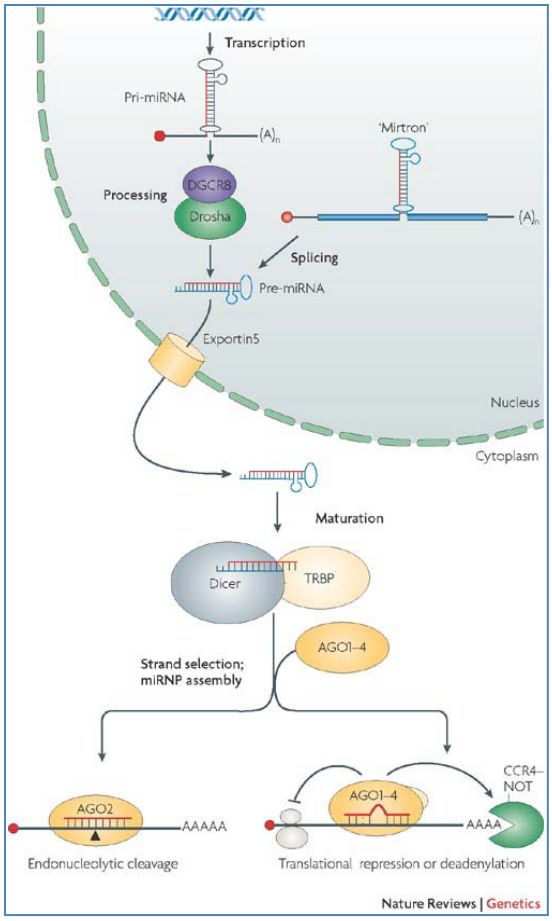


Figure 6.1 Schematic of miRNA biogenesis.
Pre-miRNAs may arise by direct transcription of pri-miRNAs, which are then processed by Drosha/DGCR8 (DiGeorge syndrome critical region gene 8) in the nucleus. Alternatively, pre-miRNAs may arise directly by splicing of mRNA introns, known as "mirtrons." Pre-miRNAs are exported to the cytoplasm via exportin 5, where they are processed by the enzyme complex of Dicer/TRBP (TAR RNA binding protein). The -5p and -3p miRNA strands are then separated and one or both is loaded onto AGO (Argonaute) proteins for biological action on target mRNA, either by targeting it for degradation (endonucleolytic cleavage), known as the RNA-Induced Silencing Complex (RISC) or translation repression or deadenylation (by CCR4-NOT deadenylation complex). *©2013 Nature Publishing Group. Reprinted by permission from: Filipowicz W et al. Nature Reviews Genetics 2008;9:102-114.*

some miRNAs are formed by splicing of introns from existing mRNAs (Berezikov, Chung et al. 2007, Sibley, Seow et al. 2012). Pri-miRNAs are cleaved by the enzyme Drosha to liberate a precursor (pre)-miRNA (Lee, Ahn et al. 2003, Gregory, Yan et al. 2004, Kim, Han et al. 2009)

and following exportin 5-mediated nuclear export (Lund, Guttinger et al. 2004), the pre-miRNA is cleaved by the Dicer enzyme into a double-stranded RNA (Hutvagner, McLachlan et al. 2001, Ketting, Fischer et al. 2001). The 5' (-5p) and 3' (-3p) strands are then separated into mature miRNAs and loaded onto the Argonaute (AGO) proteins to perform the miRNA's inhibitory function, and both may function as active miRNAs (Yang, Phillips et al. 2011). In addition, miRNAs may activate translation by mediating interactions between the fragile X mental retardation-related protein 1 (FXR1), AGO proteins, and mRNA. This occurs in quiescent cells in culture (Vasudevan, Tong et al. 2007) and in immature xenopus laevis oocytes (Mortensen, Serra et al. 2011), and may relate to enhanced mRNA stability, although the exact mechanism has not yet been elucidated.

Several miRNAs have been reported to be involved in cell fate decisions. One key regulator of airway epithelial mucociliary differentiation is miR-449a, which has been shown to be endogenously increased during differentiation of nasal epithelial cells in ALI culture (Lize, Herr et al. 2010), and to function by inhibiting the Delta/Notch pathway, to allow induction of ciliated cell differentiation (Marcet, Chevalier et al. 2011, Marcet, Chevalier et al. 2011). (Williams, Larner-Svensson et al. 2009, Yick, Zwinderman et al. 2013). MiR-449a expression was decreased in bronchial brushings from asthmatic patients (Solberg, Ostrin et al. 2012), making it a prime candidate to explain the lack of mucociliary differentiation observed in our asthmatic-derived ALI cultures. Furthermore, miR-203 is induced in suprabasal keratinocytes during epidermal development, restricting p63 expression to the basal cell layer (Lena, Shalom-Feuerstein et al. 2008, Yi, Poy et al. 2008). Jardim et al. described decreased miR-203 expression in bronchial brushings from asthmatic donors (Jardim, Dailey et al. 2012), which may imply a mechanism for the expanded populations of basal cells we observed in asthmatic airways. With

regards to miRNA regulation of epithelial differentiation in asthma, previous transcriptional comparisons between epithelial cells from healthy and asthmatic donors have been conducted on bronchial brushings (Jardim, Dailey et al. 2012, Solberg, Ostrin et al. 2012) and bronchial biopsies. To our knowledge no studies have yet compared the miRNA transcriptome of airway epithelial cells from healthy and asthmatic donors throughout differentiation in ALI culture.

We hypothesized that distinct miRNA and mRNA expression profiles are responsible for defective mucociliary differentiation of airway epithelial cells from asthmatic donors. Furthermore, as EIB is postulated to involve defective epithelial water transport (Anderson and Daviskas 2000) and shows features of increased epithelial fragility (Hallstrand, Moody et al. 2005), we hypothesized that there will be differences in mRNA and miRNA expression between asthmatic donors with and without EIB. We sequenced both large and small RNA in our ALI cultures of airway epithelial cells from healthy control, exercise induce bronchonstriction (EIB) (+) and EIB(-) asthmatic donors at days 0, 5, 11 and 20 of differentiation. Our aims were to first compare expression of known developmental and cell-type specific genes and miRNAs in a hypothesis-driven approach, followed by a non-hypothesis-driven global analysis of mRNAs and miRNAs that are differentially-expressed between disease groups during differentiation, and to identify important miRNA-mRNA interactions that regulate mucociliary differentiation *in vitro*.

6.2 Methods and materials

6.2.1 RNA sequencing

RNA extraction. Airway epithelial cells from healthy control (n=5), EIB(-) asthmatic (n=5) and EIB(+) asthmatic (n=5) donors were cultured at air-liquid interface as described in **Chapter 5.2.2**. Total RNA was collected at days 0, 5, 11 and 20. Briefly, inserts were rinsed in PBS and

incubated with 700 uL Qiazol lysis reagent for five minutes at room temperature and lysate collected by pipetting vigorously. Samples were homogenized by vortexing for one minute and then stored on ice, followed by storage at -80°C. MiRNEasy kits (Qiagen) were used according to the manufacturer's directions to isolate both small and large RNA fractions in 40 µL RNase-free distilled water. RNA concentration was assessed with the NanoDrop 8000 spectrophotometer (Thermo Scientific). All samples had excellent RNA quality (RNA Integrity Number (RIN) >9) as determined with the 2100 Agilent Bioanalyzer RNA6000 Nano kit (Agilent Technologies Inc., Santa Clara, USA). Aliquots of 2 µg total RNA were set aside for sequencing library creation.

Library preparation. As shown in **Figure 6.2,** sequencing libraries were created for both large and small RNA samples isolated at days 0, 5, 11 and 20 for all subjects (total = 60 samples). Large RNA libraries were prepared using Illumina® TruSeq® RNA Sample Preparation Kit v2. Briefly, mRNA was isolated using magnetic beads-based poly(A) selection, fragmented, and reverse transcription preformed using random primers, followed by second- strand synthesis to create double-stranded cDNA fragments. These cDNA fragments were then end-repaired by adding a single 'A' base, and ligated to Illumina® Paired-End sequencing adapters. The products were then purified and PCR-amplified to create the final cDNA library. Small RNA libraries were prepared using Illumina® TruSeq® Small RNA Sample Preparation

Kit v2. Briefly, 3' and 5' adapter sequences were sequentially ligated to miRNAs, and the products were PCR-amplified and gel purified to create the final cDNA library.

Sequencing and quality control. Large and small RNA samples were multiplexed so that four or eight samples (respectively) were added per lane of a flowcell. cDNA libraries were sequenced on an Illumina HiSeq 2000, generating 100-base paired-end reads (for large RNA) and 36-base

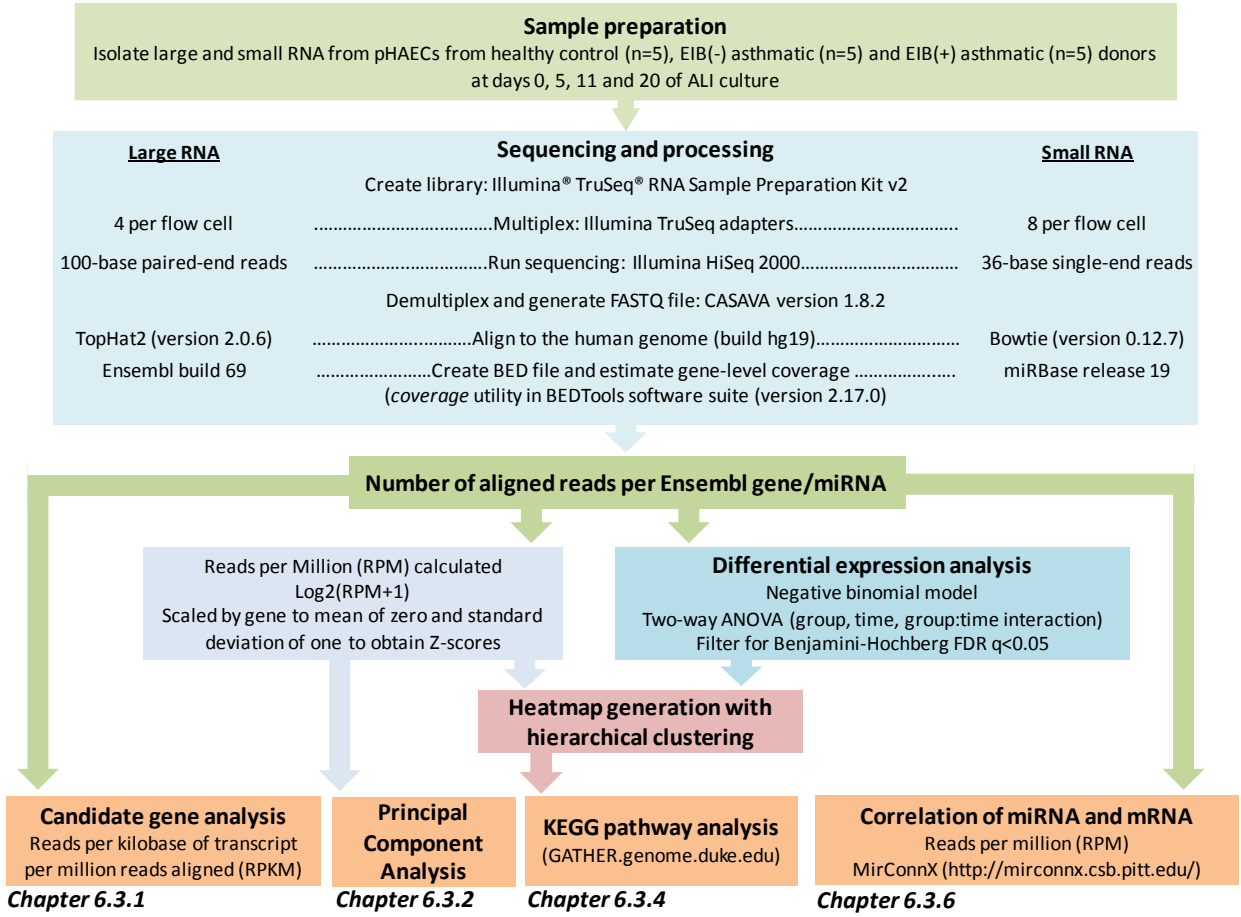


Figure 6.2 Schematic of data analysis.
Total RNA was extracted at days 0, 5, 11 and 20 of ALI cultures of primary human airway epithelial cells from healthy control, EIB(-) and EIB(+) asthmatic donors. Sequencing libraries were prepared for large and small RNA and the number of aligned reads per Ensembl gene and per miRBase ID was determined as described in **Section 6.2.1**. Expression of 61 candidate genes was expressed as reads per kilobase of transcript per million reads (RPKM) (see results in **Section 6.3.1**). Reads per million reads aligned (RPM) were used to create z-scores for Principal Component Analysis of large and small RNA reads (see **Section 6.3.2**). Differential expression of large and small RNA was analyzed by two-way ANOVA of raw aligned reads fit to a negative binomial model. Ensembl genes with a false discovery rate of less than 0.05 for the group:time interaction term were hierarchically clustered into four groups based on similarity of expression (see **Section 6.3.3** for details of expression). KEGG pathway analysis of the four clusters of genes was carried out using the GATHER web tool (see **Section 6.3.4**). Analysis of miRNA regulatory networks was carried out using mirConnX (see **Section 6.3.6**).

single-end reads (for small RNA). CASAVA version 1.8.2 was used to de-multiplex combined samples and generate FASTQ files, which are file formats combining nucleotide sequences with quality information. The following settings were used: the last base of each raw read was trimmed off, and reads that failed Illumina's chastity filter, meaning that the ratio of the brightest intensity to the sum of the two brightest intensities was less than 0.6 for at least two of the first 25 cycles, were removed.

Large RNA data processing and alignment to genome. Large RNA reads that passed chastity filtering were aligned to the human genome (build hg19) using TopHat2 (version 2.0.6) (Kim, Pertea et al. 2013), by setting the estimated mean and standard deviation of the insert size to -25 and 50 bases, respectively. A BED file (a tab-delimited text file with defined features) of Ensembl Gene (ENSG) loci was constructed from Ensembl build 69, by merging the unique exons from all Ensembl transcripts within each ENSG record into one metagene. This BED file included 55,841 unique ENSG loci mapping to chromosomes 1-22, X, Y and the mitochondrial chromosome. The BED file was used to determine the number of reads aligning to each Ensembl Gene (metagene) locus with the coverage utility in the BEDTools software suite (version 2.17.0) (Quinlan and Hall 2010).

Small RNA data processing and alignment to genome. Small RNA reads that passed chastity filtering were processed using the fastx-clipper tool in the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) to remove the Illumina adapter sequence (TGGAATTCTCGGGTGCCAAGG) from the 3' end of each read. Reads that were ≥ 15 nucleotides long were then aligned to hg19 using Bowtie (version 0.12.7) (Langmead, Trapnell et al. 2009), allowing up to 10 alignments and up to three mismatches per read. Reads aligning to the genome were then filtered to remove any alignments with more than one mismatch, and a

BAM (Binary Sequence Alignment/Map (SAM)) file was created using SAMtools (version 0.1.18) (Li, Handsaker et al. 2009). The number of reads aligning to each miRNA was determined using a BED file of mature miRNA loci constructed from release 19 of miRBase (Kozomara and Griffiths-Jones 2011) and the coverage utility in the BEDTools software suite (version 2.17.0) (Quinlan and Hall 2010).

6.2.2 Candidate gene analysis

We selected 61 candidate genes based on literature reports of cell type-specific expression (basal, goblet or ciliated cells), a role in transcriptional regulation of cell fate decisions (ie. SOX2, FOXA1, SPDEF), and signaling pathways involved in epithelial differentiation (notch, EGFR, TGFβ and wnt/β-catenin) (**Table 6.1).** All statistical analysis and visualization was performed using the R environment for statistical computing (version 2.15.1, available online at http://www.R-project.org/) (R Development Core Team 2011). RNA abundance was normalized to the total length (in kilobases) of all exons corresponding to each Ensembl Gene and to the number of aligned reads in each sample (in millions) to obtain reads per kilobase per million reads (RPKM) values. A two-way ANOVA was performed for each candidate gene to obtain uncorrected p-values for group, time and group:time interaction using Prism version 5.04 (GraphPad Inc., San Diego, CA). To correct for multiple comparisons, a Pearson correlation matrix of the 61 genes across all samples was created using the R command cor.test. The correlation matrix was analyzed using the Matrix Spectral Decomposition (matSpD) engine to determine the effective sample size (http://gump.qimr.edu.au/general/daleN/matSpD/, based on the methods of (Nyholt 2004)). The threshold pre-correction p-value of 0.0027 was determined according to the estimate proposed by Li and Ji (Li and Ji 2005).

6.2.3 Differential expression analysis

Differential expression of each transcript (Ensembl Gene IDs or mature microRNAs) was assessed by creating negative binomial general linearized models using the glm.nb function in the MASS package (Venables and Ripley 2002). A two-way ANOVA was performed, resulting in p-values for group, time and group:time interaction, for each feature. Features were then filtered by three separate criteria: 1) there must be at least one aligned read in at least one sample from every subject; 2) the median number of aligned reads across all samples from all subjects must be at least 20; and 3) the negative binomial model must converge for all effects in the model (group, time, and group:time interaction). These filters reduced the number of Ensembl genes to 21,876 and the number of mature microRNAs to 437. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg 1995). An FDR $q<0.05$ was considered statistically significant for this analysis.

6.2.4 Principal component analysis

Raw coverage values were normalized by the total number of aligned reads in each sample to obtain a value of aligned reads per million reads aligned (RPM), and a pseudocount of 1 was added before $\log_2$-transformation. The resulting log2(RPM+1) values were scaled by gene to a mean of zero and a standard deviation of one across all samples to obtain z-scores for the 21,876 Ensembl genes and 437 miRNAs. Principal Component Analysis (PCA) was performed on these z-scores using the 'prcomp' function in the R software.

6.2.5 Hierarchical clustering

Z-scores were trimmed to a minimum of -2 and a maximum of +2. The 1564 Ensembl genes with a $q<0.05$ in the group:time interaction term were then partitioned into the four main clusters of genes showing similar changes in gene expression during differentiation using hierarchical clustering of the trimmed z-score values with the complete linkage method and Euclidean inter-gene distances. A heatmap was constructed using R software to visualize these clusters.

6.2.6 Pathway analysis

To assess the biological significance of identified genes, we determined if the genes identified were linked to specific cellular functions using the online tool GATHER (Gene Annotation Tool to Help Explain Relationships; gather.genome.duke.edu) (Chang and Nevins 2006). We considered a KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway or Gene Ontology (GO) significantly enriched if it had a Bayes factor of ≥ 3 and a FDR less than 0.05.

6.2.7 miRNA-mRNA interactions

To identify significant mRNA-miRNA relationships during differentiation, the miRConnX web server was used (Huang, Athanassiou et al. 2011) with the following settings: reads per million reads aligned (RPM) for all mRNAs (21876 Ensembl IDs) and miRNAs (437 accession IDs) that passed filtering criteria, prior network weight of 0.3, Pearson correlation, and a regulation strength threshold of 0.95. MirConnX calculates miRNA-mRNA correlation strengths and then fits these undirected interactions to a directed network based upon prior knowledge of transcription factor binding, miRNA target prediction from several algorithms, the species of interest, and evidence from the literature. Mature miRNAs, including -3p and -5p forms, and

genomic duplicates were collapsed by mirConnX into a single hub by taking the median value of all isoforms. Networks were downloaded in XGMML format and visualized in Cytoscape 3.0.2 (Kohl, Wiese et al. 2011).

6.3 Results

6.3.1 Analysis of candidate developmental genes

For our initial analysis of gene transcription profiles inducing mucociliary differentiation in our ALI cultures, we selected 61 candidate genes. The candidates were selected based on literature reports of cell type-specific expression (basal, goblet or ciliated cells), a role in transcriptional regulation of cell fate decisions (ie. SOX2, FOXA1, SPDEF), and signaling pathways involved in epithelial differentiation (notch, EGFR, TGFβ and wnt/β-catenin) (**Table 6.1).** As many of these genes are expected to be co-expressed during differentiation, a Bonferroni correction would likely result in false negatives. To address this issue, a correlation matrix of the 61 genes was created (**Appendix A.1**) and analyzed using the Matrix Spectral Decomposition (matSpD) engine (http://gump.qimr.edu.au/general/ daleN/matSpD/). According to the estimate proposed by Li and Ji (Li and Ji 2005), the effective sample size was determined to be 20, and therefore a pre-correction p-value of$\leq$0.0026 was considered statistically significant for this experiment. This value represents a corrected p-value threshold of 0.05.

(Following pages)
Table 6.1 Analysis of expression of candidate genes during mucociliary differentiation *in vitro*.
$ Log_2(day20/day0): Log_2(fold change) of reads per million (RPM) in the control group only. Log_2 fold changes with a positive value indicate upregulation during differentiation, while negative values indicate downregulation. A log_2 value of ± 1 indicates a 2-fold change.
† P-values ≤ 0.0026 are considered to be statistically significant (**bold**), which equates to a corrected p-value of less than 0.05 based on calculation of effective sample size.

Gene	Description	Log_2(day 20/day0)$^{\$}$	Two-way ANOVA p-values†		
			Group	Time	Group:Time
Basal cell					
CD109	CD109 molecule	-1.8	0.85	**0.0001**	0.09
ITGA6	integrin, alpha 6	-1.2	0.58	**0.0001**	0.02
KRT5	keratin 5	-0.8	0.93	**0.0001**	0.14
ΔNp63	tumor protein p63	-1.4	0.42	**0.0001**	0.35
Goblet cell					
MUC1	mucin 1, cell surface associated	1.1	0.36	**0.0001**	0.75
MUC5AC	mucin 5AC, oligomeric mucus/gel-forming	10.8	0.31	**0.0001**	0.11
MUC5B	mucin 5B, oligomeric mucus/gel-forming	11.7	1.00	**0.0001**	0.50
RAB27A	RAB27A, member RAS oncogene family	0.1	0.46	**0.0001**	0.02
TFF1	trefoil factor 1	3.0	0.21	**0.001**	0.32
TFF3	trefoil factor 3	7.2	0.89	**0.0001**	0.99
Ciliated cell					
DNAH9	dynein, axonemal, heavy chain 9	11.2	0.48	**0.0001**	0.45
DNAI1	dynein, axonemal, intermediate chain 1	14.5	0.48	**0.0001**	0.67
FOXJ1	forkhead box J1	10.4	0.35	**0.0001**	0.43
MCIN	multiciliate cell differentiation 1	4.5	0.28	**0.0001**	0.45
MYB	v-myb myeloblastosis viral oncogene homolog (avian)	4.0	0.73	**0.0001**	0.87
RFX3	regulatory factor X, 3 (influences HLA class II expression)	2.4	0.73	**0.0001**	0.84
SPAG8	sperm associated antigen 8	5.5	0.26	**0.0001**	0.26
TEKT1	tektin 1	13.8	0.73	**0.0001**	0.97
Notch pathway					
DLK2	delta-like 2 homolog (Drosophila)	0.8	0.24	**0.0001**	0.08
DLL1	delta-like 1 (Drosophila)	0.9	0.66	**0.0001**	0.09
JAG1	jagged 1	-2.1	0.21	**0.0001**	0.68
JAG2	jagged 2	0.6	0.54	**0.0001**	0.01
NOTCH1	notch 1	-1.9	0.98	**0.0001**	0.01
NOTCH2	notch 2	-0.3	0.91	**0.001**	0.66
NOTCH3	notch 3	-1.1	0.61	**0.0001**	0.20
Transcriptional regulators of cell fate					
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	0.9	0.11	**0.0001**	0.01
FOXA1	forkhead box A1	0.3	0.97	**0.0001**	0.68
FOXA2	forkhead box A2	-1.4	0.19	**0.0001**	0.56
FOXA3	forkhead box A3	7.9	0.65	**0.0001**	0.94
FOXM1	forkhead box M1	-2.4	0.17	**0.0001**	0.16
KLF5	Kruppel-like factor 5 (intestinal)	0.1	0.55	**0.0001**	0.35

Gene	Description	Log_2(day 20/day0)$^{\$}$	Two-way ANOVA p-values†		
			Group	Time	Group:Time
NKX2-1	NK2 homeobox 1	0.7	0.35	**0.0001**	0.06
SOX2	SRY (sex determining region Y)-box 2	2.9	0.15	**0.0001**	0.05
SOX9	SRY (sex determining region Y)-box 9	-2.1	0.69	**0.0001**	**0.0003**
SPDEF	SAM pointed domain containing ets transcription factor	1.6	0.16	**0.0001**	0.51
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	0.5	0.19	**0.0001**	**0.001**
EGFR family					
AREG	amphiregulin	-2.70	0.10	**0.0001**	0.004
EGF	epidermal growth factor	2.13	0.72	**0.0001**	0.57
EGFR	epidermal growth factor receptor	-2.00	0.08	**0.0001**	**0.0015**
EREG	epiregulin	-3.50	0.69	**0.0001**	0.41
HBEGF	heparin-binding EGF-like growth factor	-2.03	0.19	**0.0001**	0.014
TGFβ family					
BMP2	bone morphogenetic protein 2	-2.24	0.35	**0.0001**	0.02
BMP4	bone morphogenetic protein 4	3.00	0.89	**0.0001**	0.64
BMP7	bone morphogenetic protein 7	3.76	0.56	**0.0001**	0.63
BMPR2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	-0.88	0.92	**0.0001**	0.003
INHBA	inhibin, beta A	-2.45	0.33	**0.0001**	0.15
SMAD3	SMAD family member 3	-0.79	0.45	**0.0001**	0.36
TGFB1	transforming growth factor, beta 1	-2.17	0.84	**0.0001**	0.01
TGFB2	transforming growth factor, beta 2	-0.43	0.53	0.0847	0.14
TGFBR1	transforming growth factor, beta receptor 1	-1.33	0.13	**0.0001**	**0.0001**
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	-0.57	0.53	**0.0001**	0.39
Wnt family					
AXIN2	axin 2	5.35	0.26	**0.0001**	0.08
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	-0.23	0.49	**0.0001**	0.27
DKK1	dickkopf 1 homolog (Xenopus laevis)	-2.11	0.14	**0.0001**	0.03
DKK3	dickkopf 3 homolog (Xenopus laevis)	-1.61	0.33	**0.0001**	0.68
FRAT1	frequently rearranged in advanced T-cell lymphomas	2.93	0.08	**0.0001**	**0.001**
FZD10	frizzled family receptor 10	0.46	0.31	**0.0001**	0.04
GATA6	GATA binding protein 6	-1.28	0.55	**0.0001**	**0.001**
GSK3B	glycogen synthase kinase 3 beta	-0.81	1.00	**0.0001**	0.01
SFRP2	secreted frizzled-related protein 2	5.17	0.46	**0.0001**	0.59
WNT4	wingless-type MMTV integration site family, member 4	0.96	0.14	**0.0001**	0.07

We reported a striking defect in ciliogenesis in both EIB(-) and EIB(+) cultures in **Chapter 5**, and thus we queried several genes related to ciliary function and ciliogenesis. These included structural components of the dynein motor (DNAH9, DNAI1), cytoskeletal proteins tektin (TEKT1) and sperm associated antigen (SPAG8), as well as transcription factors involved in ciliogenesis (FOXJ1, MCIN, MYB, RFX3). As expected, expression of all of the genes in this category increased throughout differentiation. Although none passed the threshold p-value for group:time interaction, the expression at day 20 of FOXJ1 (**Figure 6.3A**), DNAI1 (**Figure 6.3B**), and SPAG8 (**Figure 6.3C**) were significantly correlated with the percentage of ciliated cells at the apical surface as observed by histology in **Chapter 5**. These correlation values (r^2 <0.55) suggest that there are additional regulators of ciliogenesis beyond these three candidate genes.

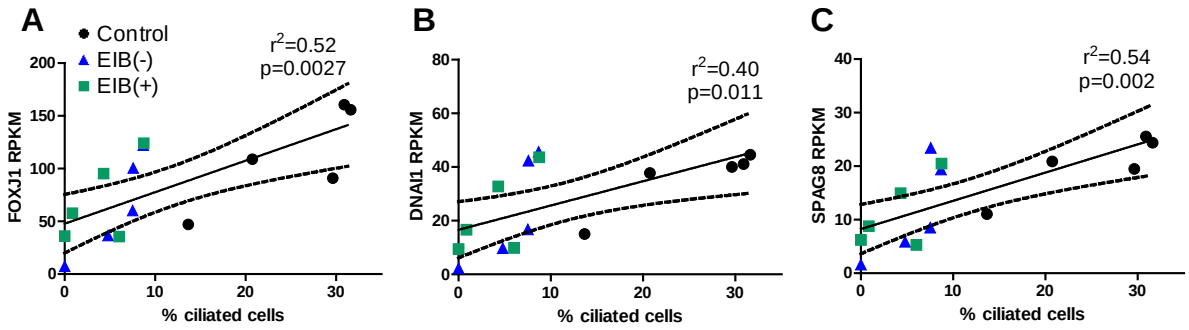


Figure 6.3 Cilia-related gene expression is correlated with the percentage of ciliated cells.
Expression of **(A)** FOXJ1, **(B)** DNAI1 and **(C)** SPAG8 expressed in reads per kilobase of transcript per million reads (RPKM) at day 20 was correlated with percentage of ciliated cells on the apical surface of ALI cultures as determined by histology in **Chapter 5**. Solid lines indicate linear regression across all samples, while dashed lines indicate 95% confidence intervals. The correlation measure r^2 and the p-value are indicated for each graph. Symbols denote donor disease group; black circles – control, blue triangles – EIB(-), green squares – EIB(+).

While we found increased numbers of cytokeratin-5-expressing basal cells in EIB(-) cultures by immunohistochemistry (**Chapter 5**), we found no difference between groups for basal cell-restricted genes, including CD109, ΔNp63, CK-5 (KRT5) and integrin α6 (ITGA6) (Dvorak, Tilley et al. 2011). Basal cell genes decreased during differentiation, as would be expected as basal cells constitute a smaller proportion of the total cell population in differentiated compared to monolayer cultures. As expected, the expression of goblet cell-specific genes increased over time in ALI culture, including mucins (MUC5AC, MUC5B, MUC1), mucin-associated secretory peptides (TFF1 and TFF3) and a GTPase involved in trafficking of secretory granules (RAB27A) (Dvorak, Tilley et al. 2011). In line with our histology findings in **Chapter 5**, none of these goblet cell-specific genes were differentially expressed between disease groups during differentiation.

Expression of transcriptional regulators of cell fate, and the notch, TGFβ, EGFR and wnt/β-catenin families changed significantly during *in vitro* differentiation of airway epithelial cells from healthy donors. Several of these genes were aberrantly expressed in our asthmatic-derived ALI cultures at discrete timepoints, with TGFBR1, EGFR and GATA6 being significantly downregulated in asthmatic donors at day 0 (**Figure 6.4A**), while FRAT1 and STAT6 expression was significantly lower in asthmatic-derived ALI cultures at day 20 (**Figure 6.4B**).

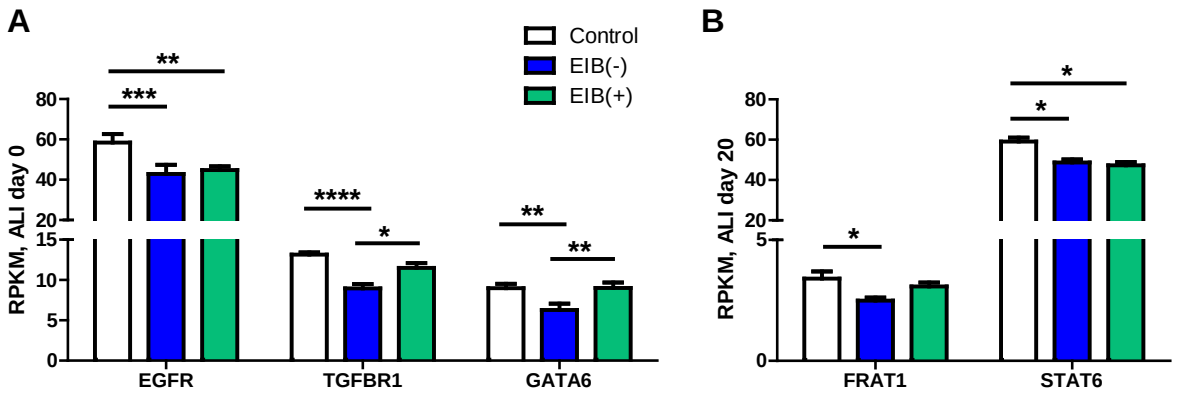


Figure 6.4 Developmental genes are differentially expressed between disease subgroups in ALI cultures

(A) Expression of EGFR, TGFBR1, and GATA6 was significantly lower in asthmatic subgroups at day 0, while **(B)** FRAT1 and STAT6 were significantly decreased in asthmatic-derived ALI cultures at day 20. Data are shown as mean ± SEM of reads per kilobase of transcript per million reads (RPKM). Two-way ANOVA with Bonferroni post-test was used to assess statistical significance of each gene during differentiation, with the following p-values: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

In this candidate gene analysis we were able to replicate several candidate molecules previously described to be involved in mucociliary differentiation, and further demonstrate that their expression is altered in asthmatic-derived epithelium. However, these analyses do not capture the full transcriptional profile and the regulatory mechanisms underlying aberrant differentiation in EIB(-) and EIB(+) asthmatic-derived ALI cultures. The subsequent results therefore focus on a global analysis of gene expression to identify genes and biological pathways that are differentially-expressed during differentiation of ALI cultures derived from controls and EIB(-) and EIB(+) asthmatic subjects.

6.3.2 Global analysis of RNA sequencing

Sequencing of large RNAs from ALI cultures detected reads that mapped to more than 55,000 unique genomic regions, while the sequencing of small RNAs detected reads that mapped to 1319 miRNAs. After excluding genes that displayed either very low expression (median counts <20), zero counts in all timepoints from any one donor, and those that were unable to be fit to the negative binomial model used for differential gene expression analysis, we detected 21,876 unique Ensembl gene identities and 437 mature miRNAs. For a global overview of the variance throughout ALI differentiation and between donors, we performed principal components analysis (PCA). Visualization of mRNAs that passed filtering criteria indicated that undifferentiated ALI (day 0, red) samples were clearly segregated from differentiated ALI samples (days 5-20) along PC1, which together with PC2 accounted for 46% of the variance in our samples (**Figure 6.5A**). This segregation is likely due to the homogeneous basal cell phenotype at day 0. At day 5, samples appeared to group together (green), but samples at days 11 (blue) and 20 (purple) largely overlapped, possibly owing to different rates of differentiation in individual ALI cultures. In contrast, the PCA for miRNAs demonstrated clear segregation for each differentiation timepoint, suggesting that distinct miRNA regulatory processes are activated throughout mucociliary differentiation (**Figure 6.5B**). The PCA analysis was not able to distinguish mRNA or miRNA expression between control (circles), EIB (-, triangles) or EIB (+, squares) asthmatic-derived ALI cultures at any of the differentiation timepoints.

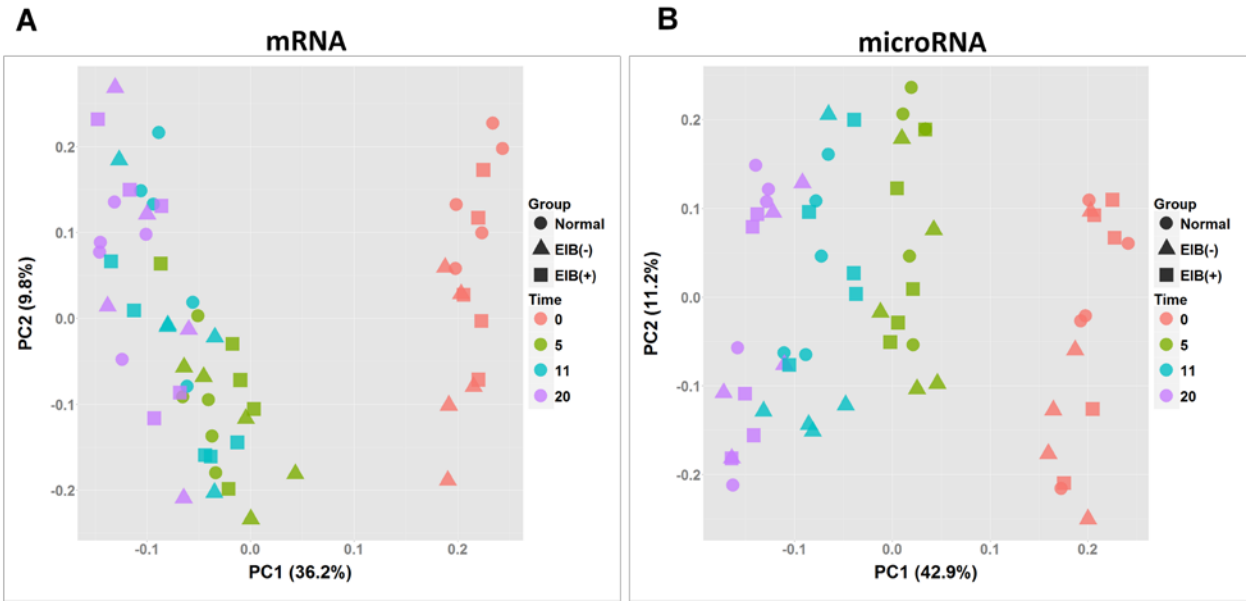


Figure 6.5 Principal component analysis of mRNA and miRNA in all air-liquid interface cultures. PCA was used to assess variance of **(A)** mRNA and **(B)** miRNA between samples, using reads per million (RPM) of all features that passed filtering criteria. Donor groups are differentiated by symbol, and timepoints by colour. X-axes are principal component (PC) 1, y-axes PC2, and the contribution to overall variance is given as a percentage in brackets.

6.3.3 Differential gene expression during ALI culture from asthmatic donors

To assess differential gene expression differences between groups (group), over time (time) as well as the interaction of the two terms (group:time interaction), we used two-way ANOVA and a Benjamini-Hochberg FDR corrected p-value (q) less than 0.05 was considered statistically significant (**Table 6.2**). As expected, the majority of genes (18,527 of 21,876) changed significantly over time over 20 days of ALI differentiation, which is reflective of the large number of transcriptional changes that occur during mucociliary differentiation. Between groups, 2561 genes were differentially expressed regardless of time, indicating that these genes are either unrelated to the differentiation process or are persistently different between groups. Finally, 1564 genes were differentially expressed between groups during differentiation of epithelial cells

(group:time interaction). As we were mostly interested in the mechanisms underlying aberrant epithelial differentiation in asthma, these 1564 genes were selected for further analysis.

	Total genes	Two-way ANOVA term	FDR q<0.05
mRNA	21,876	Group	2561
		Time	18,527
		Group:Time interaction	**1564**

Table 6.2 Overview of differentially-expressed genes for each analysis term.

To separate the 1564 differentially expressed genes into distinct gene expression patterns, hierarchical clustering was performed using the complete linkage method with Euclidean intergene distances, which separated the genes into four distinct cultures (see heatmap in **Figure 6.6**). To better visualize the expression kinetics within each cluster, the three most significant genes by group:time FDR q-value are shown graphically in **Figure 6.7**. In cluster 1 (demarked in red), all 390 RNAs decreased in expression in control cultures during differentiation and were downregulated in EIB(-) cultures at day 0. Of the top three significant mRNAs in this cluster, C5orf51 is a validated protein coding gene with no known function, ITCH is an E3 ubiquitin ligase that targets proteins for degradation, and AIDA is a scaffold protein that has a similar structure to focal adhesion-associated proteins (Zhang and Aravind 2010), and tethers proteins, such as those involved in the Wnt signaling cascade, to microtubules (Rui, Xu et al. 2007). Interestingly, two genes from our candidate gene analysis, BMPR2 (bone morphogenetic protein receptor, type II) and TGFBR1 (transforming growth factor beta receptor, type 1) were also present in cluster 1, and followed similar kinetics.

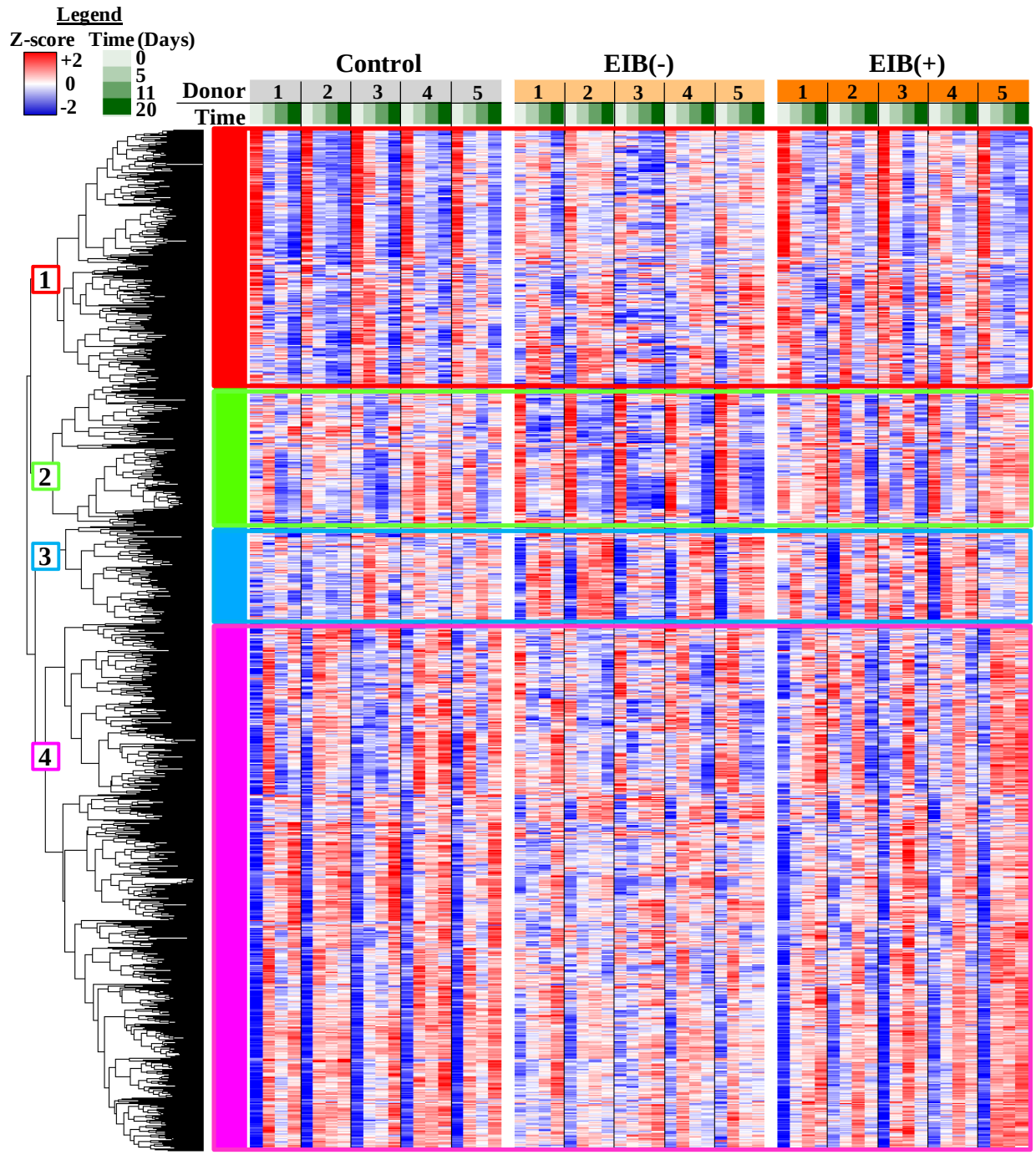
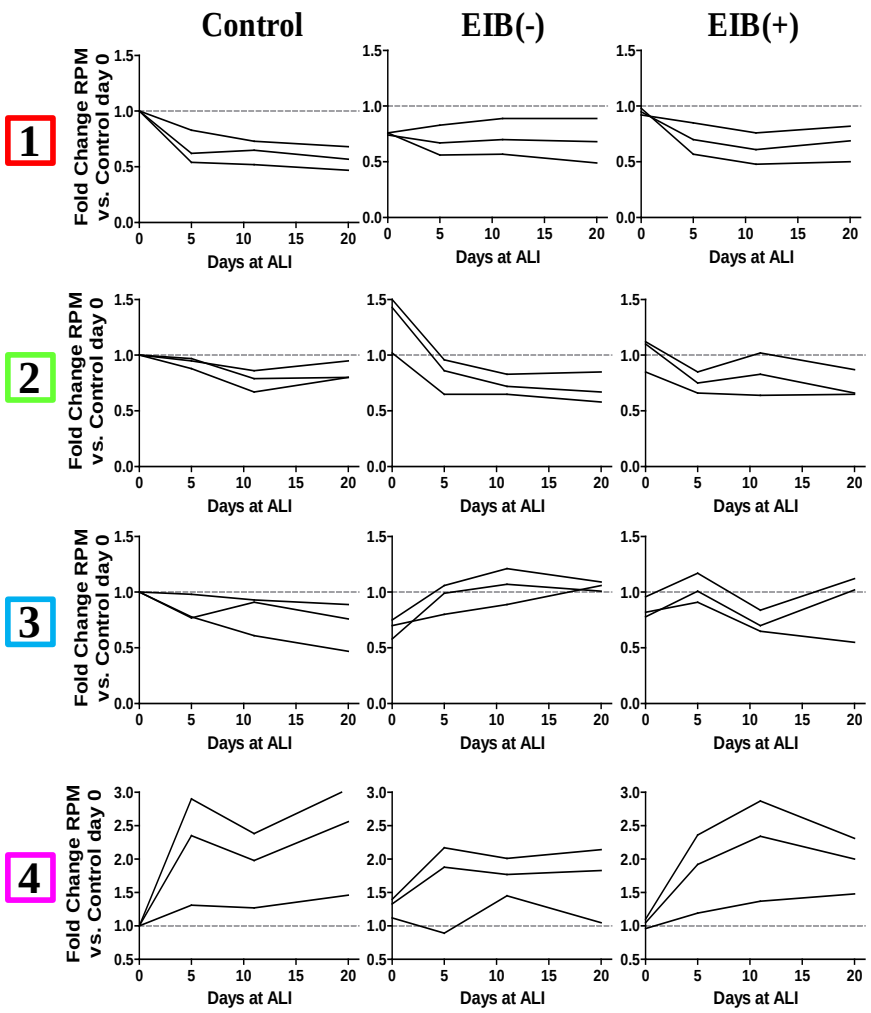


Figure 6.6 Hierarchical clustering of differentially-expressed genes into four groups.
The 1564 differentially-expressed genes by group:time analysis were hierarchically clustered into four groups as indicated by numbered boxes on the dendrogram at left. Each row of the heatmap represents one gene, while each column represents one sample. Red indicates expression (Z-score) above the median expression for a given gene, and blue indicates expression below the median.

Cluster 2 (demarked in green) contained 218 genes that also decreased during differentiation in the control group, but with lowest expression at day 11 (**Figure 6.7**). Expression of cluster 2 genes was elevated at day 0 in the EIB(-) group, and followed a biphasic expression pattern in EIB(+) cultures, with lowest expression at days 0 and 11. The two most significant RNAs identified were the pseudogenes AC067950.1 (also known as ribosomal protein S20 pseudogene 10; RPS20P10) and FAM207BP, while the third, DDX41, is a putative RNA helicase with unknown function.

Of the 148 genes in cluster 3 (demarked in blue), expression of these RNAs was lowest at day 0, but increased during differentiation in EIB(-) cultures and was biphasic in expression in EIB(+) cultures, with peaks at days 5 and 20. The most significant gene was CEACAMP3 (carcinoembryonic antigen-related cell adhesion molecule pseudogene 3), a pseudogene of the CEACAM group, which in general encodes human glycoproteins related to immunoglobulins (Kuespert, Pils et al. 2006). CTC-366B18.2 is a novel antisense gene with no known function, while DNAJB14 is a validated, protein coding gene that may be enriched in ciliated cells (McClintock, Glasser et al. 2008).

Finally, cluster 4 (demarked in pink) was the largest group, with 808 genes that followed a biphasic expression pattern in the control group, characterized by peaks at days 5 and 20. This expression pattern was dampened in the EIB(-) group, and was notably delayed in the EIB(+) group. The top three genes in this pathway were the protein-coding genes SULT6B1 (sulfotransferase family, cytosolic, 6B, member 1), ZNF428 (zinc finger protein 428), and SRRM5 (serine/arginine repetitive matrix 5). The latter two genes arise from overlapping loci on chromosome 19, with ZNF428 on the reverse strand and SRRM5 on the forward strand. Several genes from our candidate list were also present in cluster 4, including the goblet cell-specific



Symbol	Description	Group:Time FDR q
C5orf51	chromosome 5 open reading frame 51	**$5.2x10^{-4}$**
ITCH	itchy E3 ubiquitin protein ligase	**$8.1x10^{-4}$**
AIDA	axin interactor, dorsalization associated	**$8.1x10^{-4}$**
AC067950.1	*ribosomal protein S20 pseudogene 10	**$<1x10^{-16}$**
FAM207BP	family with sequence similarity 207, member B, pseudogene	**$3.5x10^{-3}$**
DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	**$3.6x10^{-3}$**
CEACAMP3	carcinoembryonic antigen-related cell adhesion molecule pseudogene 3	**$<1x10^{-16}$**
CTC-366B18.2	*novel antisense gene	**$8.8x10^{-4}$**
DNAJB14	DnaJ (Hsp40) homolog, subfamily B, member 14	**$3.5x10^{-3}$**
SULT6B1	sulfotransferase family, cytosolic, 6B, member 1	**$1.7x10^{-5}$**
ZNF428	zinc finger protein 428	**$1.4x10^{-4}$**
SRRM5	serine/arginine repetitive matrix 5	**$2.3x10^{-4}$**

Figure 6.7 Expression kinetics of the three most significant genes in each cluster.
The top three genes in each cluster were identified by group:time FDR q-value and were plotted as fold change vs. control day 0 (shown as dashed horizontal line). Gene symbol, gene description and the Group:Time FDR q value for the selected genes are shown at right. *Indicates gene descriptions that were obtained from Ensembl.org.

gene MUC5B, the wnt family member FRAT1, and ciliated cell-associated genes DNAI1, DNAH9 and TEKT1. Additionally, several other ciliated-cell related genes were identified, including DNAH17 (dynein, axonemal, heavy chain 17), KLC4 (kinesin light chain 4), CROCCP3 (ciliary rootlet coiled-coil, rootletin pseudogene 3) and GAPDHS (GAPDH, spermatogenic). Expression of these ciliary genes increased dramatically during differentiation in control cultures, but all were significantly decreased in EIB(-) and EIB(+) cultures at day 20 (**Appendix A.2)**.

6.3.4 Metabolic pathways are differentially expressed in asthmatic ALI cultures

To determine the biological significance of these 1564 genes, we used the web-based program GATHER (Chang and Nevins 2006)) to identify KEGG pathways that were enriched in each gene cluster (**Table 6.3**). Again to better visualize the expression kinetics of RNAs within each cluster, all genes identified by the GATHER analysis for group:time FDR q-value are also shown graphically in **Figure 6.8**.

In cluster 1 (red), the KEGG pathways involved in regulation of actin cytoskeleton, tight junction, and focal adhesion were identified, all of which are known to be dynamically modulated during cellular reorganization, polarization and differentiation. These genes decreased during differentiation in control cultures, but were decreased at day 0 in the EIB(-) asthmatic group.

Cluster 3 (blue) was significantly enriched for the insulin signaling and mitogen activated protein kinase (MAPK) signaling pathways. Genes in these signaling pathways did not change during differentiation in control cultures, but in both asthmatic groups there was decreased

Cluster	Total # genes	KEGG Pathway Annotation	# genes in pathway	Bayes factor	FDR
1	390	hsa04810: Regulation of actin cytoskeleton	12	69	0.016
		hsa04530: Tight junction	8	24	0.022
		path:hsa04510: Focal adhesion	11	9	0.045
2	218	hsa00190: Oxidative phosphorylation	4	26	*0.052*
3	148	hsa04910: Insulin signaling pathway	6	270	0.0041
		hsa04010: MAPK signaling pathway	6	18	0.033
4	808	hsa00190: Oxidative phosphorylation	18	$1x10^{7}$	$8.5x10^{-8}$
		hsa00240: Pyrimidine metabolism	12	9228	$5.1x10^{-5}$
		path:hsa00230: Purine metabolism	11	14	0.025

Table 6.3 KEGG pathways enriched in each cluster of differentially-expressed genes.

expression at day 0 and a general increase in expression during differentiation (**Figure 6.8**). In addition, EIB(+) cultures showed markedly biphasic gene expression.

Finally, cluster 4 (pink) was significantly enriched for three pathways, including oxidative phosphorylation, pyrimidine metabolism and purine metabolism, which were significantly different in expression between disease groups (**Figure 6.8**). Genes identified in the oxidative phosphorylation pathway encoded protein subunits of the electron transport chain, including cytochrome C oxidase (COX) and NADH dehydrogenase (NDUF), suggesting a robust effect on cellular energy (ATP) production. Interestingly, the top-ranked KEGG pathway in cluster 2 (green) was also oxidative phosphorylation and included four additional genes related to cellular energy production, although this did not reach the statistical cutoff of FDR<0.05 (denoted with italics in **Table 6.3**). The altered expression of mitochondrial metabolic

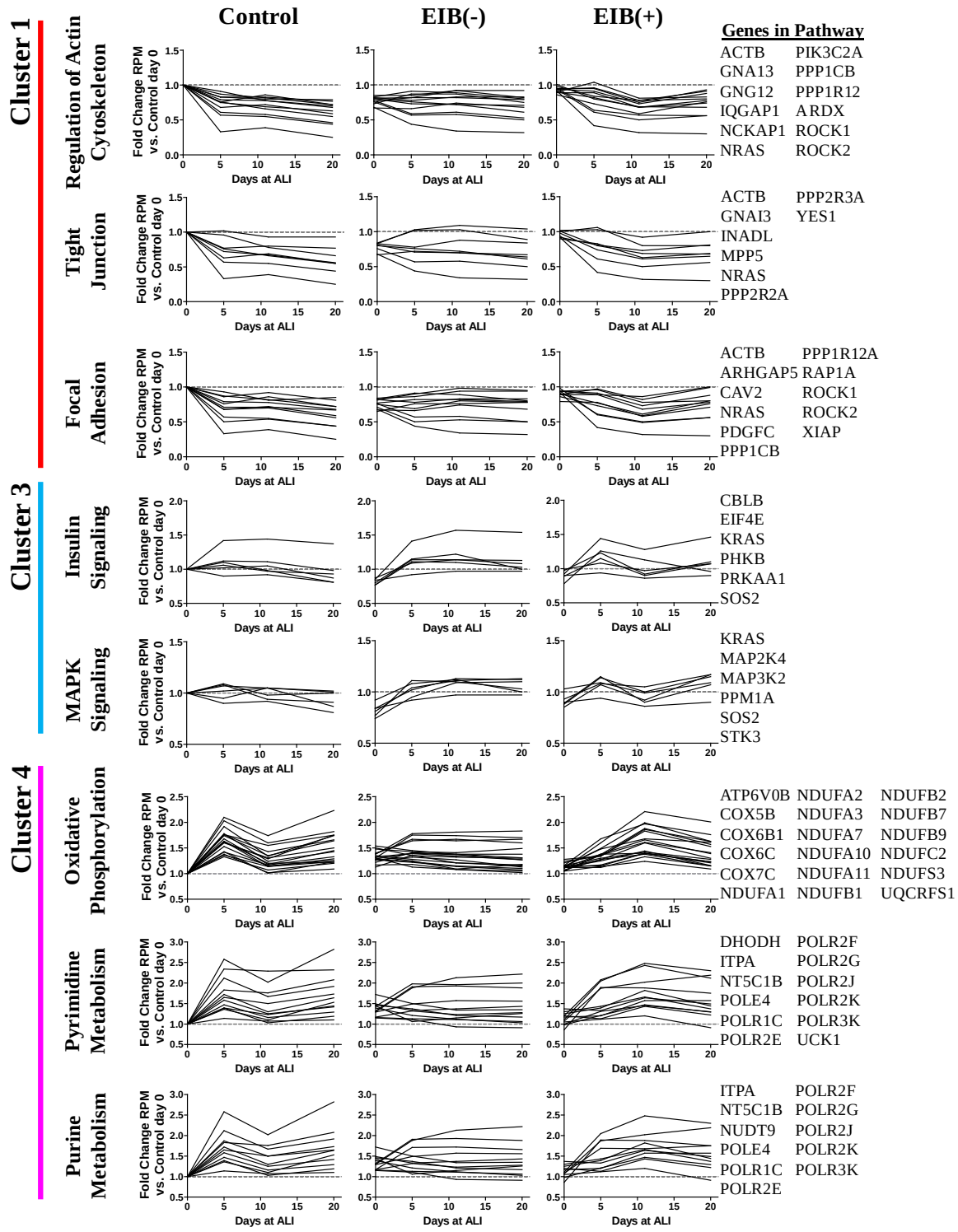


Figure 6.8 Expression kinetics of differentially-expressed KEGG pathways.
Kinetics of pathway expression in each cluster are expressed as mean fold change compared to the mean RPM in control day 0 (dashed horizontal line) for control (left column), EIB(-) (middle column) and EIB(+) (right column) groups. Genes in each pathway are listed at right.

subunits does not appear to reflect a decreased number of mitochondria in asthma, as expression of voltage-dependent anion channel 1 (VDAC1), a surrogate marker of mitochondrial mass, did not differ between groups (data not shown). The pyrimidine and purine metabolism pathways in cluster 4 contained several common genes, the majority of which encode subunits of RNA polymerases (POLR). The genes in these three identified pathways followed remarkably similar kinetics, however while the control group had biphasic expression, with peaks at days 5 and 20, there was a complete lack of induction in EIB(-) cultures, and delayed induction, until day 11, in EIB(+) cultures. Overall, the identified pathways suggest distinct metabolic and transcriptional abnormalities in the EIB(-) and EIB(+) subgroups compared to control cultures during mucociliary differentiation.

6.3.5 Differential expression of microRNA during mucociliary differentiation

MicroRNAs represent an important endogenous mechanism for post-transcriptional regulation of mRNA function, and can either suppress or activate a given target (Vasudevan, Tong et al. 2007). We first queried the expression of two candidate miRNA molecules, miR-449a and miR-203a. We found that expression of miR-449a increased during mucociliary differentiation, (**Appendix A.3A**), while miR-203 expression decreased during differentiation (**Appendix A.3B**). Neither miRNA differed significantly in expression between donor groups.

To identify potential mechanisms underlying the differential RNA expression in asthmatic-derived ALI cultures, we assessed global expression of miRNAs. We again used a negative binomial model with two-way ANOVA to assess differential gene expression between groups (group), over time in culture (time) and the interaction of the two terms (group:time interaction) (**Table 6.4**). As expected, most miRNAs (382 of 437) changed significantly over

time in ALI culture, and we found 62 miRNAs that were differentially expressed between groups, regardless of time. However, we did not find any miRNAs that were significant in the group:time interaction analysis by FDR q-value.

	Total miRNAs	Two-way ANOVA term	FDR q<0.05
miRNA	437	Group	62
		Time	382
		Group:Time Interaction	**0**

Table 6.4 Overview of differentially-expressed miRNAs for each analysis term.

6.3.6 MiRNA regulation of mucociliary differentiation *in vitro*

As no miRNAs were significantly different during differentiation between groups, we next proposed to identify key miRNA regulators of mucociliary differentiation in our ALI samples. We used the web application mirConnX (Huang, Athanassiou et al. 2011) to find correlations between all 21,876 genes and 437 miRNAs in all samples across all timepoints. Within our data we identified a network of 34 miRNA hubs, which demonstrated interactions with 990 genes and 20 transcription factors, for a total of 1603 interactions. In order to address the question of which miRNAs were contributing to the process of differentiation in airway epithelial cells, we focused our attention on those miRNAs that increased or decreased in expression by ≥ 2 -fold between days 0 and 20. Of the miRNAs that passed this cut-off we found 12 miRNA hubs that decreased in expression (coloured blue in **Figure 6.9**) and 8 that increased in expression (coloured red in **Figure 6.10**; see **Appendix A.4** for the complete list of miRNA hubs).

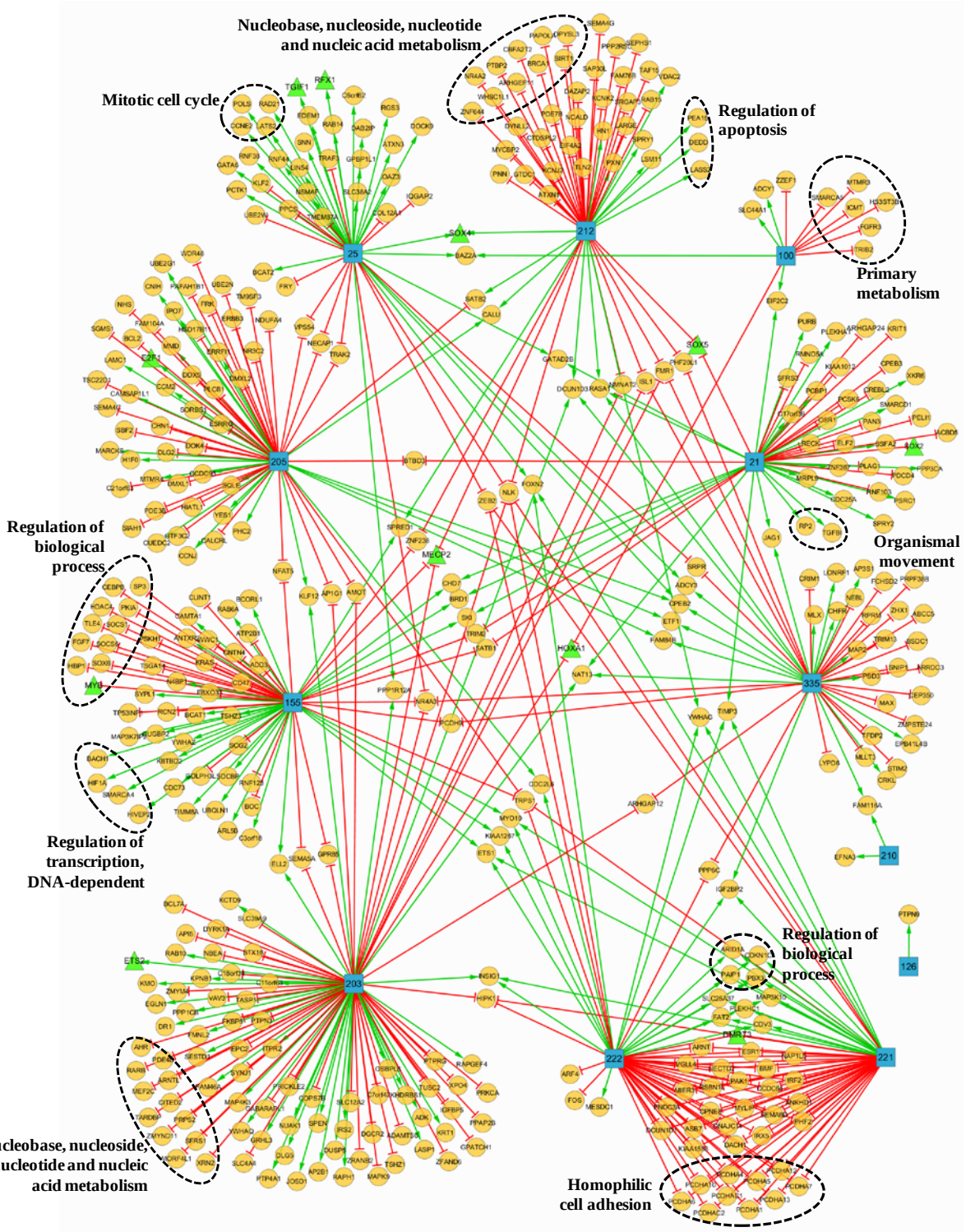


Figure 6.9 Regulatory networks of miRNAs that decreased by ≥2-fold during differentiation.
MiRNA hubs are shown as blue squares, transcription factors as green triangles, and genes as yellow circles. Red lines indicate negative regulation (suppression), while green lines indicate positive regulation (activation) of targets. Targets that were enriched for specific gene ontologies are shown in dashed circles and labeled accordingly.

To determine the biological relevance of each of these miRNA hubs, we identified gene ontologies enriched in the target networks using the GATHER web tool (Chang and Nevins 2006). MiRNAs that were more highly expressed in monolayer culture activated targets belonging to gene ontologies such as mitotic cell cycle (miR-25), apoptosis (miR-212), organismal movement (miR-21), transcription (miR-155) and biological processes (miR-221 and miR-222), but suppressed targets such as nucleic acid metabolism (miR-212 and miR-203), homophilic cell adhesion (miR-221 and miR-222), regulation of biological process (miR-155) and primary metabolism (miR-100) (**Figure 6.9** and **Appendix A.5**). In contrast, miRNAs that were more highly expressed in differentiated cultures suppressed proliferation-related gene ontologies such as mitotic cell cycle (miR-32), cell proliferation (miR-141) and regulation of physiological process (miR-200a), and activated targets involved in regulation of transcription (miR-32 and miR-141) and RNA splicing (miR-190b) (**Figure 6.10** and **Appendix A.6**). Taken together, these findings suggest that multiple, temporally-regulated miRNAs act together to coordinate the transition from a proliferative, submerged culture to a metabolically active, pseudostratified epithelium.

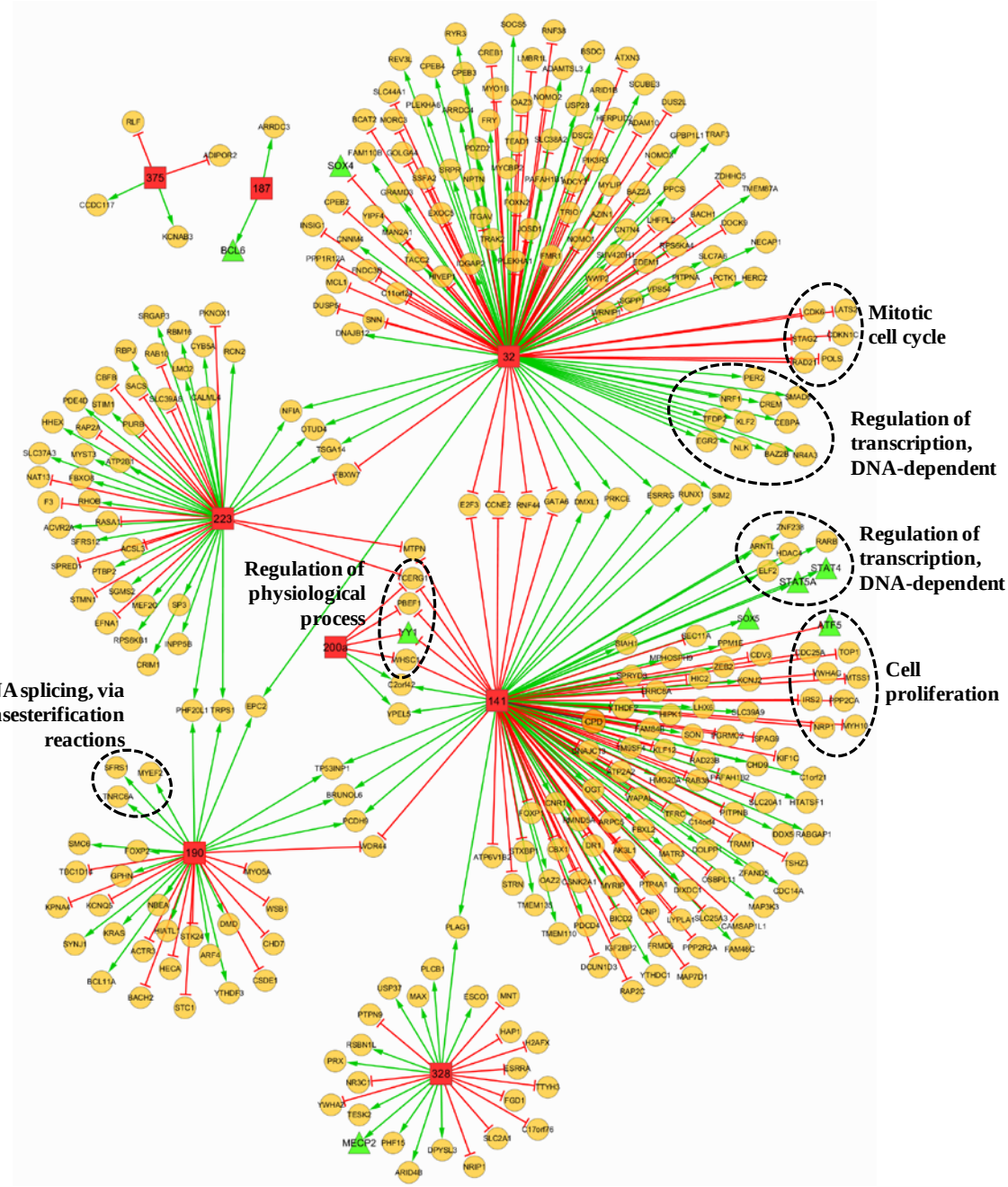


Figure 6.10 Regulatory networks of miRNAs that increased by ≥2-fold during differentiation.
MiRNA hubs are shown as red squares, transcription factors as green triangles, and genes as yellow circles. Red lines indicate negative regulation (suppression), while green lines indicate positive regulation (activation) of targets. Targets that were enriched for specific gene ontologies are shown in dashed circles and labeled accordingly.

6.4 Discussion

In this study we describe the first comprehensive analysis of mRNA and miRNA expression at multiple timepoints during mucociliary differentiation of airway epithelial cells derived from healthy and asthmatic donors with two clinical disease phenotypes, based on exercise-induced bronchoconstriction (EIB) status. Specifically, we identified a signature of 1564 genes that are differentially expressed between donor groups during differentiation. Interestingly, among these genes, oxidative phosphorylation and transcription-related metabolic pathways were strongly induced in control cultures during ALI differentiation, but were completely absent in EIB(-) cultures, and delayed in EIB(+) cultures. In addition, actin cytoskeleton and junction-related pathways were notably decreased in EIB(-) cultures at day 0, as were insulin and MAPK signaling pathways. We also identified multiple miRNA hubs that regulate gene expression related to the transition between a proliferative, monolayer state and a metabolically active state in the differentiated epithelium. Overall, we have identified distinct RNA expression profiles in airway epithelial cells from two asthmatic phenotypes, which may underpin the aberrant differentiation observed in disease.

Our finding that expression of >80% of all genes and miRNAs changed significantly during differentiation is supported by previous studies showing clear separation of differentiated epithelium and submerged monolayers of basal cells (Ross, Dailey et al. 2007, Hackett, Shaykhiev et al. 2011). Basal cells are crucial for epithelial differentiation, as they act as key progenitor cells of the airway epithelium (Hackett, Shaheen et al. 2008, Rock, Onaitis et al. 2009). The basal cell transcriptome includes multiple extracellular matrix and adhesive proteins required for basal cells to maintain strong contacts with the basement membrane and with other cells (Hackett, Shaykhiev et al. 2011). The ability to form adherens and tight junctions, as well as

the ability to reorganize the actin cytoskeleton, is necessary for the polarization of epithelial cells transitioning from a squamous phenotype to a columnar phenotype during differentiation (Mege, Gavard et al. 2006). In asthmatic-derived epithelial cells, and in particular those from EIB(-) donors, we found multiple actin cytoskeleton-related pathways to be downregulated in submerged culture (day 0). Exposure of basal epithelial cells to air at the onset of mucociliary differentiation appears to initiate transcriptional events associated with a cellular repair response (Ross, Dailey et al. 2007), suggesting that an inability of basal cells to respond to extracellular stressors may underlie the failure of the asthmatic epithelium to differentiate. In agreement with this, we also found expression of genes in the insulin and MAPK signaling pathways to be decreased in asthmatic-derived epithelial cells at day 0, suggesting impaired signal transduction in response to growth factors. Furthermore, bioinformatics analysis of several publicly-available data sets found that the inositol signaling pathway was defective in asthma in response to stressors such as ozone and viral infection (Agrawal, Sinha et al. 2009).

Perhaps most striking was the markedly abnormal pattern of induction of the oxidative phosphorylation pathway, including several subunits of the mitochondrial electron transport chain, in EIB(-) and EIB(+) cultures. Proliferative cells use the inefficient method of aerobic glycolysis to produce ATP, whereas differentiated cells use oxidative phosphorylation for cellular energy requirements, suggesting that induction of this pathway is necessary for appropriate mucociliary differentiation (Warburg 1956, Vander Heiden, Cantley et al. 2009). Mitochondria are found concentrated in the apical and basolateral domains of differentiated airway epithelial cells and form barriers to restrict calcium-dependent signaling functions (Ribeiro, Paradiso et al. 2003). Stem cells tend to favour glycolysis over oxidative phosphorylation for energy production (Simsek, Kocabas et al. 2010, Stringari, Edwards et al.

2012), which correlates with our finding of increased numbers of basal cells in asthmatic ALI cultures *in vitro* (**Chapter 5** and Hackett, Warner et al. 2009). In addition to inefficient energy production, reduced oxidative phosphorylation can also lead to damaging leakage of reactive oxygen species (Favre, Zhdanov et al. 2010). Pezzulo et al. previously reported that epithelial cells *in vitro* had much higher expression of several oxidative phosphorylation genes than did bronchial brushings, and suggested that this was an artifact of the nutrients available to the cells (Pezzulo, Starner et al. 2011). However, the dynamic expression of these genes during differentiation in control cultures, and the striking differences between disease groups, suggests otherwise.

Altered expression of the oxidative phosphorylation pathway may indicate mitochondrial dysfunction in asthma. Mitochondrial dysfunction was observed in a mouse model of allergic asthma, as evidenced by reduced expression of cytochrome c oxidase in the bronchial epithelium, decreased ATP levels in the lung and mitochondrial structural abnormalities (Mabalirajan, Dinda et al. 2008). To further explore this idea, mitochondrial morphology in the airway epithelium in asthma could be explored by transmission electron microscopy. Additionally, metabolic imaging of the ratios of NADH to NAD+ in living cells can provide a measure of oxidative phosphorylation versus glycolysis and may provide important functional information (Stringari, Edwards et al. 2012). We also found that the pyrimidine and purine metabolism pathways followed the same expression pattern as the oxidative phosphorylation pathway. Although these pathways are important for regulating cellular ATP levels, we found that the majority of the genes encode RNA polymerases. *De novo* transcription is clearly necessary to assemble new cellular components for the formation of ciliated and goblet cells during differentiation. As a whole, our findings suggest impaired sensing of the extracellular

environment, aberrant intracellular signaling, and depressed metabolic activity in EIB(-) cultures throughout differentiation, and a delayed onset of several of these pathways in EIB(+) cultures. Importantly, this is the first description of distinct transcriptional profiles throughout mucociliary differentiation in the specific clinical subgroups of EIB(+) and EIB(-) asthma.

Endogenous miRNAs play an important role in regulating cellular RNA abundance. Differentiation of many epithelial cell types is regulated by miRNAs, including the gut (Tsuchiya, Oku et al. 2009, Nguyen, Dalmasso et al. 2010) and the skin (Lena, Shalom-Feuerstein et al. 2008, Yi, Poy et al. 2008). The conducting airway is no exception; miR-449a in particular is induced upon mucociliary differentiation of airway epithelial cells *in vitro* (Lize, Herr et al. 2010, Marcet, Chevalier et al. 2011). A recent study of epithelial cells obtained by bronchial brushing found that the miR-34/449 family was repressed in asthma and that this could be recapitulated by treatment of ALI cultures with IL-13 (Solberg, Ostrin et al. 2012). In our study, members of the miR-449 family were not significantly downregulated in asthmatic-derived cultures. This could be due to differences in asthma severity or clinical phenotype between our two studies. However, based on the finding that miR-449 was restored *in vivo* after inhaled corticosteroid treatment but was repressed following treatment of ALI cultures with IL-13 (Solberg, Ostrin et al. 2012), we suggest that downregulation of miR-449 is a response to the T_H2 inflammatory environment rather than an inherent feature of the epithelium in asthma.

We identified a number of miRNA regulatory networks involved in mucociliary differentiation of airway epithelial cells. Two studies to our knowledge have compared the miRNA signature in monolayer and differentiated cultures in healthy donors. The first was a sequencing-based analysis of miRNA expression in nasal epithelial cells from three healthy donors at days 0, 7, 14 and 21 of ALI culture (Marcet, Chevalier et al. 2011). The authors of that

study identified several miRNAs whose expression increased or decreased significantly throughout differentiation, but only characterized the role of miR-449 in the development of multiciliated cells. Three of the miRNAs that they identified as downregulated during differentiation were also present in our mirConnX analysis: miR-205, mR-21 and miR-210 (Marcet, Chevalier et al. 2011). However, the overall role of these miRNAs in mucociliary differentiation is unclear, as only miR-21 had targets that were enriched for a specific function (activating organismal movement). Martinez-Anton and colleagues recently carried out a microarray-based analysis of miRNA and mRNA expression in day 0 compared to day 28 in ALI cultures derived from one healthy donor and described several miRNAs that were up- or downregulated during differentiation (Martinez-Anton, Sokolowska et al. 2013). However, their study did not directly correlate expression of the miRNAs and mRNAs to identify regulatory networks. We again identified common miRNAs that were up- (miR-200) or downregulated (miR-100 and miR-155) during differentiation. Interestingly, Martinez-Anton et al. had identified that several miRNAs were predicted to inhibit cell proliferation (Martinez-Anton, Sokolowska et al. 2013), which we also found to be the case in our direct analysis of miRNA-mRNA interactions.

Several of the miRNA regulatory hubs that we identified have been shown to be overexpressed in bronchial brushings from asthmatic compared to non-asthmatic donors (miR-155, -335, -100) (Jardim, Dailey et al. 2012), although the predicted targets or consequences of this were not explored. MiR-375 was upregulated during differentiation in our ALI culture system, and although it had few predicted targets, has been shown to regulate TSLP release from human bronchial epithelial cells in response to environmental exposures (Bleck, Grunig et al. 2013). We had also hypothesized that miR-203 would be upregulated during differentiation, as it

is known to be upregulated in suprabasal cells during epidermal stratification and acts to inhibit p63 expression in those cells (Lena, Shalom-Feuerstein et al. 2008, Yi, Poy et al. 2008). However, this was not the case, perhaps owing to the fact that the initial submerged stage of ALI culture in fact promotes a squamous phenotype in which miR-203 has a stronger role (Hackett, Shaykhiev et al. 2011).

We also did not identify any miRNAs that targeted the oxidative phosphorylation or pyrimidine metabolism pathways, which may be due to the fact that we focused on those miRNAs that changed with time only, regardless of donor group. We did not in fact find any miRNAs that were significant for group:time interaction, which may be due to our sample size. It is also possible that alternative epigenetic methods, such as DNA methylation or histone modifications, may be responsible for the differential gene expression we observed in asthmatic-derived ALI cultures, and would therefore require future studies to analyze these potential transcriptional mechanisms. Additionally, the mirConnX tool has limitations as it is based on prior knowledge of miRNA and mRNA interactions, and thus it is possible that we did not identify all of the important miRNA hubs. Further analyses will likely focus on elucidating novel regulatory interactions that are not informed by prior knowledge. In future, we will also aim to identify those miRNAs with the largest magnitude of difference between donor groups at specific stages of culture.

As global gene expression analysis may over correct and miss subtle changes in gene expression, we additionally conducted a candidate gene analysis for all genes previously shown to be altered during differentiation. We confirmed that basal cell genes KRT5, ΔNP63, CD109 and ITGA6 decrease with time during differentiation as previously reported by Dvorak et al (Dvorak, Tilley et al. 2011). Interestingly, we did not observe any differences in expression of

basal cell-related genes in our asthmatic ALI cultures, despite our observation of an expanded population of CK-5-expressing basal cells in EIB(-) cultures (**Chapter 5**). This suggests that post-transcriptional or post-translational modifications may regulate CK-5 protein expression in these cells. In addition, the ciliated cell-related genes we identified in our global analysis of differential expression were significantly decreased in EIB(-) and EIB(+) groups equally. This correlation of gene expression and histological evidence further validates our finding of defective ciliogenesis in both asthmatic subgroups.

During mucociliary differentiation, we found that expression of all members of the EGFR, TGFβ and wnt/β-catenin pathways changed in agreement with the findings of Ross and colleagues (Ross, Dailey et al. 2007), but that a number of genes were downregulated in asthmatic-derived ALI cultures. Increased EGFR expression and activation occurs in damaged or repairing epithelium, and is positively correlated with asthma severity *in vivo* (Puddicombe, Polosa et al. 2000, Polosa, Puddicombe et al. 2002, Hamilton, Puddicombe et al. 2005), which appears to contradict our findings. Decreased expression of the Wnt pathway member GATA6 is consistent with the increased numbers of basal cells we observed in EIB(-) cultures, as decreased GATA6 expression in the epithelium results in impaired differentiation in the postnatal mouse airway (Zhang, Goss et al. 2008) and colon (Beuling, Aronson et al. 2012). Our observation of decreased STAT6 expression in asthmatic ALI cultures may have implications for epithelial responses to T_H2 cytokines, as activation of STAT6 by the T_H2 cytokines IL-4 and IL-13 is required for SPDEF-mediated goblet cell hyperplasia (Park, Korfhagen et al. 2007). Previous studies have found increased STAT6 expression in the bronchial epithelium of severe asthmatics (Mullings, Wilson et al. 2001), but no difference in the epithelium of stable asthmatics (Tomita, Caramori et al. 2012). Overall, decreased expression of several candidate genes involved in

growth factor and cytokine signaling may indicate an inappropriate epithelial response to external stimuli in asthma.

As with all experimental studies, ours had both strengths and weaknesses. Most transcriptional studies of primary human airway epithelial cells to date have used microarray technology. Microarray studies are robust, but are limited to specific probes for previously-identified transcripts, and probe binding affinity may lead to false negative readings (Ross, Dailey et al. 2007). In the present study, we used RNA deep sequencing, which allows us to identify the entire RNA transcriptional population. Previous studies have assessed mRNA and/or miRNA profiles either during differentiation of cells from healthy donors (Ross, Dailey et al. 2007, Lize, Herr et al. 2010) or to compare monolayer (Kicic, Hallstrand et al. 2010) or well-differentiated cells from asthmatic and healthy donors (Jardim, Dailey et al. 2012, Solberg, Ostrin et al. 2012). Our experimental approach was more comprehensive, as we included epithelial cells from well-phenotyped human donors at multiple timepoints throughout ALI differentiation. One drawback to this comprehensive approach was that we were limited to only five donors per group. However, this sample size with multiple timepoints provides robust results across several methods of analyzing differential expression (Soneson and Delorenzi 2013). Our use of the negative binomial distribution to model our data is the preferred method for RNA sequencing experiments, as it enables us to more accurately accommodate the overdispersion (increased variance) between biological replicates than does a Poisson distribution (Oberg, Bot et al. 2012).

Although there are advanced computational approaches to sequencing data, the technology is not without limitation. For example, sequencing-based analysis of some miRNAs is complicated by existence of multiple precursors for a given mature miRNA. For example, let-

7a-5p can arise from three separate precursors (let-7a-1, let-7a-2 and let-7a-3), leading to the potential for overrepresentation of let-7a-5p in our final data. For this reason, validation of our findings by alternate methods is still necessary. RT-qPCR has been shown to correlate well with RNA sequencing data in diverse tissues including human airway biopsies (Yick, Zwinderman et al. 2013), bovine muscle cells (Lee, Malik et al. 2014) and human placenta (Saben, Zhong et al. 2014). While *in vitro* cell culture does not completely recapitulate the *in vivo* environment, several studies have shown strong correlations between ALI cultures and bronchial brushings for both mRNA (Dvorak, Tilley et al. 2011, Pezzulo, Starner et al. 2011) and miRNA expression (Solberg, Ostrin et al. 2012). Our use of a pure epithelial population also minimizes confusion about the cellular origin of specific mRNAs and miRNAs, which is an acknowledged caveat of using bronchial brushings or biopsies (Pezzulo, Starner et al. 2011, Yick, Zwinderman et al. 2013). It is possible that our finding of decreased expression of many pathways in EIB(-) cultures may reflect an overall decrease in transcription in this donor group. However, comparison of RNA yield in a fixed cell number in monolayer culture did not reveal any differences between donor groups (**Appendix A.7)**. In addition, RNA libraries were created using the same amount of input material per sample. This indicates that although the pathways we have identified were unable to be induced in EIB(-) cultures and were delayed in EIB(+) cultures, there may in fact be as-yet-unidentified groups of RNAs that are more highly induced in these cultures.

In summary, we describe here a novel and comprehensive analysis of the transcriptional events associated with airway epithelial cell differentiation from asthmatic and healthy donors *in vitro*. We have identified several metabolic and signaling pathways that are differentially expressed during differentiation between healthy, EIB(-) asthmatic and EIB(+) asthmatic donors.

While expression of the oxidative phosphorylation, transcription, actin cytoskeleton and insulin and MAPK signaling pathways were more notably dysregulated in the EIB(-) group, genes related to ciliogenesis were equally downregulated in both asthmatic subgroups. This suggests that future studies of well-phenotyped asthmatic donors are necessary to expand our understanding of disease heterogeneity and pathogenesis, and identify potential avenues for molecular therapies.

Chapter 7: Conclusions

Asthma is a serious global health problem with significant morbidity and mortality, and disease prevalence continues to rise (Centers for Disease and Prevention 2011). Despite effective symptom management in many individuals, there is no “cure” for the disease. The role of the airway epithelium as the first point of contact with the inhaled environment, and its role in modulating airway inflammation and remodeling is now a topic of great interest (Holgate 2007, Kato and Schleimer 2007, Lambrecht and Hammad 2012). Multiple epithelial abnormalities occur in asthma, including downregulation of junctional proteins E-cadherin (Trautmann, Kruger et al. 2005, de Boer, Sharma et al. 2008, Hackett, Singhera et al. 2011) and ZO-1 (Xiao, Puddicombe et al. 2011), dysregulated expression of repair molecules EGFR (Puddicombe, Polosa et al. 2000), CD44 (Lackie, Baker et al. 1997), and ki-67 (Fedorov, Wilson et al. 2005, Cohen, E et al. 2007), loss of columnar epithelial cells, and basement membrane thickening (Barbato, Turato et al. 2006). There is also evidence that many of these alterations occur early in disease, perhaps even prior to the onset of symptoms and diagnosis of asthma (Fedorov, Wilson et al. 2005, Barbato, Turato et al. 2006). Therefore, understanding the molecular signatures of mucociliary differentiation in the setting of wound repair in asthma may hold great promise for minimizing the progression of airway remodeling leading to airflow limitation and acute inflammatory episodes.

7.1 In vitro models of epithelial basal cell plasticity

Maintenance and repair of the pseudostratified airway epithelium relies upon the progenitor capacity of basal cells (Schoch, Lori et al. 2004, Hong, Reynolds et al. 2004a, Hong, Reynolds et al. 2004b), which are capable of both self-renewal and differentiation into ciliated, serous and goblet cells (Hackett, Shaheen et al. 2008, Rock, Onaitis et al. 2009). In this role, the basal cell is crucial for wound repair in response to injury (Hong, Reynolds et al. 2004a, Hong, Reynolds et al. 2004b, Rock, Onaitis et al. 2009, Hegab, Nickerson et al. 2012, Musah, Chen et al. 2012). This function necessitates a high degree of plasticity in order for the cell to spread, migrate, proliferate and finally differentiate to complete the restitution of a patent epithelium (Erjefalt, Erjefalt et al. 1995). In this thesis, we have used three distinct *in vitro* models of human airway epithelial basal cell plasticity – epithelial-mesenchymal transition, repair of mechanical scratch wounds, and differentiation at air-liquid interface – which together provide a complete view of epithelial repair and the basal cell's role in this process.

Epithelial-mesenchymal transition (EMT) in response to TGFβ$_1$

TGFβ$_1$ expression is increased in asthmatic airways, and it has been suggested that plasticity between the epithelial and mesenchymal compartments may contribute to airway remodeling (Holgate, Davies et al. 2003, Boxall, Holgate et al. 2006). In this direction, in **Chapter 3** we exposed epithelial cells to the pro-fibrotic mediator TGFβ$_1$, which led to dissolution of epithelial contacts, loss of cell polarity and the acquisition of mesenchymal characteristics including expression of EDA-fibronectin and synthesis of extracellular matrix proteins. This phenotypic shift mimics the initial stages of wound repair.

In both monolayer culture, which represents the basal cell phenotype, as well as in differentiated cultures at air-liquid interface, which mimics the *in vivo* structure of the epithelium, we described the basal cells as the exclusive cell type that undergoes TGFβ_1-induced EMT (Hackett, Warner et al. 2009). This suggests that basal cells are intrinsically plastic and able to differentiate into multiple cell types including a migratory and matrix secretory cell type indicative of a mesenchymal cell. Interestingly, the EMT response was more pronounced in ALI cultures from asthmatic donors, primarily owing to an expanded population of p63- and CK-5-expressing basal cells. These findings have distinct implications for airway remodeling. First, inflammatory mediators released in the airways (ie. TGFβ) have true repercussions for epithelial basal cell function, including loss of polarity and adhesion junctions. Secondly, decreased expression of junctional proteins such as E-cadherin and ZO-1 *in vivo* may in fact demonstrate that epithelial plasticity is active in asthma (Hackett, Singhera et al. 2011, Xiao, Puddicombe et al. 2011).

Wound repair

Following damage, cuboidal basal epithelial cells spread, migrate and proliferate to cover the wound site. Monolayer cultures model cuboidal basal airway epithelial cells (Hackett, Shaykhiev et al. 2011), and using a scratch wound assay in monolayer cultures we assessed the ability of basal cells to migrate and proliferate to restore a mechanical wound. Specifically, we assessed the role of the transcription factor ΔNp63α, which is known to be absolutely critical for differentiation of epithelial tissues including the conducting airways (Mills, Zheng et al. 1999, Yang, Schweitzer et al. 1999, Daniely, Liao et al. 2004). In **Chapter 4** we found that inhibition of ΔNp63α impaired restitution of scratch wounds in monolayer culture, which we attributed

primarily to decreased proliferation (Warner, Hackett et al. 2013). Despite our demonstration of increased numbers of p63-expressing basal cells in the asthmatic epithelium, delayed onset of epithelial restitution has been demonstrated both in differentiated ALI (Hackett, Singhera et al. 2011) and monolayer cultures (Stevens, Kicic et al. 2008) derived from asthmatic donors. These findings suggest that p63 expression is necessary, but not sufficient, for the early stages of epithelial wound repair involving proliferation and migration. Importantly, while we previously described increased numbers of p63-expressing basal cells in the airways of asthmatic subjects, in this chapter we demonstrated that protein expression within basal cells derived from asthmatic and non-asthmatic donors is not different. This finding indicates that rather than a per-cell increase in p63 expression, there is a defect in the ability of epithelial basal cells to undergo normal mucociliary differentiation in asthma.

Differentiation in air-liquid interface (ALI) culture

Air-liquid interface culture is a system in which airway epithelial basal cells are first cultured submerged to promote proliferation, and then exposed to air at the apical surface to initiate differentiation. This process recapitulates *in vivo* wound repair from the post-migratory phase onward. In **Chapter 5** we found that basal cells from healthy donors were able to self-renew and give rise to both ciliated and goblet cells. However, basal epithelial cells from asthmatic donors were impaired in the transition to a ciliated but not goblet cell phenotype, which manifested as an expansion of the basal cell population. These findings suggest that basal cell differentiation, also termed the “resolution phase” of epithelial repair, is intrinsically defective in asthma, leading to the maintenance of excessive numbers of basal cells and the formation of fewer ciliated cells. In combination with our observation of shorter cilia in asthmatic-derived ALI cultures, our findings

suggest impaired mucociliary clearance in asthma due to ciliary defects (Afzelius 2004) which is supported by previous literature (O'Riordan, Zwang et al. 1992, Daviskas, Anderson et al. 2005). This may leave the epithelium more susceptible to inhaled particulate matter, viruses, aeroallergens and subsequent damage.

Molecular regulation of basal cell plasticity

To understand the molecular determinants of basal cell differentiation, in **Chapter 4** we focused on the basal cell transcription factor p63 as a candidate gene, as it has previously been demonstrated to regulate several genes involved in repair, adhesion, proliferation and cytoskeletal structure in other epithelial tissues. We demonstrated that in human airway epithelial cells, the ΔNp63α isoform positively regulates the expression of EGFR, β-catenin and jagged1, which have well documented roles in epithelial repair (Warner, Hackett et al. 2013). Inhibition of ΔNp63α expression within airway basal cells resulted in reduced proliferation and migration (Warner, Hackett et al. 2013). The essential role of ΔNp63α in airway epithelial differentiation was later demonstrated by Arason et al., as inhibition of ΔNp63α expression resulted in loss of the ability of basal cells to differentiate in ALI culture (Arason, Jonsdottir et al. 2014).

While candidate gene approaches allow for rapid phenotyping of cellular responses to gene manipulation, this method relies on prior knowledge and does not take into account the modulation of gene networks that occurs during highly specialized and integrated cellular responses. Thus in **Chapter 6**, we used an unbiased RNA sequencing approach to provide a comprehensive analysis of mRNA and miRNA expression during differentiation of airway epithelial cells from asthmatic and non-asthmatic donors.

We identified that the most significant differentially-expressed pathways between asthmatic and non-asthmatic donor groups were responsible for cellular energy production and RNA transcription. The strong induction of these pathways in cells from healthy donors suggests peaking energy requirements as the cells initiate cell-type specific functions such as ciliogenesis. Indeed, transcription of cilia components occurs early in differentiation, between days 4-10 (Ross, Dailey et al. 2007). Therefore the inability to initiate metabolic processes early is a likely contributor to the epithelial phenotype we have observed in asthma. In line with this idea, mitochondria are concentrated in the apical domains of differentiated epithelial cells to provide energy for cellular functions (Ribeiro, Paradiso et al. 2003). Mitochondrial dysfunction in asthma has been suggested in several studies (Mabalirajan, Dinda et al. 2008, Perez, Hill et al. 2010, Xu 2010, Dranka, Benavides et al. 2011), and a very recent study found several asthma-associated polymorphisms in mitochondrial genes encoding subunits of the electron transport chain (Flaquer, Heinzmann et al. 2014). If metabolism is dampened in the airway epithelium in asthma, or the switch from glycolysis to oxidative phosphorylation cannot be made, the epithelium may be metabolically unable to sustain cell types with high energy requirements. This may contribute to the observed deficiency in differentiation of basal cells into ciliated cells in asthma.

We found several actin-related pathways to be decreased prior to the onset of differentiation in asthmatic-derived ALI cultures. This is in line with findings of decreased expression of several adhesion molecules in the asthmatic epithelium, including tight junction protein ZO-1 (Xiao, Puddicombe et al. 2011) and adherens junction protein E-cadherin (Hackett, Singhera et al. 2011, Hiejink, Kies et al. 2007, Trautmann, Kruger et al. 2005). The suppressed ability to form intercellular adhesions or reorganize the actin cytoskeleton also impacts the ability to initiate and maintain apical-basal polarity and undergo subsequent differentiation (Shin,

Fogg et al. 2006, Herder, Swiercz et al. 2013). The actin cytoskeleton is also intimately involved in signal transduction (Rodriguez-Boulan, Kreitzer et al. 2005). We identified numerous growth factor receptors and signaling pathways that were differentially regulated between donor groups, including EGFR, TGFβ, Wnt/β-catenin, and insulin signaling. MAPK signaling, which lies downstream of several growth factor receptors, was also affected. This suggests that epithelial cells from asthmatic donors may be unable to respond to pro-differentiation cues *in vitro* due to the combined detrimental effects of impaired polarization, junction formation and growth factor signaling.

Overall, our findings suggest that lineage commitment and molecular reprogramming in basal cells are skewed in asthma to favour epithelial plasticity in response to fibrotic stimuli such as $TGF\beta_1$, rather than mucociliary differentiation, as shown in **Figure 7.1**. This may be a result of several regulatory mechanisms, including the inability to form junctions and rearrange the cytoskeleton, impaired growth factor sensing and signaling, and the high energy requirements of differentiating into more specialized epithelial cell types, which likely act together to result in the observed phenotypic differences in disease. However, whether there is a single overarching reason for this, or if it is the result of multiple shifts in the epithelial response that culminate in an inability to differentiate, remains unknown.

MiRNAs are attractive as potential regulators of epithelial differentiation, as they are known to regulate mRNA stability and translation. As noted in **Chapter 6**, principal component analyses demonstrated that miRNAs segregated clearly by time during ALI differentiation, suggesting that they are tightly regulated during differentiation. In this thesis, we have taken the comprehensive approach of assessing both miRNA and mRNA expression temporally to elucidate a putative regulatory network within our bronchial epithelial cultures that regulates the

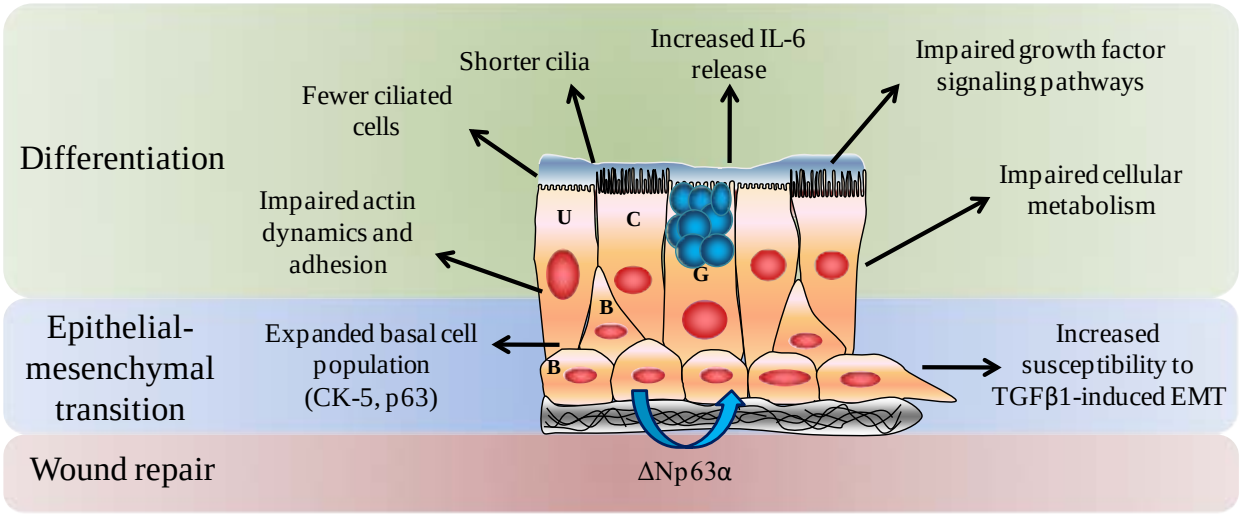


Figure 7.1 Regulation of epithelial basal cell plasticity is altered in asthma.
Using three distinct models of epithelial plasticity *in vitro*, we have identified numerous alterations in the airway epithelial basal cell in asthma. In response to $TGF\beta_1$, basal cells undergo molecular reprogramming, losing expression of epithelial characteristics and gaining mesenchymal characteristics. We identified an expanded population of basal cells expressing cytokeratin (CK)-5 and p63 in the epithelium in asthma, which leads to an overall increase in the response to $TGF\beta_1$. In monolayer culture, ΔNp63α is required for wound repair, and also regulates expression of several repair-related genes, including epidermal growth factor receptor, β-catenin and jagged1. During differentiation, air-liquid interface cultures from EIB(+) and EIB(-) asthmatic donors have fewer ciliated cells and increased production of IL-6, and EIB(-) donors additionally had shorter cilia and retained increased numbers of basal cells. In addition, both phenotypes demonstrated dysregulation of RNA in pathways involved in actin dynamics and cellular adhesion, metabolism, and growth factor signaling. Overall, epithelial plasticity was notably altered in asthma.

transition between proliferation and differentiation. Although several groups have now identified distinct miRNA profiles in bronchial brushings (Jardim, Dailey et al. 2012, Solberg, Ostrin et al. 2012) and biopsies (Yick, Zwinderman et al. 2013) from asthmatic compared to healthy donors, in this study we did not identify any miRNAs that were significantly different in our group:time statistical analysis. This may suggest that other factors, such as epigenetic or genetic regulation, may underlie aberrant basal cell differentiation in asthma.

Several studies have demonstrated epithelial abnormalities in children with asthma, suggesting that genetic or epigenetic predisposition may contribute to disease pathogenesis

(Barbato, Turato et al. 2006, Stevens, Kicic et al. 2008, Kicic, Hallstrand et al. 2010, Parker, Sarlang et al. 2010, Lopez-Guisa, Powers et al. 2012, Stefanowicz, Hackett et al. 2012). Stefanowicz et al. showed differential methylation of several genes in asthmatic compared to healthy or atopic pediatric donors (Stefanowicz, Hackett et al. 2012). In addition, many asthma-susceptibility single nucleotide polymorphisms (SNPs) have been identified that have implications for the epithelium (Palmer, Irvine et al. 2006, He, Hallstrand et al. 2009, Moffatt, Gut et al. 2010). Whether epigenetic alterations or SNPs have direct implications on airway differentiation is unknown at this time but is an exciting avenue of future study.

7.2 The airway epithelial phenotype in EIB(+) asthma

While we showed that ALI cultures from EIB(+) and EIB(-) asthmatics were both markedly deficient in ciliated cells, our findings provide clear evidence of distinct epithelial differences between EIB(-) and EIB(+) asthmatics. The EIB(+) phenotype demonstrated less severe remodeling and were able, although delayed, to induce metabolic pathways during differentiation. For several reasons, we had hypothesized that EIB(+) asthmatics would have marked epithelial dysregulation and damage. Firstly, the mechanism of EIB is postulated to involve defective epithelial water transport (Anderson and Daviskas 2000). Secondly, EIB(+) asthmatics have increased epithelial fragility as evidenced by higher numbers of columnar epithelial cells in induced sputum (Hallstrand, Moody et al. 2005). Together, these findings suggest that EIB(+) asthmatics would have a fragile epithelium with defective solute transport.

Recent evidence has identified increased numbers of intraepithelial mast cells in EIB(+) asthma, which may suggest a role for epithelial-derived mast cell chemokines in EIB pathogenesis (Lai, Altemeier et al. 2014). Interestingly, the mast cells were described to express

markers that have been associated with the "T_H2 high" phenotype of asthma (Woodruff, Boushey et al. 2007, Dougherty, Sidhu et al. 2010, Lai, Altemeier et al. 2014), which is more amenable to the action of inhaled corticosteroids than the non-T_H2 high phenotype (Woodruff, Boushey et al. 2007). This may suggest that airway inflammation in EIB(+) patients is more effectively controlled by corticosteroid therapy than in EIB(-) patients, which could result in the lesser degree of airway remodeling we have observed. Related to this idea, there may also be differences in the extent of tissue damage caused by exacerbations in EIB(+) and EIB(-) individuals. In one study of asthmatic patients, exercise- and allergen-induced bronchoconstriction resulted in a similar initial decrease in $_{FEV1}$, but only the allergen-exposed group showed an increase in airway responsiveness and epithelial loss 48 hours after challenge (Tateishi, Motojima et al. 1996), suggesting that EIB leads to a transient inflammatory response rather than excessive and long lasting airway damage and associated remodeling. However, two previous studies had demonstrated that 30-50% of patients who have EIB experience a second fall in FEV_1 in the first eight hours after exercise challenge (Boulet, Legris et al. 1987, Chhabra and Ojha 1998). Although it is possible that our study was simply underpowered to detect moderate morphological changes, the robust transcriptional differences identified in **Chapter 6** suggest that there may indeed be a less severe defect in differentiation in EIB(+) asthmatics. Future inquiries of the epithelial phenotype in EIB(+) asthmatic patients are necessary to better understand the contribution of the epithelium to disease pathogenesis.

7.3 Strengths and limitations of our experimental models

The models described in this thesis relied on the use of airway epithelial cells isolated from human donors. We feel that this is a great strength of our work. Immortalized cell lines are

robust models to answer many specific questions, but in order to query the inherent disease processes it is essential to characterize human cellular and tissue responses. We utilized monolayer cell culture as a model of epithelial basal cells (Hackett, Shaykhiev et al. 2011), which has the caveat that it lacks other cell types that participate in epithelial repair and remodeling, such as infiltrating cells from the vasculature and matrix-secreting fibroblasts. In order to fully understand the differences in epithelial plasticity in health and disease, we utilized airway epithelial cells obtained from well-phenotyped human donors that were collected at multiple timepoints throughout mucociliary differentiation. While this limited the number of donors to five per donor group, this experimental protocol was more comprehensive than previous transcriptional analyses that utilized cultures derived from one (Dvorak, Tilley et al. 2011) or three (Ross, Dailey et al. 2007, Marcet, Chevalier et al. 2011) independent donors. Another caveat of the *in vitro* ALI model is that some *in vivo* features of the asthmatic airway, such as mucous metaplasia, are not observed *in vitro* and rather appear to be a response to EGFR activation by its ligands or by T_H2 cytokines such as IL-13 (Shim, Dabbagh et al. 2001). However, our approach has nevertheless identified specific defects in epithelial differentiation and lineage commitment in asthma, and our use of an isolated culture system gives confidence that our findings truly represent the transcriptional events that occur in the epithelium.

Experimental animal models of allergic airways disease, such as house dust mite or ovalbumin exposure murine models, are able to provide important insight into disease mechanisms, but are known to lack key features that are relevant to the present study (Bates, Rincon et al. 2009, Holmes, Solari et al. 2011). Firstly, the murine respiratory system does not fully represent human airway biology, particularly because the pseudostratified airway epithelium is restricted to the mouse trachea rather than extending to the distal airways

(illustrated in (Rackley and Stripp 2012)). In addition, there is currently no murine model that is able to fully recapitulate the complexity and individual variability of human asthma (Hirota, Hackett et al. 2011), including the existence of multiple different subphenotypes of disease and the contribution of genetic susceptibility. As the basis of the experiments presented in this thesis was to understand whether inherent epithelial alterations may underlie asthma pathogenesis, our experimental approach has generated important findings concerning the role of the epithelial basal cell in asthma, which has direct clinical relevance to human disease.

7.4 Therapeutic implications of basal cell plasticity

We have identified an undifferentiated epithelial phenotype in asthma, identified by increased numbers of basal cells that are less able to differentiate into ciliated cells, potentially due to impaired energy production and mRNA transcription. This phenotype suggests a number of therapeutic avenues. As the basal cell is responsible for the restitution and maintenance of the airway epithelium, mechanisms to restore its ability to differentiate may prove beneficial for patient health. This could be attempted using targeted gene therapy to re-introduce expression of genes that are downregulated in asthma, such as those necessary for formation of cell-cell junctions and epithelial polarization (ie. E-cadherin), differentiation of ciliated cells (ie. foxj1, dyneins), or cellular metabolism (ie. NADH dehydrogenase, RNA polymerase). The difficulty with this approach is the identification of a single target gene, as we have demonstrated several pathways that are critically dysregulated in epithelial cells from diseased individuals. EGF has been suggested as a mechanism to restore epithelial barrier integrity in asthma (Swindle, Collins et al. 2009), as it has shown to be effective in the treatment of intestinal barrier defects in inflammatory bowel disease (Sinha, Nightingale et al. 2003). However there is an obvious need

to carefully balance proliferative potential with differentiation to allow a rapid and complete repair response but avoid risks such as metaplasia or progenitor exhaustion by inducing terminal differentiation.

MicroRNAs have already been identified as promising therapeutic candidates. For example, the let-7 miRNA family has been identified as a therapeutic candidate in both cancer and bronchial hyperresponsiveness (Kumar, Ahmad et al. 2011, Levanen, Bhakta et al. 2013). Although we identified several miRNA regulatory networks that appear to regulate basal cell transition between proliferation and differentiation, we were as yet unable to discern striking differences between healthy and asthmatic donors. However, evidence from the literature has identified a number of miRNAs that are differentially expressed in asthmatic bronchial brushings (Jardim, Dailey et al. 2012), in exosomes from bronchoalveolar lavage fluid (Levanen, Bhakta et al. 2013), and in bronchial biopsies (Yick, Zwinderman et al. 2013). Further exploration into the miRNA expression patterns in the airway epithelium will assist our understanding of abnormalities in asthma and provide exciting therapeutic targets. If individual targets were identified, a pertinent direction would be to create therapeutic complementary short RNAs (antagomirs) or miRNA mimics that could be delivered by inhalation. Our RNA sequencing data set also provides the potential to identify novel long non-coding (lnc) RNAs. Some lncRNAs, such as terminal differentiation-induced ncRNA (TINCR), have already been shown to aid in stabilization of differentiation-related mRNAs (Kretz, Siprashvili et al. 2013). Exogenous administration or knockdown of such lncRNAs may enable the epithelium to be reprogrammed to allow differentiation in response to damage and/or ALI culture conditions.

Antioxidant therapy is an intriguing concept for treatment of asthma. Inflammation in asthma leads to production of reactive oxygen species and reactive nitrogen species, resulting in

oxidative damage (Rahman 2002). Additionally, antioxidant defenses may be compromised in individuals with asthma (Bucchieri, Puddicombe et al. 2002, Fitzpatrick, Baena-Cagnani et al. 2012, Piacentini, Polimanti et al. 2013). Our findings of altered expression of oxidative phosphorylation enzymes also support this concept. Several potential antioxidant therapies have been proposed, including ascorbic acid (Mickleborough 2008, Fanucchi, Bracher et al. 2012). A systematic review of the therapeutic effect of ascorbic acid on asthma and EIB was unable to show any clinical benefit of therapy, though there may have been some improvement in EIB (Milan, Hart et al. 2013). Cysteine donors such as N-acetylcysteine have also shown benefit, reducing RSV-induced ciliary defects in human bronchial epithelial cells *in vitro* (Mata, Sarrion et al. 2012), as well as showing efficacy *in vivo* in EIB (Baumann, Rundell et al. 2005), and settings of pulmonary inflammation (Meyer, Buhl et al. 1994, Behr, Maier et al. 1997, Antonicelli, Parmentier et al. 2002, Blesa, Cortijo et al. 2003). Treatment with low-dose doxycycline has also proven useful for reducing cellular oxidative stress (Muroya, Chang et al. 2012, Parvu, Alb et al. 2013, Antonio, Ceron et al. 2014). To restore cellular energy production, mitochondrial transfer may also be an exciting avenue of study. Mitochondria transfer from induced pluripotent mesenchymal stem cells to epithelial cells, with preservation of ATP, was found to occur *in vitro* via formation of tunneling nanotubes (Li, Zhang et al. 2014). Assessing the potential of antioxidants and therapies to redirect epithelial differentiation would be of great interest moving forward.

An obvious difficulty with the aformentioned concepts is that early treatment is optimal. A thirteen year follow-up study of adult patients with mild asthma who were randomized to early or delayed-onset inhaled corticosteroid (budesonide) therapy found that the patients that had delayed onset of therapy had more airway inflammation and less optimal disease control

(Haahtela, Tamminen et al. 2009). The truth remains that until we can identify susceptible individuals early in life and provide treatments that minimize or halt disease progression, asthma will remain a significant global health burden. In the coming years, it is our hope that dysregulated epithelial differentiation will be addressed as an early biomarker and a potential therapeutic avenue in asthma management.

7.5 Summary

In conclusion, this thesis has provided evidence of altered plasticity of epithelial basal cells in asthma, which has implications for the reparative potential of the epithelium. We have demonstrated that the asthmatic epithelium is more able to differentiate to a mesenchymal-like cell through the actions of TGFβ_1. In contrast, asthmatic-derived airway epithelial basal cells were impaired in the ability to undergo ciliated cell differentiation and demonstrated altered regulation of several pathways involved in barrier formation and cellular metabolism. Furthermore, we identified distinct phenotypic and transcriptional differences between asthmatics with and without exercise-induced bronchoconstriction, which may suggest more prominent epithelial dysregulation in the EIB(-) phenotype. Our findings contribute to the growing body of evidence that epithelial repair is aberrant in asthma and provide mechanistic insight as to potential therapeutic targets for future work.

7.6 Suggested future directions

As with most scientific studies, we are left with a number of interesting questions that could be addressed in future studies.

1. Confirm expression and function of differentially expressed pathways

We identified several pathways that were differentially expressed in ALI cultures from healthy control, EIB(-) and EIB(+) asthmatic donors. In future, we must first confirm our findings at the RNA level using an alternate method such as RT-qPCR. Additionally, we would assess expression at the protein level in formalin-fixed paraffin-embedded sections or protein lysates for cellular components, or in apical washes or basal media for secreted proteins. To explore the functional significance of genes, miRNAs, or pathways identified, we would then aim to disrupt or reintroduce expression of these candidate molecules to assess whether this would enable asthmatic-derived pHAECs to undergo appropriate mucociliary differentiation. To confirm that our findings hold true in a separate population of asthmatic individuals, we would also analyze expression of these various molecules in biobanked human donor airway tissue and epithelial cells.

The identification of the oxidative phosphorylation pathway as aberrantly expressed in asthma is particularly intriguing. A novel mechanism to study bioenergetics in airway epithelial cells at air-liquid interface was recently described, in which cells were cultured on an in-house engineered insert for the Seahorse Extracellular Flux (XF24) Analyzer (Seahorse Bioscience Inc. North Billerica, MA) (Xu, Janocha et al. 2014). This technology enables measurements of oxygen consumption rate and extracellular acidification rate as indicators of cellular respiration and glycolysis, respectively (Xu, Janocha et al. 2014). This system would be of great interest to compare the metabolic regulation in differentiating epithelial cell cultures from asthmatic and healthy donors.

It is possible that the pathways we have identified are regulated by common transcription factors or epigenetic mechanisms. To identify such master regulators, we could utilize the Encyclopedia of DNA Elements (ENCODE) database, which uses data from chromatin immunoprecipitation (ChIP)-DNA sequencing experiments to identify transcription factors that are likely to regulate the genes in the user's input list (Consortium, Bernstein et al. 2012). In our case, we would search for regulators of specific pathways or clusters of genes.

2. Assess cilia function in EIB(+) and EIB(-) ALI cultures.

In addition to our findings here, other groups have also reported observations consistent with aberrant ciliated cell differentiation in asthma, including fewer ciliated cells in ALI cultures from pediatric donors (Parker, Sarlang et al. 2010) and ciliary dysfunction and structural abnormalities in *ex vivo* epithelial strips collected by brushing of severe asthmatics (Thomas, Rutman et al. 2010). We would aim to determine cilia beat frequency by microscopy, cilia ultrastructure by transmission electron microscopy, and expression at the protein level of cilia components using immunoblot and immunohistochemistry.

3. Explore phenotype-specific epithelial differences in ALI culture.

As described above, we observed more pronounced features of airway remodeling and transcriptional dysregulation in EIB(-) as compared to EIB(+) asthmatic epithelial cells, despite increased epithelial fragility as evidenced by increased numbers of columnar epithelial cells in induced sputum (Hallstrand, Moody et al. 2005). Furthermore, increased numbers of intraepithelial mast cells have been demonstrated in the airways of EIB(+) individuals (Lai,

Altemeier et al. 2014). Future studies will aim to elucidate mechanisms underlying these differences by assessing:

a) Baseline production of immunomodulatory factors such chemokines and growth factors, including known mast cell chemoattractants such as stem cell factor (SCF) (Al-Muhsen, Shablovsky et al. 2004), IL-33 and TSLP (Lai, Altemeier et al. 2014);

b) Expression of epithelial junction proteins relating to increased loss of airway epithelial cells and impaired transepithelial water transport from EIB(+) donors; and

c) Expression of aquaporins and solute transporters to assess the flux of solvents/solutes in EIB(+) epithelial cells in ALI culture, in the absence of exercise-induced drying of the airway surface liquid. Initial studies could focus on gene expression using the RNA sequencing data and RT-qPCR of RNA samples from ALI cultures, followed by analysis of protein expression by immunoblot of cell lysates and immunohistochemistry of FFPE tissue of ALI cultures.

References

Abdala-Valencia, H., J. Earwood, S. Bansal, M. Jansen, G. Babcock, B. Garvy, M. Wills-Karp and J. M. Cook-Mills (2007). "Nonhematopoietic NADPH oxidase regulation of lung eosinophilia and airway hyperresponsiveness in experimentally induced asthma." Am J Physiol Lung Cell Mol Physiol **292**(5): L1111-1125.

Abdel-Rahman, A. M., S. A. el-Sahrigy and S. I. Bakr (2006). "A comparative study of two angiogenic factors: vascular endothelial growth factor and angiogenin in induced sputum from asthmatic children in acute attack." Chest **129**(2): 266-271.

Acloque, H., M. S. Adams, K. Fishwick, M. Bronner-Fraser and M. A. Nieto (2009). "Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease." J Clin Invest **119**(6): 1438-1449.

Afzelius, B. A. (2004). "Cilia-related diseases." J Pathol **204**(4): 470-477.

Agrawal, A., A. Sinha, T. Ahmad, J. Aich, P. Singh, A. Sharma and B. Ghosh (2009). "Maladaptation of critical cellular functions in asthma: bioinformatic analysis." Physiol Genomics **40**(1): 1-7.

Al-Muhsen, S., S. Letuve, A. Vazquez-Tello, M. A. Pureza, H. Al-Jahdali, A. S. Bahammam, Q. Hamid and R. Halwani (2013). "Th17 cytokines induce pro-fibrotic cytokines release from human eosinophils." Respir Res **14**: 34.

Al-Muhsen, S. Z., G. Shablovsky, R. Olivenstein, B. Mazer and Q. Hamid (2004). "The expression of stem cell factor and c-kit receptor in human asthmatic airways." Clin Exp Allergy **34**(6): 911-916.

Allahverdian, S., N. Harada, G. K. Singhera, D. A. Knight and D. R. Dorscheid (2008). "Secretion of IL-13 by airway epithelial cells enhances epithelial repair via HB-EGF." Am J Respir Cell Mol Biol **38**(2): 153-160.

Alvi, A. J., H. Clayton, C. Joshi, T. Enver, A. Ashworth, M. Vivanco, T. C. Dale and M. J. Smalley (2003). "Functional and molecular characterisation of mammary side population cells." Breast Cancer Res **5**(1): R1-8.

Anderson, S. D. and E. Daviskas (2000). "The mechanism of exercise-induced asthma is." J Allergy Clin Immunol **106**(3): 453-459.

Ando, S., H. Otani, Y. Yagi, K. Kawai, H. Araki, S. Fukuhara and C. Inagaki (2007). "Proteinase-activated receptor 4 stimulation-induced epithelial-mesenchymal transition in alveolar epithelial cells." Respir Res **8**: 31.

Antonicelli, F., M. Parmentier, E. M. Drost, N. Hirani, I. Rahman, K. Donaldson and W. MacNee (2002). "Nacystelyn inhibits oxidant-mediated interleukin-8 expression and NF-kappaB nuclear binding in alveolar epithelial cells." Free Radic Biol Med **32**(6): 492-502.

Antonio, R. C., C. S. Ceron, E. Rizzi, E. B. Coelho, J. E. Tanus-Santos and R. F. Gerlach (2014). "Antioxidant effect of doxycycline decreases MMP activity and blood pressure in SHR." Mol Cell Biochem **386**(1-2): 99-105.

Apponi, L. H., A. H. Corbett and G. K. Pavlath (2011). "RNA-binding proteins and gene regulation in myogenesis." Trends Pharmacol Sci **32**(11): 652-658.

Arason, A. J., H. R. Jonsdottir, S. Halldorsson, B. E. Benediktsdottir, J. T. Bergthorsson, S. Ingthorsson, O. Baldursson, S. Sinha, T. Gudjonsson and M. K. Magnusson (2014). "deltaNp63 has a role in maintaining epithelial integrity in airway epithelium." PLoS One **9**(2): e88683.

Araya, J., S. Cambier, J. A. Markovics, P. Wolters, D. Jablons, A. Hill, W. Finkbeiner, K. Jones, V. C. Broaddus, D. Sheppard, A. Barzcak, Y. Xiao, D. J. Erle and S. L. Nishimura (2007). "Squamous metaplasia amplifies pathologic epithelial-mesenchymal interactions in COPD patients." J Clin Invest **117**(11): 3551-3562.

Bai, J., X. S. Liu, Y. J. Xu, Z. X. Zhang, M. Xie and W. Ni (2007). "Extracellular signal-regulated kinase activation in airway smooth muscle cell proliferation in chronic asthmatic rats." Sheng Li Xue Bao **59**(3): 311-318.

Bai, T. R. and D. A. Knight (2005). "Structural changes in the airways in asthma: observations and consequences." Clin Sci (Lond) **108**(6): 463-477.

Bakin, A. V., C. Rinehart, A. K. Tomlinson and C. L. Arteaga (2002). "p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration." J Cell Sci **115**(Pt 15): 3193-3206.

Baluk, P., C. G. Lee, H. Link, E. Ator, A. Haskell, J. A. Elias and D. M. McDonald (2004). "Regulated angiogenesis and vascular regression in mice overexpressing vascular endothelial growth factor in airways." Am J Pathol **165**(4): 1071-1085.

Bamberger, C., A. Hafner, H. Schmale and S. Werner (2005). "Expression of different p63 variants in healing skin wounds suggests a role of p63 in reepithelialization and muscle repair." Wound Repair Regen **13**(1): 41-50.

Bandapalli, O. R., S. Dihlmann, R. Helwa, S. Macher-Goeppinger, J. Weitz, P. Schirmacher and K. Brand (2009). "Transcriptional activation of the beta-catenin gene at the invasion front of colorectal liver metastases." J Pathol **218**(3): 370-379.

Baraket, M., B. G. Oliver, J. K. Burgess, S. Lim, G. G. King and J. L. Black (2012). "Is low dose inhaled corticosteroid therapy as effective for inflammation and remodeling in asthma? A randomized, parallel group study." Respir Res **13**: 11.

Barbato, A., G. Turato, S. Baraldo, E. Bazzan, F. Calabrese, C. Panizzolo, M. E. Zanin, R. Zuin, P. Maestrelli, L. M. Fabbri and M. Saetta (2006). "Epithelial damage and angiogenesis in the airways of children with asthma." Am J Respir Crit Care Med **174**(9): 975-981.

Barbato, A., G. Turato, S. Baraldo, E. Bazzan, F. Calabrese, M. Tura, R. Zuin, B. Beghe, P. Maestrelli, L. M. Fabbri and M. Saetta (2003). "Airway inflammation in childhood asthma." Am J Respir Crit Care Med **168**(7): 798-803.

Barbieri, C. E., L. J. Tang, K. A. Brown and J. A. Pietenpol (2006). "Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis." Cancer Res **66**(15): 7589-7597.

Baren, J. M., E. D. Boudreaux, B. E. Brenner, R. K. Cydulka, B. H. Rowe, S. Clark and C. A. Camargo, Jr. (2006). "Randomized controlled trial of emergency department interventions to improve primary care follow-up for patients with acute asthma." Chest **129**(2): 257-265.

Bartis, D., N. Mise, R. Y. Mahida, O. Eickelberg and D. R. Thickett (2014). "Epithelial-mesenchymal transition in lung development and disease: does it exist and is it important?" Thorax **69**(8): 760-765.

Basbaum, C. B., B. Jany and W. E. Finkbeiner (1990). "The serous cell." Annu Rev Physiol **52**: 97-113.

Bates, J. H., M. Rincon and C. G. Irvin (2009). "Animal models of asthma." Am J Physiol Lung Cell Mol Physiol **297**(3): L401-410.

Bates, R. C. and A. M. Mercurio (2003). "Tumor necrosis factor-alpha stimulates the epithelial-to-mesenchymal transition of human colonic organoids." Mol Biol Cell **14**(5): 1790-1800.

Bauer, T., N. Motosugi, K. Miura, H. Sabe and T. Hiiragi (2008). "Dynamic rearrangement of surface proteins is essential for cytokinesis." Genesis **46**(3): 152-162.

Baumann, J. M., K. W. Rundell, T. M. Evans and A. M. Levine (2005). "Effects of cysteine donor supplementation on exercise-induced bronchoconstriction." Med Sci Sports Exerc **37**(9): 1468-1473.

Bedouch, P., C. A. Marra, J. M. FitzGerald, L. D. Lynd and M. Sadatsafavi (2012). "Trends in asthma-related direct medical costs from 2002 to 2007 in British Columbia, Canada: a population based-cohort study." PLoS One **7**(12): e50949.

Behr, J., K. Maier, B. Degenkolb, F. Krombach and C. Vogelmeier (1997). "Antioxidative and clinical effects of high-dose N-acetylcysteine in fibrosing alveolitis. Adjunctive therapy to maintenance immunosuppression." Am J Respir Crit Care Med **156**(6): 1897-1901.

Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing." Journal of the Royal Statistical Society Series B-Methodological **57**(1): 289-300.

Berezikov, E., W. J. Chung, J. Willis, E. Cuppen and E. C. Lai (2007). "Mammalian mirtron genes." Mol Cell **28**(2): 328-336.

Bernacki, S. H., A. L. Nelson, L. Abdullah, J. K. Sheehan, A. Harris, C. W. Davis and S. H. Randell (1999). "Mucin gene expression during differentiation of human airway epithelia in vitro. Muc4 and muc5b are strongly induced." Am J Respir Cell Mol Biol **20**(4): 595-604.

Beuling, E., B. E. Aronson, L. M. Tran, K. A. Stapleton, E. N. ter Horst, L. A. Vissers, M. P. Verzi and S. D. Krasinski (2012). "GATA6 is required for proliferation, migration, secretory cell maturation, and gene expression in the mature mouse colon." Mol Cell Biol **32**(17): 3392-3402.

Bhattacharya, S., J. D. Jackson, A. V. Das, W. B. Thoreson, C. Kuszynski, J. James, S. Joshi and I. Ahmad (2003). "Direct identification and enrichment of retinal stem cells/progenitors by Hoechst dye efflux assay." Invest Ophthalmol Vis Sci **44**(6): 2764-2773.

Birkaya, B., K. Ortt and S. Sinha (2007). "Novel in vivo targets of DeltaNp63 in keratinocytes identified by a modified chromatin immunoprecipitation approach." BMC Mol Biol **8**: 43.

Blahna, M. T. and A. Hata (2013). "Regulation of miRNA biogenesis as an integrated component of growth factor signaling." Curr Opin Cell Biol **25**(2): 233-240.

Blaineau, C., M. Tessier, P. Dubessay, L. Tasse, L. Crobu, M. Pages and P. Bastien (2007). "A novel microtubule-depolymerizing kinesin involved in length control of a eukaryotic flagellum." Curr Biol **17**(9): 778-782.

Bleck, B., G. Grunig, A. Chiu, M. Liu, T. Gordon, A. Kazeros and J. Reibman (2013). "MicroRNA-375 regulation of thymic stromal lymphopoietin by diesel exhaust particles and ambient particulate matter in human bronchial epithelial cells." J Immunol **190**(7): 3757-3763.

Blesa, S., J. Cortijo, M. Mata, A. Serrano, D. Closa, F. Santangelo, J. M. Estrela, J. Suchankova and E. J. Morcillo (2003). "Oral N-acetylcysteine attenuates the rat pulmonary inflammatory response to antigen." Eur Respir J **21**(3): 394-400.

Blume, C., E. J. Swindle, P. Dennison, N. P. Jayasekera, S. Dudley, P. Monk, H. Behrendt, C. B. Schmidt-Weber, S. T. Holgate, P. H. Howarth, C. Traidl-Hoffmann and D. E. Davies (2013). "Barrier responses of human bronchial epithelial cells to grass pollen exposure." Eur Respir J **42**(1): 87-97.

Borthwick, D. W., M. Shahbazian, Q. T. Krantz, J. R. Dorin and S. H. Randell (2001). "Evidence for stem-cell niches in the tracheal epithelium." Am J Respir Cell Mol Biol **24**(6): 662-670.

Borthwick, L. A., A. Gardner, A. De Soyza, D. A. Mann and A. J. Fisher (2012). "Transforming Growth Factor-beta1 (TGF-beta1) Driven Epithelial to Mesenchymal Transition (EMT) is Accentuated by Tumour Necrosis Factor alpha (TNFalpha) via Crosstalk Between the SMAD and NF-kappaB Pathways." Cancer Microenviron **5**(1): 45-57.

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

Boulet, L. P., C. Legris, H. Turcotte and J. Hebert (1987). "Prevalence and characteristics of late asthmatic responses to exercise." J Allergy Clin Immunol **80**(5): 655-662.

Boxall, C., S. T. Holgate and D. E. Davies (2006). "The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma." Eur Respir J **27**(1): 208-229.

Braun-Fahrlander, C. (2003). "Environmental exposure to endotoxin and other microbial products and the decreased risk of childhood atopy: evaluating developments since April 2002." Curr Opin Allergy Clin Immunol **3**(5): 325-329.

Breuer, R., T. G. Christensen, E. C. Lucey, P. J. Stone and G. L. Snider (1987). "An ultrastructural morphometric analysis of elastase-treated hamster bronchi shows discharge followed by progressive accumulation of secretory granules." Am Rev Respir Dis **136**(3): 698-703.

Broekema, M., W. Timens, J. M. Vonk, F. Volbeda, M. E. Lodewijk, M. N. Hylkema, N. H. Ten Hacken and D. S. Postma (2011). "Persisting remodeling and less airway wall eosinophil activation in complete remission of asthma." Am J Respir Crit Care Med **183**(3): 310-316.

Brusselle, G. G., C. Vanderstichele, P. Jordens, R. Deman, H. Slabbynck, V. Ringoet, G. Verleden, I. K. Demedts, K. Verhamme, A. Delporte, B. Demeyere, G. Claeys, J. Boelens, E. Padalko, J. Verschakelen, G. Van Maele, E. Deschepper and G. F. Joos (2013). "Azithromycin for prevention of exacerbations in severe asthma (AZISAST): a multicentre randomised double-blind placebo-controlled trial." Thorax **68**(4): 322-329.

Bucchieri, F., S. M. Puddicombe, J. L. Lordan, A. Richter, D. Buchanan, S. J. Wilson, J. Ward, G. Zummo, P. H. Howarth, R. Djukanovic, S. T. Holgate and D. E. Davies (2002). "Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis." Am J Respir Cell Mol Biol **27**(2): 179-185.

Cabral, A. L., G. M. Conceicao, C. H. Fonseca-Guedes and M. A. Martins (1999). "Exercise-induced bronchospasm in children: effects of asthma severity." Am J Respir Crit Care Med **159**(6): 1819-1823.

Caliskan, M., Y. A. Bochkov, E. Kreiner-Moller, K. Bonnelykke, M. M. Stein, G. Du, H. Bisgaard, D. J. Jackson, J. E. Gern, R. F. Lemanske, Jr., D. L. Nicolae and C. Ober (2013). "Rhinovirus wheezing illness and genetic risk of childhood-onset asthma." N Engl J Med **368**(15): 1398-1407.

Camara, J. and G. Jarai (2010). "Epithelial-mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-alpha." Fibrogenesis Tissue Repair **3**(1): 2.

Candi, E., A. Rufini, A. Terrinoni, D. Dinsdale, M. Ranalli, A. Paradisi, V. De Laurenzi, L. G. Spagnoli, M. V. Catani, S. Ramadan, R. A. Knight and G. Melino (2006). "Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice." Cell Death Differ **13**(6): 1037-1047.

Cano, A., M. A. Perez-Moreno, I. Rodrigo, A. Locascio, M. J. Blanco, M. G. del Barrio, F. Portillo and M. A. Nieto (2000). "The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression." Nat Cell Biol **2**(2): 76-83.

Capetandes, A., M. Zhuang, F. N. Haque, L. Xie and M. Frieri (2007). "Vascular endothelial growth factor is increased by human pulmonary cells stimulated with Dermatophagoides sp. extract." Allergy Asthma Proc **28**(3): 324-330.

Cardoso, W. V. and J. Lu (2006). "Regulation of early lung morphogenesis: questions, facts and controversies." Development **133**(9): 1611-1624.

Carroll, D. K., J. S. Brugge and L. D. Attardi (2007). "p63, cell adhesion and survival." Cell Cycle **6**(3): 255-261.

Carroll, D. K., J. S. Carroll, C. O. Leong, F. Cheng, M. Brown, A. A. Mills, J. S. Brugge and L. W. Ellisen (2006). "p63 regulates an adhesion programme and cell survival in epithelial cells." Nat Cell Biol **8**(6): 551-561.

Carroll, N. G., S. Mutavdzic and A. L. James (2002). "Distribution and degranulation of airway mast cells in normal and asthmatic subjects." Eur Respir J **19**(5): 879-885.

Carver, E. A., R. Jiang, Y. Lan, K. F. Oram and T. Gridley (2001). "The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition." Mol Cell Biol **21**(23): 8184-8188.

Centers for Disease, C. and Prevention (2011). "Vital signs: asthma prevalence, disease characteristics, and self-management education: United States, 2001--2009." MMWR Morb Mortal Wkly Rep **60**(17): 547-552.

Chang, J. T. and J. R. Nevins (2006). "GATHER: a systems approach to interpreting genomic signatures." Bioinformatics **22**(23): 2926-2933.

Chatham, M., E. R. Bleecker, P. L. Smith, R. R. Rosenthal, P. Mason and P. S. Norman (1982). "A comparison of histamine, methacholine, and exercise airway reactivity in normal and asthmatic subjects." Am Rev Respir Dis **126**(2): 235-240.

Chhabra, S. K. and U. C. Ojha (1998). "Late asthmatic response in exercise-induced asthma." Ann Allergy Asthma Immunol **80**(4): 323-327.

Chin, L. Y., Y. Bosse, C. Pascoe, T. L. Hackett, C. Y. Seow and P. D. Pare (2012). "Mechanical properties of asthmatic airway smooth muscle." Eur Respir J **40**(1): 45-54.

Chu, H. W., S. Balzar, G. J. Seedorf, J. Y. Westcott, J. B. Trudeau, P. Silkoff and S. E. Wenzel (2004). "Transforming growth factor-beta2 induces bronchial epithelial mucin expression in asthma." Am J Pathol **165**(4): 1097-1106.

Ciulla, M. M., G. Acquistapace, L. Toffetti, R. Paliotti, S. Ferrero, F. Magrini and P. Braidotti (2009). Ki67 cytoplasmic expression: observations in normal tissue from heart atrial appendages of healthy rats. Cell Cycle. United States. **8:** 2125.

Cohen, L., X. E, J. Tarsi, T. Ramkumar, T. K. Horiuchi, R. Cochran, S. DeMartino, K. B. Schechtman, I. Hussain, M. J. Holtzman and M. Castro (2007). "Epithelial cell proliferation contributes to airway remodeling in severe asthma." Am J Respir Crit Care Med **176**(2): 138-145.

Collins, P. D., S. Marleau, D. A. Griffiths-Johnson, P. J. Jose and T. J. Williams (1995). "Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo." J Exp Med **182**(4): 1169-1174.

Collison, A., L. Hatchwell, N. Verrills, P. A. Wark, A. P. de Siqueira, M. Tooze, H. Carpenter, A. S. Don, J. C. Morris, N. Zimmermann, N. W. Bartlett, M. E. Rothenberg, S. L. Johnston, P. S. Foster and J. Mattes (2013). "The E3 ubiquitin ligase midline 1 promotes allergen and rhinovirus-induced asthma by inhibiting protein phosphatase 2A activity." Nat Med **19**(2): 232-237.

Consortium, E. P., B. E. Bernstein, E. Birney, I. Dunham, E. D. Green, C. Gunter and M. Snyder (2012). "An integrated encyclopedia of DNA elements in the human genome." Nature **489**(7414): 57-74.

Crapo, R. O., R. Casaburi, A. L. Coates, P. L. Enright, J. L. Hankinson, C. G. Irvin, N. R. MacIntyre, R. T. McKay, J. S. Wanger, S. D. Anderson, D. W. Cockcroft, J. E. Fish and P. J. Sterk (2000). "Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999." Am J Respir Crit Care Med **161**(1): 309-329.

Crook, T., J. M. Nicholls, L. Brooks, J. O'Nions and M. J. Allday (2000). "High level expression of deltaN-p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)?" Oncogene **19**(30): 3439-3444.

Crosby, L. M. and C. M. Waters (2010). "Epithelial repair mechanisms in the lung." Am J Physiol Lung Cell Mol Physiol **298**(6): L715-731.

Daniely, Y., G. Liao, D. Dixon, R. I. Linnoila, A. Lori, S. H. Randell, M. Oren and A. M. Jetten (2004). "Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium." Am J Physiol Cell Physiol **287**(1): C171-181.

Danilov, A. V., D. Neupane, A. S. Nagaraja, E. V. Feofanova, L. A. Humphries, J. DiRenzo and M. Korc (2011). "DeltaNp63alpha-mediated induction of epidermal growth factor receptor promotes pancreatic cancer cell growth and chemoresistance." PLoS One **6**(10): e26815.

Darby, I., O. Skalli and G. Gabbiani (1990). "Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing." Lab Invest **63**(1): 21-29.

Davenport, E. A. and P. Nettesheim (1996). "Regulation of mucociliary differentiation of rat tracheal epithelial cells by type I collagen gel substratum." Am J Respir Cell Mol Biol **14**(1): 19-26.

Davies, D. E. (2009). "The role of the epithelium in airway remodeling in asthma." Proc Am Thorac Soc **6**(8): 678-682.

Davies, D. E., J. Wicks, R. M. Powell, S. M. Puddicombe and S. T. Holgate (2003). "Airway remodeling in asthma: new insights." J Allergy Clin Immunol **111**(2): 215-225; quiz 226.

Davies, M., M. Robinson, E. Smith, S. Huntley, S. Prime and I. Paterson (2005). "Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways." J Cell Biochem **95**(5): 918-931.

Davis, B. N., A. C. Hilyard, G. Lagna and A. Hata (2008). "SMAD proteins control DROSHA-mediated microRNA maturation." Nature **454**(7200): 56-61.

Daviskas, E., S. D. Anderson, J. Shaw, S. Eberl, J. P. Seale, I. A. Yang and I. H. Young (2005). "Mucociliary clearance in patients with chronic asthma: effects of beta agonists." Respirology **10**(4): 426-435.

de Boer, W. I., H. S. Sharma, S. M. Baelemans, H. C. Hoogsteden, B. N. Lambrecht and G. J. Braunstahl (2008). "Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation." Can J Physiol Pharmacol **86**(3): 105-112.

de Jong, P. M., M. A. van Sterkenburg, S. C. Hesseling, J. A. Kempenaar, A. A. Mulder, A. M. Mommaas, J. H. Dijkman and M. Ponec (1994). "Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface." Am J Respir Cell Mol Biol **10**(3): 271-277.

de Kluijver, J., J. A. Schrumpf, C. E. Evertse, J. K. Sont, P. J. Roughley, K. F. Rabe, P. S. Hiemstra, T. Mauad and P. J. Sterk (2005). "Bronchial matrix and inflammation respond to inhaled steroids despite ongoing allergen exposure in asthma." Clin Exp Allergy **35**(10): 1361-1369.

Devulapalli, C. S., K. C. Lodrup Carlsen, G. Haland, M. C. Munthe-Kaas, M. Pettersen, P. Mowinckel and K. H. Carlsen (2007). "No evidence that early use of inhaled corticosteroids reduces current asthma at 10 years of age." Respir Med **101**(8): 1625-1632.

Di Como, C. J., M. J. Urist, I. Babayan, M. Drobnjak, C. V. Hedvat, J. Teruya-Feldstein, K. Pohar, A. Hoos and C. Cordon-Cardo (2002). "p63 expression profiles in human normal and tumor tissues." Clin Cancer Res **8**(2): 494-501.

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

Dougherty, R. H., S. S. Sidhu, K. Raman, M. Solon, O. D. Solberg, G. H. Caughey, P. G. Woodruff and J. V. Fahy (2010). "Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma." J Allergy Clin Immunol **125**(5): 1046-1053 e1048.

Dranka, B. P., G. A. Benavides, A. R. Diers, S. Giordano, B. R. Zelickson, C. Reily, L. Zou, J. C. Chatham, B. G. Hill, J. Zhang, A. Landar and V. M. Darley-Usmar (2011). "Assessing bioenergetic function in response to oxidative stress by metabolic profiling." Free Radic Biol Med **51**(9): 1621-1635.

Drewelus, I., C. Gopfert, C. Hippel, A. Dickmanns, K. Damianitsch, T. Pieler and M. Dobbelstein (2010). "p63 antagonizes Wnt-induced transcription." Cell Cycle **9**(3): 580-587.

Dvorak, A., A. E. Tilley, R. Shaykhiev, R. Wang and R. G. Crystal (2011). "Do airway epithelium air-liquid cultures represent the in vivo airway epithelium transcriptome?" Am J Respir Cell Mol Biol **44**(4): 465-473.

El-Zein, M., M. E. Parent, J. Siemiatycki and M. C. Rousseau (2014). "History of allergic diseases and lung cancer risk." Ann Allergy Asthma Immunol.

Ellenrieder, V., S. F. Hendler, W. Boeck, T. Seufferlein, A. Menke, C. Ruhland, G. Adler and T. M. Gress (2001). "Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation." Cancer Res **61**(10): 4222-4228.

Engelhardt, J. F., H. Schlossberg, J. R. Yankaskas and L. Dudus (1995). "Progenitor cells of the adult human airway involved in submucosal gland development." Development **121**(7): 2031-2046.

Erjefalt, J. S., I. Erjefalt, F. Sundler and C. G. Persson (1995). "In vivo restitution of airway epithelium." Cell Tissue Res **281**(2): 305-316.

Erjefalt, J. S., M. Korsgren, M. C. Nilsson, F. Sundler and C. G. Persson (1997a). "Association between inflammation and epithelial damage-restitution processes in allergic airways in vivo." Clin Exp Allergy **27**(11): 1344-1355.

Erjefalt, J. S., M. Korsgren, M. C. Nilsson, F. Sundler and C. G. Persson (1997b). "Prompt epithelial damage and restitution processes in allergen challenged guinea-pig trachea in vivo." Clin Exp Allergy **27**(12): 1458-1470.

Evans, M. J., M. V. Fanucchi, L. A. Miller, M. A. Carlson, S. J. Nishio and D. M. Hyde (2010). "Reduction of collagen VII anchoring fibrils in the airway basement membrane zone of infant rhesus monkeys exposed to house dust mite." Am J Physiol Lung Cell Mol Physiol **298**(4): L543-547.

Evans, M. J., M. V. Fanucchi, C. G. Plopper and D. M. Hyde (2010). "Postnatal development of the lamina reticularis in primate airways." Anat Rec (Hoboken) **293**(6): 947-954.

Evans, M. J. and C. G. Plopper (1988). "The role of basal cells in adhesion of columnar epithelium to airway basement membrane." Am Rev Respir Dis **138**(2): 481-483.

Fanucchi, M. V., A. Bracher, S. F. Doran, G. L. Squadrito, S. Fernandez, E. M. Postlethwait, L. Bowen and S. Matalon (2012). "Post-exposure antioxidant treatment in rats decreases airway hyperplasia and hyperreactivity due to chlorine inhalation." Am J Respir Cell Mol Biol **46**(5): 599-606.

Faratian, D., A. Munro, C. Twelves and J. M. Bartlett (2009). Membranous and cytoplasmic staining of Ki67 is associated with HER2 and ER status in invasive breast carcinoma. Histopathology. England. **54:** 254-257.

Favre, C., A. Zhdanov, M. Leahy, D. Papkovsky and R. O'Connor (2010). "Mitochondrial pyrimidine nucleotide carrier (PNC1) regulates mitochondrial biogenesis and the invasive phenotype of cancer cells." Oncogene **29**(27): 3964-3976.

Fedorov, I. A., S. J. Wilson, D. E. Davies and S. T. Holgate (2005). "Epithelial stress and structural remodelling in childhood asthma." Thorax **60**(5): 389-394.

Filipowicz, W., S. N. Bhattacharyya and N. Sonenberg (2008). "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" Nat Rev Genet **9**(2): 102-114.

Finkbeiner, W. E. (1999). "Physiology and pathology of tracheobronchial glands." Respir Physiol **118**(2-3): 77-83.

Firszt, R., D. Francisco, T. D. Church, J. M. Thomas, J. L. Ingram and M. Kraft (2013). "Interleukin-13 induces collagen type-1 expression through matrix metalloproteinase-2 and transforming growth factor-beta1 in airway fibroblasts in asthma." Eur Respir J.

Fitzpatrick, A. M., C. E. Baena-Cagnani and L. B. Bacharier (2012). "Severe asthma in childhood: recent advances in phenotyping and pathogenesis." Curr Opin Allergy Clin Immunol **12**(2): 193-201.

Flaquer, A., A. Heinzmann, S. Rospleszcz, B. Mailaparambil, H. Dietrich, K. Strauch and R. Grychtol (2014). "Association study of mitochondrial genetic polymorphisms in asthmatic children." Mitochondrion **14**(1): 49-53.

Folkard, S. G., J. Westwick and A. B. Millar (1997). "Production of interleukin-8, RANTES and MCP-1 in intrinsic and extrinsic asthmatics." Eur Respir J **10**(9): 2097-2104.

Ford, J. R. and M. Terzaghi-Howe (1992). "Basal cells are the progenitors of primary tracheal epithelial cell cultures." Exp Cell Res **198**(1): 69-77.

Foresi, A., G. Bertorelli, A. Pesci, A. Chetta and D. Olivieri (1990). "Inflammatory markers in bronchoalveolar lavage and in bronchial biopsy in asthma during remission." Chest **98**(3): 528-535.

Forino, M., R. Torregrossa, M. Ceol, L. Murer, M. D. Vella, D. D. Prete, A. D'Angelo and F. Anglani (2006). "TGFbeta1 induces epithelial-mesenchymal transition, but not myofibroblast transdifferentiation of human kidney tubular epithelial cells in primary culture." Int J Exp Pathol **87**(3): 197-208.

Freishtat, R. J., A. M. Watson, A. S. Benton, S. F. Iqbal, D. K. Pillai, M. C. Rose and E. P. Hoffman (2010). "Asthmatic Airway Epithelium is Intrinsically Inflammatory and Mitotically Dyssynchronous." Am J Respir Cell Mol Biol.

Freishtat, R. J., A. M. Watson, A. S. Benton, S. F. Iqbal, D. K. Pillai, M. C. Rose and E. P. Hoffman (2011). "Asthmatic airway epithelium is intrinsically inflammatory and mitotically dyssynchronous." Am J Respir Cell Mol Biol **44**(6): 863-869.

Fulcher, M. L., S. Gabriel, K. A. Burns, J. R. Yankaskas and S. H. Randell (2005). "Well-differentiated human airway epithelial cell cultures." Methods Mol Med **107**: 183-206.

Gal, A., T. Sjoblom, L. Fedorova, S. Imreh, H. Beug and A. Moustakas (2008). "Sustained TGF beta exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis." Oncogene **27**(9): 1218-1230.

Galli, S. J., J. Kalesnikoff, M. A. Grimbaldeston, A. M. Piliponsky, C. M. Williams and M. Tsai (2005). "Mast cells as "tunable" effector and immunoregulatory cells: recent advances." Annu Rev Immunol **23**: 749-786.

Garcia-Zepeda, E. A., M. E. Rothenberg, R. T. Ownbey, J. Celestin, P. Leder and A. D. Luster (1996). "Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia." Nat Med **2**(4): 449-456.

Ge, Q., L. M. Moir, J. L. Black, B. G. Oliver and J. K. Burgess (2010). "TGFβ1 induces IL-6 and inhibits IL-8 release in human bronchial epithelial cells: The role of Smad2/3." Journal of Cellular Physiology **225**(3): 846-854.

Giangreco, A., E. N. Arwert, I. R. Rosewell, J. Snyder, F. M. Watt and B. R. Stripp (2009). "Stem cells are dispensable for lung homeostasis but restore airways after injury." Proc Natl Acad Sci U S A **106**(23): 9286-9291.

Giangreco, A., L. Lu, C. Vickers, V. H. Teixeira, K. R. Groot, C. R. Butler, E. V. Ilieva, P. J. George, A. G. Nicholson, E. K. Sage, F. M. Watt and S. M. Janes (2012). "beta-Catenin

determines upper airway progenitor cell fate and preinvasive squamous lung cancer progression by modulating epithelial-mesenchymal transition." J Pathol **226**(4): 575-587.

Gizycki, M. J., E. Adelroth, A. V. Rogers, P. M. O'Byrne and P. K. Jeffery (1997). "Myofibroblast involvement in the allergen-induced late response in mild atopic asthma." Am J Respir Cell Mol Biol **16**(6): 664-673.

Global Initiative for Asthma, P. (2012a, December 2012). "Global Strategy for Asthma Management and Prevention." Retrieved July 12, 2013, from http://www.ginasthma.org/local/uploads/files/GINA_Report_March13.pdf.

Global Initiative for Asthma, P. (2012b, December 2012). "Pocket Guide for Asthma Management and Prevention." Retrieved July 12, 2013, from http://www.ginasthma.org/local/uploads/files/GINA_Pocket2013_May15.pdf.

Gomperts, B. N., J. A. Belperio, P. N. Rao, S. H. Randell, M. C. Fishbein, M. D. Burdick and R. M. Strieter (2006). "Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury." J Immunol **176**(3): 1916-1927.

Gras, D., A. Bourdin, I. Vachier, L. de Senneville, C. Bonnans and P. Chanez (2012). "An ex vivo model of severe asthma using reconstituted human bronchial epithelium." J Allergy Clin Immunol **129**(5): 1259-1266 e1251.

Gray, T. E., K. Guzman, C. W. Davis, L. H. Abdullah and P. Nettesheim (1996). "Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells." Am J Respir Cell Mol Biol **14**(1): 104-112.

Green, F. H., J. C. Butt, A. L. James and N. G. Carroll (2006). "Abnormalities of the bronchial arteries in asthma." Chest **130**(4): 1025-1033.

Greenburg, G. and E. D. Hay (1982). "Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells." J Cell Biol **95**(1): 333-339.

Gregory, R. I., T. P. Chendrimada, N. Cooch and R. Shiekhattar (2005). "Human RISC couples microRNA biogenesis and posttranscriptional gene silencing." Cell **123**(4): 631-640.

Gregory, R. I., K. P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch and R. Shiekhattar (2004). "The Microprocessor complex mediates the genesis of microRNAs." Nature **432**(7014): 235-240.

Greiling, D. and R. A. Clark (1997). "Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix." J Cell Sci **110 (Pt 7)**: 861-870.

Guilbert, T. W., W. J. Morgan, R. S. Zeiger, D. T. Mauger, S. J. Boehmer, S. J. Szefler, L. B. Bacharier, R. F. Lemanske, Jr., R. C. Strunk, D. B. Allen, G. R. Bloomberg, G. Heldt, M. Krawiec, G. Larsen, A. H. Liu, V. M. Chinchilli, C. A. Sorkness, L. M. Taussig and F. D. Martinez (2006). "Long-term inhaled corticosteroids in preschool children at high risk for asthma." N Engl J Med **354**(19): 1985-1997.

Guillot, C. and T. Lecuit (2013). "Adhesion disengagement uncouples intrinsic and extrinsic forces to drive cytokinesis in epithelial tissues." Dev Cell **24**(3): 227-241.

Guseh, J. S., S. A. Bores, B. Z. Stanger, Q. Zhou, W. J. Anderson, D. A. Melton and J. Rajagopal (2009). "Notch signaling promotes airway mucous metaplasia and inhibits alveolar development." Development **136**(10): 1751-1759.

Haahtela, T., K. Tamminen, T. Kava, L. P. Malmberg, P. Rytila, K. Nikander, T. Persson and O. Selroos (2009). "Thirteen-year follow-up of early intervention with an inhaled corticosteroid in patients with asthma." J Allergy Clin Immunol **124**(6): 1180-1185.

Haby, M. M., S. D. Anderson, J. K. Peat, C. M. Mellis, B. G. Toelle and A. J. Woolcock (1994). "An exercise challenge protocol for epidemiological studies of asthma in children: comparison with histamine challenge." Eur Respir J **7**(1): 43-49.

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

Hackett T-L, W. S., Shaheen F, Pechkovsky DV, Argenteri R, Murray L, Kicic A, Stick S, Knight DA (2008). "TGF-beta-induces epithelial-mesenchymal-transition in primary human bronchial epithelial cells from normal and asthmatic subjects." Am J Respir Crit Care Med **177:A97**: 239.

Hackett, T. L. (2012). "Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma." Curr Opin Allergy Clin Immunol **12**(1): 53-59.

Hackett, T. L., H. G. de Bruin, F. Shaheen, M. van den Berge, A. J. van Oosterhout, D. S. Postma and I. H. Heijink (2013). "Caveolin-1 controls airway epithelial barrier function. Implications for asthma." Am J Respir Cell Mol Biol **49**(4): 662-671.

Hackett, T. L., F. Shaheen, A. Johnson, S. Wadsworth, D. V. Pechkovsky, D. B. Jacoby, A. Kicic, S. M. Stick and D. A. Knight (2008). "Characterization of side population cells from human airway epithelium." Stem Cells **26**(10): 2576-2585.

Hackett, T. L., G. K. Singhera, F. Shaheen, P. Hayden, G. R. Jackson, R. G. Hegele, S. Van Eeden, T. R. Bai, D. R. Dorscheid and D. A. Knight (2011). "Intrinsic phenotypic differences of asthmatic epithelium and its inflammatory responses to respiratory syncytial virus and air pollution." Am J Respir Cell Mol Biol **45**(5): 1090-1100.

Hackett, T. L., S. M. Warner, D. Stefanowicz, F. Shaheen, D. V. Pechkovsky, L. A. Murray, R. Argentieri, A. Kicic, S. M. Stick, T. R. Bai and D. A. Knight (2009). "Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1." Am J Respir Crit Care Med **180**(2): 122-133.

Hajj, R., T. Baranek, R. Le Naour, P. Lesimple, E. Puchelle and C. Coraux (2007). "Basal cells of the human adult airway surface epithelium retain transit-amplifying cell properties." Stem Cells **25**(1): 139-148.

Hajj, R., P. Lesimple, B. Nawrocki-Raby, P. Birembaut, E. Puchelle and C. Coraux (2007). "Human airway surface epithelial regeneration is delayed and abnormal in cystic fibrosis." J Pathol **211**(3): 340-350.

Halayko, A. J., T. Tran, S. Y. Ji, A. Yamasaki and R. Gosens (2006). "Airway smooth muscle phenotype and function: interactions with current asthma therapies." Curr Drug Targets **7**(5): 525-540.

Halim, T. Y., R. H. Krauss, A. C. Sun and F. Takei (2012). "Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation." Immunity **36**(3): 451-463.

Hallstrand, T. S., E. Y. Chi, A. G. Singer, M. H. Gelb and W. R. Henderson, Jr. (2007). "Secreted phospholipase A2 group X overexpression in asthma and bronchial hyperresponsiveness." Am J Respir Crit Care Med **176**(11): 1072-1078.

Hallstrand, T. S., J. S. Debley, F. M. Farin and W. R. Henderson, Jr. (2007). "Role of MUC5AC in the pathogenesis of exercise-induced bronchoconstriction." J Allergy Clin Immunol **119**(5): 1092-1098.

Hallstrand, T. S., P. Kippelen, J. Larsson, V. Bougault, J. C. van Leeuwen, J. M. Driessen and J. D. Brannan (2013). "Where to from here for exercise-induced bronchoconstriction: the unanswered questions." Immunol Allergy Clin North Am **33**(3): 423-442.

Hallstrand, T. S., Y. Lai, W. A. Altemeier, C. L. Appel, B. Johnson, C. W. Frevert, K. L. Hudkins, J. G. Bollinger, P. G. Woodruff, D. M. Hyde, W. R. Henderson, Jr. and M. H. Gelb (2013). "Regulation and function of epithelial secreted phospholipase A2 group x in asthma." Am J Respir Crit Care Med **188**(1): 42-50.

Hallstrand, T. S., Y. Lai, W. R. Henderson, Jr., W. A. Altemeier and M. H. Gelb (2012). "Epithelial regulation of eicosanoid production in asthma." Pulm Pharmacol Ther **25**(6): 432-437.

Hallstrand, T. S., M. W. Moody, M. L. Aitken and W. R. Henderson, Jr. (2005). "Airway immunopathology of asthma with exercise-induced bronchoconstriction." J Allergy Clin Immunol **116**(3): 586-593.

Hallstrand, T. S., M. W. Moody, M. M. Wurfel, L. B. Schwartz, W. R. Henderson, Jr. and M. L. Aitken (2005). "Inflammatory basis of exercise-induced bronchoconstriction." Am J Respir Crit Care Med **172**(6): 679-686.

Hallstrand, T. S., M. M. Wurfel, Y. Lai, Z. Ni, M. H. Gelb, W. A. Altemeier, R. P. Beyer, M. L. Aitken and W. R. Henderson (2010). "Transglutaminase 2, a novel regulator of eicosanoid production in asthma revealed by genome-wide expression profiling of distinct asthma phenotypes." PLoS One **5**(1): e8583.

Hamilton, L. M., S. M. Puddicombe, R. J. Dearman, I. Kimber, T. Sandstrom, A. Wallin, P. H. Howarth, S. T. Holgate, S. J. Wilson and D. E. Davies (2005). "Altered protein tyrosine phosphorylation in asthmatic bronchial epithelium." Eur Respir J **25**(6): 978-985.

Hammad, H., M. Chieppa, F. Perros, M. A. Willart, R. N. Germain and B. N. Lambrecht (2009). "House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells." Nat Med **15**(4): 410-416.

Hao, K., Y. Bosse, D. C. Nickle, P. D. Pare, D. S. Postma, M. Laviolette, A. Sandford, T. L. Hackett, D. Daley, J. C. Hogg, W. M. Elliott, C. Couture, M. Lamontagne, C. A. Brandsma, M.

van den Berge, G. Koppelman, A. S. Reicin, D. W. Nicholson, V. Malkov, J. M. Derry, C. Suver, J. A. Tsou, A. Kulkarni, C. Zhang, R. Vessey, G. J. Opiteck, S. P. Curtis, W. Timens and D. D. Sin (2012). "Lung eQTLs to help reveal the molecular underpinnings of asthma." PLoS Genet **8**(11): e1003029.

Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy and C. T. Weaver (2005). "Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages." Nat Immunol **6**(11): 1123-1132.

Hartsough, M. T. and K. M. Mulder (1995). "Transforming growth factor beta activation of p44mapk in proliferating cultures of epithelial cells." J Biol Chem **270**(13): 7117-7124.

Hashimoto, S., K. Matsumoto, Y. Gon, T. Nakayama, I. Takeshita and T. Horie (1999). "Hyperosmolarity-induced interleukin-8 expression in human bronchial epithelial cells through p38 mitogen-activated protein kinase." Am J Respir Crit Care Med **159**(2): 634-640.

He, J. Q., T. S. Hallstrand, D. Knight, M. Chan-Yeung, A. Sandford, B. Tripp, D. Zamar, Y. Bosse, A. L. Kozyrskyj, A. James, C. Laprise and D. Daley (2009). "A thymic stromal lymphopoietin gene variant is associated with asthma and airway hyperresponsiveness." J Allergy Clin Immunol **124**(2): 222-229.

Hegab, A. E., D. W. Nickerson, V. L. Ha, D. O. Darmawan and B. N. Gomperts (2012). "Repair and regeneration of tracheal surface epithelium and submucosal glands in a mouse model of hypoxic-ischemic injury." Respirology **17**(7): 1101-1113.

Heijink, I. H., P. M. Kies, H. F. Kauffman, D. S. Postma, A. J. van Oosterhout and E. Vellenga (2007). "Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity." J Immunol **178**(12): 7678-7685.

Heijink, I. H., D. S. Postma, J. A. Noordhoek, M. Broekema and A. Kapus (2009). "House Dust Mite-promoted Epithelial-to-mesenchymal Transition in Human Bronchial Epithelium." Am. J. Respir. Cell Mol. Biol.: 2008-0449OC.

Heijink, I. H., A. van Oosterhout and A. Kapus (2010). "Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction." Eur Respir J **36**(5): 1016-1026.

Herder, C., J. M. Swiercz, C. Muller, R. Peravali, R. Quiring, S. Offermanns, J. Wittbrodt and F. Loosli (2013). "ArhGEF18 regulates RhoA-Rock2 signaling to maintain neuro-epithelial apico-basal polarity and proliferation." Development **140**(13): 2787-2797.

Hermiston, M. L., M. H. Wong and J. I. Gordon (1996). "Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system." Genes Dev **10**(8): 985-996.

Higgins, B. G., J. R. Britton, S. Chinn, T. D. Jones, A. S. Vathenen, P. G. Burney and A. E. Tattersfield (1988). "Comparison of histamine and methacholine for use in bronchial challenge tests in community studies." Thorax **43**(8): 605-610.

Hilding, A. C. (1943). "The role of ciliary insufficiency to death from asthma and other respiratory diseases." Ann Otol Rhin Laryngol **52**: 5-19.

Hinz, B. (2010). "The myofibroblast: paradigm for a mechanically active cell." J Biomech **43**(1): 146-155.

Hirota, J. A., T. L. Hackett, M. D. Inman and D. A. Knight (2011). "Modeling asthma in mice: what have we learned about the airway epithelium?" Am J Respir Cell Mol Biol **44**(4): 431-438.

Hogan, S. P., H. F. Rosenberg, R. Moqbel, S. Phipps, P. S. Foster, P. Lacy, A. B. Kay and M. E. Rothenberg (2008). "Eosinophils: biological properties and role in health and disease." Clin Exp Allergy **38**(5): 709-750.

Hoh, R. A., T. R. Stowe, E. Turk and T. Stearns (2012). "Transcriptional program of ciliated epithelial cells reveals new cilium and centrosome components and links to human disease." PLoS One **7**(12): e52166.

Holgate, S. T. (2002). "Airway inflammation and remodeling in asthma: current concepts." Mol Biotechnol **22**(2): 179-189.

Holgate, S. T. (2007). "The epithelium takes centre stage in asthma and atopic dermatitis." Trends Immunol **28**(6): 248-251.

Holgate, S. T., D. E. Davies, P. M. Lackie, S. J. Wilson, S. M. Puddicombe and J. L. Lordan (2000). "Epithelial-mesenchymal interactions in the pathogenesis of asthma." J Allergy Clin Immunol **105**(2 Pt 1): 193-204.

Holgate, S. T., D. E. Davies, S. Puddicombe, A. Richter, P. Lackie, J. Lordan and P. Howarth (2003). "Mechanisms of airway epithelial damage: epithelial-mesenchymal interactions in the pathogenesis of asthma." Eur Respir J Suppl **44**: 24s-29s.

Holmes, A. M., R. Solari and S. T. Holgate (2011). "Animal models of asthma: value, limitations and opportunities for alternative approaches." Drug Discov Today **16**(15-16): 659-670.

Holt, P. G. and P. D. Sly (2012). "Viral infections and atopy in asthma pathogenesis: new rationales for asthma prevention and treatment." Nat Med **18**(5): 726-735.

Hong, K. U., S. D. Reynolds, A. Giangreco, C. M. Hurley and B. R. Stripp (2001). "Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion." Am J Respir Cell Mol Biol **24**(6): 671-681.

Hong, K. U., S. D. Reynolds, S. Watkins, E. Fuchs and B. R. Stripp (2004a). "Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium." Am J Pathol **164**: 577 - 588.

Hong, K. U., S. D. Reynolds, S. Watkins, E. Fuchs and B. R. Stripp (2004b). "In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations." Am J Physiol Lung Cell Mol Physiol **286**: L643 - L649.

Huang, G. T., C. Athanassiou and P. V. Benos (2011). "mirConnX: condition-specific mRNA-microRNA network integrator." Nucleic Acids Res **39**(Web Server issue): W416-423.

Huang, J., R. Olivenstein, R. Taha, Q. Hamid and M. Ludwig (1999). "Enhanced proteoglycan deposition in the airway wall of atopic asthmatics." Am J Respir Crit Care Med **160**(2): 725-729.

Huebener, P., T. Abou-Khamis, P. Zymek, M. Bujak, X. Ying, K. Chatila, S. Haudek, G. Thakker and N. G. Frangogiannis (2008). "CD44 is critically involved in infarct healing by regulating the inflammatory and fibrotic response." J Immunol **180**(4): 2625-2633.

Hutvagner, G., J. McLachlan, A. E. Pasquinelli, E. Balint, T. Tuschl and P. D. Zamore (2001). "A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA." Science **293**(5531): 834-838.

Ichikawa, T., Y. Suenaga, T. Koda, T. Ozaki and A. Nakagawara (2008). "DeltaNp63/BMP-7-dependent expression of matrilin-2 is involved in keratinocyte migration in response to wounding." Biochem Biophys Res Commun **369**(4): 994-1000.

Ingram, J. L., M. J. Huggins, T. D. Church, Y. Li, D. C. Francisco, S. Degan, R. Firszt, D. M. Beaver, N. L. Lugogo, Y. Wang, M. E. Sunday, P. W. Noble and M. Kraft (2011). "Airway fibroblasts in asthma manifest an invasive phenotype." Am J Respir Crit Care Med **183**(12): 1625-1632.

Ishikawa, H. and W. F. Marshall (2011). "Ciliogenesis: building the cell's antenna." Nat Rev Mol Cell Biol **12**(4): 222-234.

Jackson, D. J., M. D. Evans, R. E. Gangnon, C. J. Tisler, T. E. Pappas, W. M. Lee, J. E. Gern and R. F. Lemanske, Jr. (2012). "Evidence for a causal relationship between allergic sensitization and rhinovirus wheezing in early life." Am J Respir Crit Care Med **185**(3): 281-285.

James, A. (2005). "Remodelling of airway smooth muscle in asthma: what sort do you have?" Clin Exp Allergy **35**(6): 703-707.

James, A. L. and S. Wenzel (2007). "Clinical relevance of airway remodelling in airway diseases." Eur Respir J **30**(1): 134-155.

Jardim, M. J., L. Dailey, R. Silbajoris and D. Diaz-Sanchez (2012). "Distinct microRNA expression in human airway cells of asthmatic donors identifies a novel asthma-associated gene." Am J Respir Cell Mol Biol **47**(4): 536-542.

Jeffery, P. K. (1983). "Morphologic features of airway surface epithelial cells and glands." Am Rev Respir Dis **128**(2 Pt 2): S14-20.

Jeffery, P. K., A. J. Wardlaw, F. C. Nelson, J. V. Collins and A. B. Kay (1989). "Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity." Am Rev Respir Dis **140**(6): 1745-1753.

Johnson, J. R., A. Roos, T. Berg, M. Nord and J. Fuxe (2011). "Chronic respiratory aeroallergen exposure in mice induces epithelial-mesenchymal transition in the large airways." PLoS One **6**(1): e16175.

Juniper, E. F., P. A. Frith, C. Dunnett, D. W. Cockcroft and F. E. Hargreave (1978). "Reproducibility and comparison of responses to inhaled histamine and methacholine." Thorax **33**(6): 705-710.

Kaimori, A., J. Potter, J. Y. Kaimori, C. Wang, E. Mezey and A. Koteish (2007). "Transforming growth factor-beta1 induces an epithelial-to-mesenchymal transition state in mouse hepatocytes in vitro." J Biol Chem **282**(30): 22089-22101.

Kalluri, R. (2009). "EMT: when epithelial cells decide to become mesenchymal-like cells." J Clin Invest **119**(6): 1417-1419.

Kalluri, R. and E. G. Neilson (2003). "Epithelial-mesenchymal transition and its implications for fibrosis." J Clin Invest **112**(12): 1776-1784.

Karjalainen, E. M., A. Lindqvist, L. A. Laitinen, T. Kava, A. Altraja, M. Halme and A. Laitinen (2003). "Airway inflammation and basement membrane tenascin in newly diagnosed atopic and nonatopic asthma." Respir Med **97**(9): 1045-1051.

Kasai, H., J. T. Allen, R. M. Mason, T. Kamimura and Z. Zhang (2005). "TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT)." Respir Res **6**: 56.

Kato, A., S. Favoreto, Jr., P. C. Avila and R. P. Schleimer (2007). "TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells." J Immunol **179**(2): 1080-1087.

Kato, A. and R. P. Schleimer (2007). "Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity." Curr Opin Immunol **19**(6): 711-720.

Kawasaki, S., H. Tanioka, K. Yamasaki, C. J. Connon and S. Kinoshita (2006). Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. Exp Eye Res. England. **82:** 293-299.

Kay, A. B., S. Phipps and D. S. Robinson (2004). "A role for eosinophils in airway remodelling in asthma." Trends Immunol **25**(9): 477-482.

Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon and R. H. Plasterk (2001). "Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans." Genes Dev **15**(20): 2654-2659.

Kicic, A., T. S. Hallstrand, E. N. Sutanto, P. T. Stevens, M. S. Kobor, C. Taplin, P. D. Pare, R. P. Beyer, S. M. Stick and D. A. Knight (2010). "Decreased fibronectin production significantly contributes to dysregulated repair of asthmatic epithelium." Am J Respir Crit Care Med **181**(9): 889-898.

Kicic, A., E. N. Sutanto, P. T. Stevens, D. A. Knight and S. M. Stick (2006). "Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma." Am J Respir Crit Care Med **174**(10): 1110-1118.

Kilic, A., S. S. Sonar, A. O. Yildirim, H. Fehrenbach, W. A. Nockher and H. Renz (2011). "Nerve growth factor induces type III collagen production in chronic allergic airway inflammation." J Allergy Clin Immunol **128**(5): 1058-1066 e1051-1054.

Kim, D., G. Pertea, C. Trapnell, H. Pimentel, R. Kelley and S. L. Salzberg (2013). "TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions." Genome Biol **14**(4): R36.

Kim, K. K., M. C. Kugler, P. J. Wolters, L. Robillard, M. G. Galvez, A. N. Brumwell, D. Sheppard and H. A. Chapman (2006). "Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix." Proc Natl Acad Sci U S A **103**(35): 13180-13185.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat Rev Mol Cell Biol **10**(2): 126-139.

King, K. E., R. M. Ponnamperuma, M. J. Gerdes, T. Tokino, T. Yamashita, C. C. Baker and W. C. Weinberg (2006). "Unique domain functions of p63 isotypes that differentially regulate distinct aspects of epidermal homeostasis." Carcinogenesis **27**(1): 53-63.

King, K. E., R. M. Ponnamperuma, T. Yamashita, T. Tokino, L. A. Lee, M. F. Young and W. C. Weinberg (2003). "deltaNp63alpha functions as both a positive and a negative transcriptional regulator and blocks in vitro differentiation of murine keratinocytes." Oncogene **22**(23): 3635-3644.

Knight, D. A. and S. T. Holgate (2003). "The airway epithelium: structural and functional properties in health and disease." Respirology **8**(4): 432-446.

Knox, P., S. Crooks and C. S. Rimmer (1986). "Role of fibronectin in the migration of fibroblasts into plasma clots." J Cell Biol **102**(6): 2318-2323.

Kohl, M., S. Wiese and B. Warscheid (2011). "Cytoscape: software for visualization and analysis of biological networks." Methods Mol Biol **696**: 291-303.

Kojima, T., K. Takano, T. Yamamoto, M. Murata, S. Son, M. Imamura, H. Yamaguchi, M. Osanai, H. Chiba, T. Himi and N. Sawada (2008). "Transforming growth factor-beta induces epithelial to mesenchymal transition by down-regulation of claudin-1 expression and the fence function in adult rat hepatocytes." Liver Int **28**(4): 534-545.

Koster, M. I., S. Kim, A. A. Mills, F. J. DeMayo and D. R. Roop (2004). "p63 is the molecular switch for initiation of an epithelial stratification program." Genes Dev **18**(2): 126-131.

Kovacic, M. B., J. M. Myers, N. Wang, L. J. Martin, M. Lindsey, M. B. Ericksen, H. He, T. L. Patterson, T. M. Baye, D. Torgerson, L. A. Roth, J. Gupta, U. Sivaprasad, A. M. Gibson, A. M. Tsoras, D. Hu, C. Eng, R. Chapela, J. R. Rodriguez-Santana, W. Rodriguez-Cintron, P. C. Avila, K. Beckman, M. A. Seibold, C. Gignoux, S. M. Musaad, W. Chen, E. G. Burchard and G. K. Hershey (2011). "Identification of KIF3A as a novel candidate gene for childhood asthma using RNA expression and population allelic frequencies differences." PLoS One **6**(8): e23714.

Kozomara, A. and S. Griffiths-Jones (2011). "miRBase: integrating microRNA annotation and deep-sequencing data." Nucleic Acids Res **39**(Database issue): D152-157.

Kretz, M., Z. Siprashvili, C. Chu, D. E. Webster, A. Zehnder, K. Qu, C. S. Lee, R. J. Flockhart, A. F. Groff, J. Chow, D. Johnston, G. E. Kim, R. C. Spitale, R. A. Flynn, G. X. Zheng, S. Aiyer, A. Raj, J. L. Rinn, H. Y. Chang and P. A. Khavari (2013). "Control of somatic tissue differentiation by the long non-coding RNA TINCR." Nature **493**(7431): 231-235.

Kuespert, K., S. Pils and C. R. Hauck (2006). "CEACAMs: their role in physiology and pathophysiology." Curr Opin Cell Biol **18**(5): 565-571.

Kumar, M., T. Ahmad, A. Sharma, U. Mabalirajan, A. Kulshreshtha, A. Agrawal and B. Ghosh (2011). "Let-7 microRNA-mediated regulation of IL-13 and allergic airway inflammation." J Allergy Clin Immunol **128**(5): 1077-1085 e1071-1010.

Kunzmann, S., C. Schmidt-Weber, J. M. Zingg, A. Azzi, B. W. Kramer, K. Blaser, C. A. Akdis and C. P. Speer (2007). "Connective tissue growth factor expression is regulated by histamine in lung fibroblasts: potential role of histamine in airway remodeling." J Allergy Clin Immunol **119**(6): 1398-1407.

Kurita, T., G. R. Cunha, S. J. Robboy, A. A. Mills and R. T. Medina (2005). "Differential expression of p63 isoforms in female reproductive organs." Mech Dev **122**(9): 1043-1055.

Kusel, M. M., T. Kebadze, S. L. Johnston, P. G. Holt and P. D. Sly (2012). "Febrile respiratory illnesses in infancy and atopy are risk factors for persistent asthma and wheeze." Eur Respir J **39**(4): 876-882.

Labonte, I., M. Laviolette, R. Olivenstein, J. Chakir, L. P. Boulet and Q. Hamid (2008). "Quality of bronchial biopsies for morphology study and cell sampling: a comparison of asthmatic and healthy subjects." Can Respir J **15**(8): 431-435.

Lackie, P. M., J. E. Baker, U. Gunthert and S. T. Holgate (1997). "Expression of CD44 isoforms is increased in the airway epithelium of asthmatic subjects." Am J Respir Cell Mol Biol **16**(1): 14-22.

Lai, Y., W. A. Altemeier, J. Vandree, A. M. Piliponsky, B. Johnson, C. L. Appel, C. W. Frevert, D. M. Hyde, S. F. Ziegler, D. E. Smith, W. R. Henderson, Jr., M. H. Gelb and T. S. Hallstrand (2014). "Increased density of intraepithelial mast cells in patients with exercise-induced bronchoconstriction regulated through epithelially derived thymic stromal lymphopoietin and IL-33." J Allergy Clin Immunol **133**(5): 1448-1455.

Laidlaw, T. M. and J. A. Boyce (2012). "Cysteinyl leukotriene receptors, old and new; implications for asthma." Clin Exp Allergy **42**(9): 1313-1320.

Laitinen, A., A. Altraja, M. Kampe, M. Linden, I. Virtanen and L. A. Laitinen (1997). "Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid." Am J Respir Crit Care Med **156**(3 Pt 1): 951-958.

Laitinen, L. A., A. Laitinen and T. Haahtela (1993). "Airway mucosal inflammation even in patients with newly diagnosed asthma." Am Rev Respir Dis **147**(3): 697-704.

Lam, H. C., S. M. Cloonan, A. R. Bhashyam, J. A. Haspel, A. Singh, J. F. Sathirapongsasuti, M. Cervo, H. Yao, A. L. Chung, K. Mizumura, C. H. An, B. Shan, J. M. Franks, K. J. Haley, C. A. Owen, Y. Tesfaigzi, G. R. Washko, J. Quackenbush, E. K. Silverman, I. Rahman, H. P. Kim, A. Mahmood, S. S. Biswal, S. W. Ryter and A. M. Choi (2013). "Histone deacetylase 6-mediated selective autophagy regulates COPD-associated cilia dysfunction." J Clin Invest **123**(12): 5212-5230.

Lambrecht, B. N. and H. Hammad (2012). "The airway epithelium in asthma." Nat Med **18**(5): 684-692.

Langmead, B., C. Trapnell, M. Pop and S. L. Salzberg (2009). "Ultrafast and memory-efficient alignment of short DNA sequences to the human genome." Genome Biol **10**(3): R25.

LeBleu, V. S., G. Taduri, J. O'Connell, Y. Teng, V. G. Cooke, C. Woda, H. Sugimoto and R. Kalluri (2013). "Origin and function of myofibroblasts in kidney fibrosis." Nat Med **19**(8): 1047-1053.

Lee, E. J., A. Malik, S. Pokharel, S. Ahmad, B. A. Mir, K. H. Cho, J. Kim, J. C. Kong, D. M. Lee, K. Y. Chung, S. H. Kim and I. Choi (2014). "Identification of Genes Differentially Expressed in Myogenin Knock-Down Bovine Muscle Satellite Cells during Differentiation through RNA Sequencing Analysis." PLoS One **9**(3): e92447.

Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim and V. N. Kim (2003). "The nuclear RNase III Drosha initiates microRNA processing." Nature **425**(6956): 415-419.

Leir, S. H., J. E. Baker, S. T. Holgate and P. M. Lackie (2000). "Increased CD44 expression in human bronchial epithelial repair after damage or plating at low cell densities." Am J Physiol Lung Cell Mol Physiol **278**(6): L1129-1137.

Lena, A. M., R. Shalom-Feuerstein, P. R. di Val Cervo, D. Aberdam, R. A. Knight, G. Melino and E. Candi (2008). "miR-203 represses 'stemness' by repressing DeltaNp63." Cell Death Differ **15**(7): 1187-1195.

Levanen, B., N. R. Bhakta, P. Torregrosa Paredes, R. Barbeau, S. Hiltbrunner, J. L. Pollack, C. M. Skold, M. Svartengren, J. Grunewald, S. Gabrielsson, A. Eklund, B. M. Larsson, P. G. Woodruff, D. J. Erle and A. M. Wheelock (2013). "Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients." J Allergy Clin Immunol **131**(3): 894-903.

Li, C. W., L. Shi, K. K. Zhang, T. Y. Li, Z. B. Lin, M. K. Lim, F. McKeon, W. Xian and Y. Wang de (2011). "Role of p63/p73 in epithelial remodeling and their response to steroid treatment in nasal polyposis." J Allergy Clin Immunol **127**(3): 765-772 e761-762.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin and S. Genome Project Data Processing (2009). "The Sequence Alignment/Map format and SAMtools." Bioinformatics **25**(16): 2078-2079.

Li, J. and L. Ji (2005). "Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix." Heredity (Edinb) **95**(3): 221-227.

Li, X., Y. Zhang, S. C. Yeung, Y. Liang, X. Liang, Y. Ding, M. S. Ip, H. F. Tse, J. C. Mak and Q. Lian (2014). "Mitochondrial Transfer of Induced Pluripotent Stem Cells-derived MSCs to Airway Epithelial Cells Attenuates Cigarette Smoke-induced Damage." Am J Respir Cell Mol Biol(E-Pub ahead of print).

Lindsay, J., S. S. McDade, A. Pickard, K. D. McCloskey and D. J. McCance (2011). "Role of DeltaNp63gamma in epithelial to mesenchymal transition." J Biol Chem **286**(5): 3915-3924.

Liu, Y. (2004). "Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention." J Am Soc Nephrol **15**(1): 1-12.

Lize, M., C. Herr, A. Klimke, R. Bals and M. Dobbelstein (2010). "MicroRNA-449a levels increase by several orders of magnitude during mucociliary differentiation of airway epithelia." Cell Cycle **9**(22): 4579-4583.

Lloyd, C. M. and S. Saglani (2013). "T cells in asthma: influences of genetics, environment, and T-cell plasticity." J Allergy Clin Immunol **131**(5): 1267-1274; quiz 1275.

Lopez-Guisa, J. M., C. Powers, D. File, E. Cochrane, N. Jimenez and J. S. Debley (2012). "Airway epithelial cells from asthmatic children differentially express proremodeling factors." J Allergy Clin Immunol **129**(4): 990-997 e996.

Lougheed, M. D., C. Lemiere, S. D. Dell, F. M. Ducharme, J. M. Fitzgerald, R. Leigh, C. Licskai, B. H. Rowe, D. Bowie, A. Becker, L. P. Boulet and C. Canadian Thoracic Society Asthma (2010). "Canadian Thoracic Society Asthma Management Continuum--2010 Consensus Summary for children six years of age and over, and adults." Can Respir J **17**(1): 15-24.

Lu, T. X., J. Hartner, E. J. Lim, V. Fabry, M. K. Mingler, E. T. Cole, S. H. Orkin, B. J. Aronow and M. E. Rothenberg (2011). "MicroRNA-21 limits in vivo immune response-mediated activation of the IL-12/IFN-gamma pathway, Th1 polarization, and the severity of delayed-type hypersensitivity." J Immunol **187**(6): 3362-3373.

Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg and U. Kutay (2004). "Nuclear export of microRNA precursors." Science **303**(5654): 95-98.

Mabalirajan, U., A. K. Dinda, S. Kumar, R. Roshan, P. Gupta, S. K. Sharma and B. Ghosh (2008). "Mitochondrial structural changes and dysfunction are associated with experimental allergic asthma." J Immunol **181**(5): 3540-3548.

Mangiulli, M., A. Valletti, M. F. Caratozzolo, A. Tullo, E. Sbisa, G. Pesole and A. M. D'Erchia (2009). "Identification and functional characterization of two new transcriptional variants of the human p63 gene." Nucleic Acids Res **37**(18): 6092-6104.

Marcet, B., B. Chevalier, C. Coraux, L. Kodjabachian and P. Barbry (2011). "MicroRNA-based silencing of Delta/Notch signaling promotes multiple cilia formation." Cell Cycle **10**(17): 2858-2864.

Marcet, B., B. Chevalier, G. Luxardi, C. Coraux, L. E. Zaragosi, M. Cibois, K. Robbe-Sermesant, T. Jolly, B. Cardinaud, C. Moreilhon, L. Giovannini-Chami, B. Nawrocki-Raby, P. Birembaut, R. Waldmann, L. Kodjabachian and P. Barbry (2011). "Control of vertebrate multiciliogenesis by miR-449 through direct repression of the Delta/Notch pathway." Nat Cell Biol **13**(6): 693-699.

Marketos, S. G. and C. N. Ballas (1982). "Bronchial asthma in the medical literature of Greek antiquity." J Asthma **19**(4): 263-269.

Marshall, W. F. and J. L. Rosenbaum (2001). "Intraflagellar transport balances continuous turnover of outer doublet microtubules: implications for flagellar length control." J Cell Biol **155**(3): 405-414.

Martinez-Anton, A., M. Sokolowska, S. Kern, A. S. Davis, S. Alsaaty, J. K. Taubenberger, J. Sun, R. Cai, R. L. Danner, M. Eberlein, C. Logun and J. H. Shelhamer (2013). "Changes in

microRNA and mRNA Expression with Differentiation of Human Bronchial Epithelial Cells." Am J Respir Cell Mol Biol **49**(3): 384-395.

Martinez, F. D. (2007). "Asthma treatment and asthma prevention: a tale of 2 parallel pathways." J Allergy Clin Immunol **119**(1): 30-33.

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

Massion, P. P., P. M. Taflan, S. M. Jamshedur Rahman, P. Yildiz, Y. Shyr, M. E. Edgerton, M. D. Westfall, J. R. Roberts, J. A. Pietenpol, D. P. Carbone and A. L. Gonzalez (2003). "Significance of p63 amplification and overexpression in lung cancer development and prognosis." Cancer Res **63**(21): 7113-7121.

Masterson, J. C., E. L. Molloy, J. L. Gilbert, N. McCormack, A. Adams and S. O'Dea (2011). "Bone morphogenetic protein signalling in airway epithelial cells during regeneration." Cell Signal **23**(2): 398-406.

Mata, M., I. Sarrion, M. Armengot, C. Carda, I. Martinez, J. A. Melero and J. Cortijo (2012). "Respiratory syncytial virus inhibits ciliagenesis in differentiated normal human bronchial epithelial cells: effectiveness of N-acetylcysteine." PLoS One **7**(10): e48037.

Matheny, K. E., C. E. Barbieri, J. C. Sniezek, C. L. Arteaga and J. A. Pietenpol (2003). "Inhibition of epidermal growth factor receptor signaling decreases p63 expression in head and neck squamous carcinoma cells." Laryngoscope **113**(6): 936-939.

McClintock, T. S., C. E. Glasser, S. C. Bose and D. A. Bergman (2008). "Tissue expression patterns identify mouse cilia genes." Physiol Genomics **32**(2): 198-206.

McCormack, N., E. L. Molloy and S. O'Dea (2013). "Bone morphogenetic proteins enhance an epithelial-mesenchymal transition in normal airway epithelial cells during restitution of a disrupted epithelium." Respir Res **14**: 36.

McDowell, N., J. B. Gurdon and D. J. Grainger (2001). "Formation of a functional morphogen gradient by a passive process in tissue from the early Xenopus embryo." Int J Dev Biol **45**(1): 199-207.

McPherson, J. M., Piez, K.A. (1988). Collagen in dermal wound repair. The molecular and cellular biology of wound repair. R. A. F. Clark, Henson, P.M. New York, Plenum Press**:** 471-491.

Mege, R. M., J. Gavard and M. Lambert (2006). "Regulation of cell-cell junctions by the cytoskeleton." Curr Opin Cell Biol **18**(5): 541-548.

Meyer, A., R. Buhl and H. Magnussen (1994). "The effect of oral N-acetylcysteine on lung glutathione levels in idiopathic pulmonary fibrosis." Eur Respir J **7**(3): 431-436.

Mickleborough, T. D. (2008). "A nutritional approach to managing exercise-induced asthma." Exerc Sport Sci Rev **36**(3): 135-144.

Milan, S. J., A. Hart and M. Wilkinson (2013). "Vitamin C for asthma and exercise-induced bronchoconstriction." Cochrane Database Syst Rev **10**: CD010391.

Miller, M. R., J. Hankinson, V. Brusasco, F. Burgos, R. Casaburi, A. Coates, R. Crapo, P. Enright, C. P. van der Grinten, P. Gustafsson, R. Jensen, D. C. Johnson, N. MacIntyre, R. McKay, D. Navajas, O. F. Pedersen, R. Pellegrino, G. Viegi, J. Wanger and A. E. T. Force (2005). "Standardisation of spirometry." Eur Respir J **26**(2): 319-338.

Mills, A. A., B. Zheng, X. J. Wang, H. Vogel, D. R. Roop and A. Bradley (1999). "p63 is a p53 homologue required for limb and epidermal morphogenesis." Nature **398**(6729): 708-713.

Minoo, P. and R. J. King (1994). "Epithelial-mesenchymal interactions in lung development." Annu Rev Physiol **56**: 13-45.

Moffatt, M. F., I. G. Gut, F. Demenais, D. P. Strachan, E. Bouzigon, S. Heath, E. von Mutius, M. Farrall, M. Lathrop, W. O. Cookson and G. Consortium (2010). "A large-scale, consortium-based genomewide association study of asthma." N Engl J Med **363**(13): 1211-1221.

Molina-Ortiz, I., R. A. Bartolome, P. Hernandez-Varas, G. P. Colo and J. Teixido (2009). "Overexpression of E-cadherin on melanoma cells inhibits chemokine-promoted invasion involving p190RhoGAP/p120ctn-dependent inactivation of RhoA." J Biol Chem **284**(22): 15147-15157.

Molloy, E. L., A. Adams, J. B. Moore, J. C. Masterson, L. Madrigal-Estebas, B. P. Mahon and S. O'Dea (2008). "BMP4 induces an epithelial-mesenchymal transition-like response in adult airway epithelial cells." Growth Factors **26**(1): 12-22.

Mortensen, R. D., M. Serra, J. A. Steitz and S. Vasudevan (2011). "Posttranscriptional activation of gene expression in Xenopus laevis oocytes by microRNA-protein complexes (microRNPs)." Proc Natl Acad Sci U S A **108**(20): 8281-8286.

Motomura, Y., H. Morita, K. Moro, S. Nakae, D. Artis, T. A. Endo, Y. Kuroki, O. Ohara, S. Koyasu and M. Kubo (2014). "Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation." Immunity **40**(5): 758-771.

Mucenski, M. L., J. M. Nation, A. R. Thitoff, V. Besnard, Y. Xu, S. E. Wert, N. Harada, M. M. Taketo, M. T. Stahlman and J. A. Whitsett (2005). "Beta-catenin regulates differentiation of respiratory epithelial cells in vivo." Am J Physiol Lung Cell Mol Physiol **289**(6): L971-979.

Mullings, R. E., S. J. Wilson, S. M. Puddicombe, J. L. Lordan, F. Bucchieri, R. Djukanovic, P. H. Howarth, S. Harper, S. T. Holgate and D. E. Davies (2001). "Signal transducer and activator of transcription 6 (STAT-6) expression and function in asthmatic bronchial epithelium." J Allergy Clin Immunol **108**(5): 832-838.

Muroya, M., K. Chang, K. Uchida, M. Bougaki and Y. Yamada (2012). "Analysis of cytotoxicity induced by proinflammatory cytokines in the human alveolar epithelial cell line A549." Biosci Trends **6**(2): 70-80.

Musah, S., J. Chen and G. W. Hoyle (2012). "Repair of tracheal epithelium by basal cells after chlorine-induced injury." Respir Res **13**: 107.

Nakajima, M., O. Kawanami, E. Jin, M. Ghazizadeh, M. Honda, G. Asano, K. Horiba and V. J. Ferrans (1998). "Immunohistochemical and ultrastructural studies of basal cells, Clara cells and bronchiolar cuboidal cells in normal human airways." Pathol Int **48**(12): 944-953.

Natan, E. and A. C. Joerger (2012). "Structure and kinetic stability of the p63 tetramerization domain." J Mol Biol **415**(3): 503-513.

Nawshad, A., D. Lagamba, A. Polad and E. D. Hay (2005). "Transforming growth factor-beta signaling during epithelial-mesenchymal transformation: implications for embryogenesis and tumor metastasis." Cells Tissues Organs **179**(1-2): 11-23.

Neveu, W. A., J. B. Allard, O. Dienz, M. J. Wargo, G. Ciliberto, L. A. Whittaker and M. Rincon (2009). "IL-6 is required for airway mucus production induced by inhaled fungal allergens." J Immunol **183**(3): 1732-1738.

Nguyen, H. T., G. Dalmasso, Y. Yan, H. Laroui, S. Dahan, L. Mayer, S. V. Sitaraman and D. Merlin (2010). "MicroRNA-7 modulates CD98 expression during intestinal epithelial cell differentiation." J Biol Chem **285**(2): 1479-1489.

Nieto, M. A. (2013). "Epithelial plasticity: a common theme in embryonic and cancer cells." Science **342**(6159): 1234850.

Nihlberg, K., K. Larsen, A. Hultgardh-Nilsson, A. Malmstrom, L. Bjermer and G. Westergren-Thorsson (2006). "Tissue fibrocytes in patients with mild asthma: a possible link to thickness of reticular basement membrane?" Respir Res **7**: 50.

Nobre, A. R., A. Albergaria and F. Schmitt (2013). "p40: a p63 isoform useful for lung cancer diagnosis - a review of the physiological and pathological role of p63." Acta Cytol **57**(1): 1-8.

Nocker, R. E., D. F. Schoonbrood, E. A. van de Graaf, C. E. Hack, R. Lutter, H. M. Jansen and T. A. Out (1996). "Interleukin-8 in airway inflammation in patients with asthma and chronic obstructive pulmonary disease." Int Arch Allergy Immunol **109**(2): 183-191.

Nyholt, D. R. (2004). "A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other." Am J Hum Genet **74**(4): 765-769.

O'Reilly, R., N. Ullmann, S. Irving, C. J. Bossley, S. Sonnappa, J. Zhu, T. Oates, W. Banya, P. K. Jeffery, A. Bush and S. Saglani (2013). "Increased airway smooth muscle in preschool wheezers who have asthma at school age." J Allergy Clin Immunol **131**(4): 1024-1032, 1032 e1021-1016.

O'Riordan, T. G., J. Zwang and G. C. Smaldone (1992). "Mucociliary clearance in adult asthma." Am Rev Respir Dis **146**(3): 598-603.

Ober, C. and T. C. Yao (2011). "The genetics of asthma and allergic disease: a 21st century perspective." Immunol Rev **242**(1): 10-30.

Oberg, A. L., B. M. Bot, D. E. Grill, G. A. Poland and T. M. Therneau (2012). "Technical and biological variance structure in mRNA-Seq data: life in the real world." BMC Genomics **13**: 304.

Oddy, W. H., N. H. de Klerk, P. D. Sly and P. G. Holt (2002). "The effects of respiratory infections, atopy, and breastfeeding on childhood asthma." Eur Respir J **19**(5): 899-905.

Oh, J. E., R. H. Kim, K. H. Shin, N. H. Park and M. K. Kang (2011). "DeltaNp63alpha protein triggers epithelial-mesenchymal transition and confers stem cell properties in normal human keratinocytes." J Biol Chem **286**(44): 38757-38767.

Olivieri, D., A. Chetta, M. Del Donno, G. Bertorelli, A. Casalini, A. Pesci, R. Testi and A. Foresi (1997). "Effect of short-term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo-controlled study." Am J Respir Crit Care Med **155**(6): 1864-1871.

Ordonez, C. L., R. Khashayar, H. H. Wong, R. Ferrando, R. Wu, D. M. Hyde, J. A. Hotchkiss, Y. Zhang, A. Novikov, G. Dolganov and J. V. Fahy (2001). "Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression." Am J Respir Crit Care Med **163**(2): 517-523.

Orom, U. A., M. K. Lim, J. E. Savage, L. Jin, A. D. Saleh, M. P. Lisanti and N. L. Simone (2012). "MicroRNA-203 regulates caveolin-1 in breast tissue during caloric restriction." Cell Cycle **11**(7): 1291-1295.

Palmer, C. N., A. D. Irvine, A. Terron-Kwiatkowski, Y. Zhao, H. Liao, S. P. Lee, D. R. Goudie, A. Sandilands, L. E. Campbell, F. J. Smith, G. M. O'Regan, R. M. Watson, J. E. Cecil, S. J. Bale, J. G. Compton, J. J. DiGiovanna, P. Fleckman, S. Lewis-Jones, G. Arseculeratne, A. Sergeant, C. S. Munro, B. El Houate, K. McElreavey, L. B. Halkjaer, H. Bisgaard, S. Mukhopadhyay and W. H. McLean (2006). "Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis." Nat Genet **38**(4): 441-446.

Pan, X., Z. X. Wang and R. Wang (2010). "MicroRNA-21: a novel therapeutic target in human cancer." Cancer Biol Ther **10**(12): 1224-1232.

Park, B. J., S. J. Lee, J. I. Kim, S. J. Lee, C. H. Lee, S. G. Chang, J. H. Park and S. G. Chi (2000). "Frequent alteration of p63 expression in human primary bladder carcinomas." Cancer Res **60**(13): 3370-3374.

Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian and C. Dong (2005). "A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17." Nat Immunol **6**(11): 1133-1141.

Park, K. S., T. R. Korfhagen, M. D. Bruno, J. A. Kitzmiller, H. Wan, S. E. Wert, G. K. Khurana Hershey, G. Chen and J. A. Whitsett (2007). "SPDEF regulates goblet cell hyperplasia in the airway epithelium." J Clin Invest **117**(4): 978-988.

Park, K. S., J. M. Wells, A. M. Zorn, S. E. Wert, V. E. Laubach, L. G. Fernandez and J. A. Whitsett (2006). "Transdifferentiation of ciliated cells during repair of the respiratory epithelium." Am J Respir Cell Mol Biol **34**(2): 151-157.

Parker, J., S. Sarlang, S. Thavagnanam, G. Williamson, D. O'Donoghue, R. Villenave, U. Power, M. Shields, L. Heaney and G. Skibinski (2010). "A 3-D well-differentiated model of pediatric bronchial epithelium demonstrates unstimulated morphological differences between asthmatic and nonasthmatic cells." Pediatr Res **67**(1): 17-22.

Parvu, A. E., S. F. Alb, A. Craciun and M. A. Taulescu (2013). "Efficacy of subantimicrobial-dose doxycycline against nitrosative stress in chronic periodontitis." Acta Pharmacol Sin **34**(2): 247-254.

Patturajan, M., S. Nomoto, M. Sommer, A. Fomenkov, K. Hibi, R. Zangen, N. Poliak, J. Califano, B. Trink, E. Ratovitski and D. Sidransky (2002). "DeltaNp63 induces beta-catenin nuclear accumulation and signaling." Cancer Cell **1**(4): 369-379.

Payne, D. N., Y. Qiu, J. Zhu, L. Peachey, M. Scallan, A. Bush and P. K. Jeffery (2004). "Airway inflammation in children with difficult asthma: relationships with airflow limitation and persistent symptoms." Thorax **59**(10): 862-869.

Payne, D. N., A. V. Rogers, E. Adelroth, V. Bandi, K. K. Guntupalli, A. Bush and P. K. Jeffery (2003). "Early thickening of the reticular basement membrane in children with difficult asthma." Am J Respir Crit Care Med **167**(1): 78-82.

Pechkovsky, D. V., A. K. Scaffidi, T. L. Hackett, J. Ballard, F. Shaheen, P. J. Thompson, V. J. Thannickal and D. A. Knight (2008). "Transforming growth factor beta1 induces alphavbeta3 integrin expression in human lung fibroblasts via a beta3 integrin-, c-Src-, and p38 MAPK-dependent pathway." J Biol Chem **283**(19): 12898-12908.

Pellegrini, G., E. Dellambra, O. Golisano, E. Martinelli, I. Fantozzi, S. Bondanza, D. Ponzin, F. McKeon and M. De Luca (2001). "p63 identifies keratinocyte stem cells." Proc Natl Acad Sci U S A **98**(6): 3156-3161.

Perez, J., B. G. Hill, G. A. Benavides, B. P. Dranka and V. M. Darley-Usmar (2010). "Role of cellular bioenergetics in smooth muscle cell proliferation induced by platelet-derived growth factor." Biochem J **428**(2): 255-267.

Perl, A. K., S. E. Wert, D. E. Loudy, Z. Shan, P. A. Blair and J. A. Whitsett (2005). "Conditional recombination reveals distinct subsets of epithelial cells in trachea, bronchi, and alveoli." Am J Respir Cell Mol Biol **33**(5): 455-462.

Pezzulo, A. A., T. D. Starner, T. E. Scheetz, G. L. Traver, A. E. Tilley, B. G. Harvey, R. G. Crystal, P. B. McCray, Jr. and J. Zabner (2011). "The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia." Am J Physiol Lung Cell Mol Physiol **300**(1): L25-31.

Phelps, D. S. and J. Floros (1991). "Localization of pulmonary surfactant proteins using immunohistochemistry and tissue in situ hybridization." Exp Lung Res **17**(6): 985-995.

Phipps, S., N. Hansbro, C. E. Lam, S. Y. Foo, K. I. Matthaei and P. S. Foster (2009). "Allergic sensitization is enhanced in early life through toll-like receptor 7 activation." Clin Exp Allergy **39**(12): 1920-1928.

Piacentini, S., R. Polimanti, I. Simonelli, S. Donno, P. Pasqualetti, D. Manfellotto and M. Fuciarelli (2013). "Glutathione S-transferase polymorphisms, asthma susceptibility and confounding variables: a meta-analysis." Mol Biol Rep **40**(4): 3299-3313.

Pilewski, J. M., J. D. Latoche, S. M. Arcasoy and S. M. Albelda (1997). "Expression of integrin cell adhesion receptors during human airway epithelial repair in vivo." Am J Physiol **273**(1 Pt 1): L256-263.

Piriyapongsa, J., I. K. Jordan, A. B. Conley, T. Ronan and N. R. Smalheiser (2011). "Transcription factor binding sites are highly enriched within microRNA precursor sequences." Biol Direct **6**: 61.

Plopper, C. G., J. L. Alley and A. J. Weir (1986). "Differentiation of tracheal epithelium during fetal lung maturation in the rhesus monkey Macaca mulatta." Am J Anat **175**(1): 59-71.

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

Pohl, C., M. I. Hermanns, C. Uboldi, M. Bock, S. Fuchs, J. Dei-Anang, E. Mayer, K. Kehe, W. Kummer and C. J. Kirkpatrick (2009). "Barrier functions and paracellular integrity in human cell culture models of the proximal respiratory unit." Eur J Pharm Biopharm **72**(2): 339-349.

Polosa, R., S. M. Puddicombe, M. T. Krishna, A. B. Tuck, P. H. Howarth, S. T. Holgate and D. E. Davies (2002). "Expression of c-erbB receptors and ligands in the bronchial epithelium of asthmatic subjects." J Allergy Clin Immunol **109**(1): 75-81.

Puddicombe, S. M., R. Polosa, A. Richter, M. T. Krishna, P. H. Howarth, S. T. Holgate and D. E. Davies (2000). "Involvement of the epidermal growth factor receptor in epithelial repair in asthma." FASEB J **14**(10): 1362-1374.

Que, J., X. Luo, R. J. Schwartz and B. L. Hogan (2009). "Multiple roles for Sox2 in the developing and adult mouse trachea." Development **136**(11): 1899-1907.

Quinlan, A. R. and I. M. Hall (2010). "BEDTools: a flexible suite of utilities for comparing genomic features." Bioinformatics **26**(6): 841-842.

R Development Core Team, t. (2011). R: A Language and Environment for Statistical Computing. Vienna, Austria, R Foundation for Statistical Computing.

Rackley, C. R. and B. R. Stripp (2012). "Building and maintaining the epithelium of the lung." J Clin Invest **122**(8): 2724-2730.

Rahman, I. (2002). "Oxidative stress and gene transcription in asthma and chronic obstructive pulmonary disease: antioxidant therapeutic targets." Curr Drug Targets Inflamm Allergy **1**(3): 291-315.

Ramirez, R. D., S. Sheridan, L. Girard, M. Sato, Y. Kim, J. Pollack, M. Peyton, Y. Zou, J. M. Kurie, J. M. Dimaio, S. Milchgrub, A. L. Smith, R. F. Souza, L. Gilbey, X. Zhang, K. Gandia, M. B. Vaughan, W. E. Wright, A. F. Gazdar, J. W. Shay and J. D. Minna (2004). Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. Cancer Res. United States. **64:** 9027-9034.

Rawlins, E. L., T. Okubo, Y. Xue, D. M. Brass, R. L. Auten, H. Hasegawa, F. Wang and B. L. Hogan (2009). "The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium." Cell Stem Cell **4**(6): 525-534.

Redvers, R. P., A. Li and P. Kaur (2006). "Side population in adult murine epidermis exhibits phenotypic and functional characteristics of keratinocyte stem cells." Proc Natl Acad Sci U S A **103**(35): 13168-13173.

Ren, X., T. A. Shah, V. Ustiyan, Y. Zhang, J. Shinn, G. Chen, J. A. Whitsett, T. V. Kalin and V. V. Kalinichenko (2013). "FOXM1 promotes allergen-induced goblet cell metaplasia and pulmonary inflammation." Mol Cell Biol **33**(2): 371-386.

Reynolds, S. D., A. Giangreco, J. H. Power and B. R. Stripp (2000). "Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration." Am J Pathol **156**(1): 269-278.

Ribeiro, C. M., A. M. Paradiso, A. Livraghi and R. C. Boucher (2003). "The mitochondrial barriers segregate agonist-induced calcium-dependent functions in human airway epithelia." J Gen Physiol **122**(4): 377-387.

Ricciardolo, F. L., A. Di Stefano, J. H. van Krieken, J. K. Sont, A. van Schadewijk, K. F. Rabe, C. F. Donner, P. S. Hiemstra, P. J. Sterk and T. Mauad (2003). "Proliferation and inflammation in bronchial epithelium after allergen in atopic asthmatics." Clin Exp Allergy **33**(7): 905-911.

Roche, W. R., R. Beasley, J. H. Williams and S. T. Holgate (1989). "Subepithelial fibrosis in the bronchi of asthmatics." Lancet **1**(8637): 520-524.

Rock, J. R., X. Gao, Y. Xue, S. H. Randell, Y. Y. Kong and B. L. Hogan (2011). "Notch-dependent differentiation of adult airway basal stem cells." Cell Stem Cell **8**(6): 639-648.

Rock, J. R., M. W. Onaitis, E. L. Rawlins, Y. Lu, C. P. Clark, Y. Xue, S. H. Randell and B. L. Hogan (2009). "Basal cells as stem cells of the mouse trachea and human airway epithelium." Proc Natl Acad Sci U S A **106**(31): 12771-12775.

Rodilla, V., A. Villanueva, A. Obrador-Hevia, A. Robert-Moreno, V. Fernandez-Majada, A. Grilli, N. Lopez-Bigas, N. Bellora, M. M. Alba, F. Torres, M. Dunach, X. Sanjuan, S. Gonzalez, T. Gridley, G. Capella, A. Bigas and L. Espinosa (2009). "Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer." Proc Natl Acad Sci U S A **106**(15): 6315-6320.

Rodriguez-Boulan, E., G. Kreitzer and A. Musch (2005). "Organization of vesicular trafficking in epithelia." Nat Rev Mol Cell Biol **6**(3): 233-247.

Rogers, D. F. and P. J. Barnes (2006). "Treatment of airway mucus hypersecretion." Ann Med **38**(2): 116-125.

Romano, R. A., K. Smalley, C. Magraw, V. A. Serna, T. Kurita, S. Raghavan and S. Sinha (2012). "DeltaNp63 knockout mice reveal its indispensable role as a master regulator of epithelial development and differentiation." Development **139**(4): 772-782.

Rosenberger, A., H. Bickeboller, V. McCormack, D. R. Brenner, E. J. Duell, A. Tjonneland, S. Friis, J. E. Muscat, P. Yang, H. E. Wichmann, J. Heinrich, N. Szeszenia-Dabrowska, J. Lissowska, D. Zaridze, P. Rudnai, E. Fabianova, V. Janout, V. Bencko, P. Brennan, D. Mates, A. G. Schwartz, M. L. Cote, Z. F. Zhang, H. Morgenstern, S. S. Oh, J. K. Field, O. Raji, J. R. McLaughlin, J. Wiencke, L. LeMarchand, M. Neri, S. Bonassi, A. S. Andrew, Q. Lan, W. Hu, I. Orlow, B. J. Park, P. Boffetta and R. J. Hung (2012). "Asthma and lung cancer risk: a systematic investigation by the International Lung Cancer Consortium." Carcinogenesis **33**(3): 587-597.

Ross, A. J., L. A. Dailey, L. E. Brighton and R. B. Devlin (2007). "Transcriptional profiling of mucociliary differentiation in human airway epithelial cells." Am J Respir Cell Mol Biol **37**(2): 169-185.

Ross, K. R., D. A. Corey, J. M. Dunn and T. J. Kelley (2007). "SMAD3 expression is regulated by mitogen-activated protein kinase kinase-1 in epithelial and smooth muscle cells." Cell Signal **19**(5): 923-931.

Rui, Y., Z. Xu, B. Xiong, Y. Cao, S. Lin, M. Zhang, S. C. Chan, W. Luo, Y. Han, Z. Lu, Z. Ye, H. M. Zhou, J. Han, A. Meng and S. C. Lin (2007). "A beta-catenin-independent dorsalization pathway activated by Axin/JNK signaling and antagonized by aida." Dev Cell **13**(2): 268-282.

Runswick, S., T. Mitchell, P. Davies, C. Robinson and D. R. Garrod (2007). "Pollen proteolytic enzymes degrade tight junctions." Respirology **12**(6): 834-842.

Saben, J., Y. Zhong, S. McKelvey, N. K. Dajani, A. Andres, T. M. Badger, H. Gomez-Acevedo and K. Shankar (2014). "A comprehensive analysis of the human placenta transcriptome." Placenta **35**(2): 125-131.

Sacco, O., M. Silvestri, F. Sabatini, R. Sale, A. C. Defilippi and G. A. Rossi (2004). "Epithelial cells and fibroblasts: structural repair and remodelling in the airways." Paediatr Respir Rev **5 Suppl A**: S35-40.

Saglani, S., S. Lui, N. Ullmann, G. A. Campbell, R. T. Sherburn, S. A. Mathie, L. Denney, C. J. Bossley, T. Oates, S. A. Walker, A. Bush and C. M. Lloyd (2013). "IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma." J Allergy Clin Immunol **132**(3): 676-685 e613.

Saglani, S., C. Molyneux, H. Gong, A. Rogers, K. Malmstrom, A. Pelkonen, M. Makela, E. Adelroth, A. Bush, D. N. Payne and P. K. Jeffery (2006). "Ultrastructure of the reticular basement membrane in asthmatic adults, children and infants." Eur Respir J **28**(3): 505-512.

Saglani, S., D. N. Payne, J. Zhu, Z. Wang, A. G. Nicholson, A. Bush and P. K. Jeffery (2007). "Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers." Am J Respir Crit Care Med **176**(9): 858-864.

Saika, S., S. Kono-Saika, Y. Ohnishi, M. Sato, Y. Muragaki, A. Ooshima, K. C. Flanders, J. Yoo, M. Anzano, C. Y. Liu, W. W. Kao and A. B. Roberts (2004). "Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury." Am J Pathol **164**(2): 651-663.

Sarrio, D., S. M. Rodriguez-Pinilla, D. Hardisson, A. Cano, G. Moreno-Bueno and J. Palacios (2008). "Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype." Cancer Res **68**(4): 989-997.

Sasaki, Y., I. Morimoto, S. Ishida, T. Yamashita, K. Imai and T. Tokino (2001). "Adenovirus-mediated transfer of the p53 family genes, p73 and p51/p63 induces cell cycle arrest and apoptosis in colorectal cancer cell lines: potential application to gene therapy of colorectal cancer." Gene Ther **8**(18): 1401-1408.

Sato, M., Y. Muragaki, S. Saika, A. B. Roberts and A. Ooshima (2003). "Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction." J Clin Invest **112**(10): 1486-1494.

Sbisa, E., D. Catalano, G. Grillo, F. Licciulli, A. Turi, S. Liuni, G. Pesole, A. De Grassi, M. F. Caratozzolo, A. M. D'Erchia, B. Navarro, A. Tullo, C. Saccone and A. Gisel (2007). "p53FamTaG: a database resource of human p53, p63 and p73 direct target genes combining in silico prediction and microarray data." BMC Bioinformatics **8 Suppl 1**: S20.

Schmidt, M., G. Sun, M. A. Stacey, L. Mori and S. Mattoli (2003). "Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma." J Immunol **171**(1): 380-389.

Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D. M. Gorman, J. F. Bazan and R. A. Kastelein (2005). "IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines." Immunity **23**(5): 479-490.

Schoch, K. G., A. Lori, K. A. Burns, T. Eldred, J. C. Olsen and S. H. Randell (2004). "A subset of mouse tracheal epithelial basal cells generates large colonies in vitro." Am J Physiol Lung Cell Mol Physiol **286**(4): L631-642.

Semont, A., C. Demarquay, R. Bessout, C. Durand, M. Benderitter and N. Mathieu (2013). "Mesenchymal stem cell therapy stimulates endogenous host progenitor cells to improve colonic epithelial regeneration." PLoS One **8**(7): e70170.

Sherwood, L. (2006). Fundamentals of Physiology: A Human Perspective:, Cengage Learning.

Shim, J. J., K. Dabbagh, I. F. Ueki, T. Dao-Pick, P. R. Burgel, K. Takeyama, D. C. Tam and J. A. Nadel (2001). "IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils." Am J Physiol Lung Cell Mol Physiol **280**(1): L134-140.

Shin, K., V. C. Fogg and B. Margolis (2006). "Tight junctions and cell polarity." Annu Rev Cell Dev Biol **22**: 207-235.

Sibley, C. R., Y. Seow, S. Saayman, K. K. Dijkstra, S. El Andaloussi, M. S. Weinberg and M. J. Wood (2012). "The biogenesis and characterization of mammalian microRNAs of mirtron origin." Nucleic Acids Res **40**(1): 438-448.

Signoretti, S., D. Waltregny, J. Dilks, B. Isaac, D. Lin, L. Garraway, A. Yang, R. Montironi, F. McKeon and M. Loda (2000). p63 is a prostate basal cell marker and is required for prostate development. Am J Pathol. United States. **157:** 1769-1775.

Simcock, D. E., V. Kanabar, G. W. Clarke, B. J. O'Connor, T. H. Lee and S. J. Hirst (2007). "Proangiogenic activity in bronchoalveolar lavage fluid from patients with asthma." Am J Respir Crit Care Med **176**(2): 146-153.

Simsek, T., F. Kocabas, J. Zheng, R. J. Deberardinis, A. I. Mahmoud, E. N. Olson, J. W. Schneider, C. C. Zhang and H. A. Sadek (2010). "The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche." Cell Stem Cell **7**(3): 380-390.

Sinha, A., J. Nightingale, K. P. West, J. Berlanga-Acosta and R. J. Playford (2003). "Epidermal growth factor enemas with oral mesalamine for mild-to-moderate left-sided ulcerative colitis or proctitis." N Engl J Med **349**(4): 350-357.

Smith, G. H. and G. Chepko (2001). "Mammary epithelial stem cells." Microsc Res Tech **52**(2): 190-203.

Solberg, O. D., E. J. Ostrin, M. I. Love, J. C. Peng, N. R. Bhakta, L. Hou, C. Nguyen, M. Solon, C. Nguyen, A. J. Barczak, L. T. Zlock, D. P. Blagev, W. E. Finkbeiner, K. M. Ansel, J. R. Arron, D. J. Erle and P. G. Woodruff (2012). "Airway epithelial miRNA expression is altered in asthma." Am J Respir Crit Care Med **186**(10): 965-974.

Soneson, C. and M. Delorenzi (2013). "A comparison of methods for differential expression analysis of RNA-seq data." BMC Bioinformatics **14**: 91.

Song, L. and W. L. Dentler (2001). "Flagellar protein dynamics in Chlamydomonas." J Biol Chem **276**(32): 29754-29763.

Stefanowicz, D., T. L. Hackett, F. S. Garmaroudi, O. P. Gunther, S. Neumann, E. N. Sutanto, K. M. Ling, M. S. Kobor, A. Kicic, S. M. Stick, P. D. Pare and D. A. Knight (2012). "DNA methylation profiles of airway epithelial cells and PBMCs from healthy, atopic and asthmatic children." PLoS One **7**(9): e44213.

Stephens, R. E. (1997). "Synthesis and turnover of embryonic sea urchin ciliary proteins during selective inhibition of tubulin synthesis and assembly." Mol Biol Cell **8**(11): 2187-2198.

Stevens, P. T., A. Kicic, E. N. Sutanto, D. A. Knight and S. M. Stick (2008). "Dysregulated repair in asthmatic paediatric airway epithelial cells: the role of plasminogen activator inhibitor-1." Clin Exp Allergy **38**(12): 1901-1910.

Stevens, P. T., A. Kicic, E. N. Sutanto, D. A. Knight and S. M. Stick (2008). Dysregulated repair in asthmatic paediatric airway epithelial cells: the role of plasminogen activator inhibitor-1. Clin Exp Allergy. England. **38:** 1901-1910.

Strachan, D. P. (1989). "Hay fever, hygiene, and household size." BMJ **299**(6710): 1259-1260.

Stringari, C., R. A. Edwards, K. T. Pate, M. L. Waterman, P. J. Donovan and E. Gratton (2012). "Metabolic trajectory of cellular differentiation in small intestine by Phasor Fluorescence Lifetime Microscopy of NADH." Sci Rep **2**: 568.

Swindle, E. J., J. E. Collins and D. E. Davies (2009). "Breakdown in epithelial barrier function in patients with asthma: identification of novel therapeutic approaches." J Allergy Clin Immunol **124**(1): 23-34; quiz 35-26.

Talos, F., R. Schulz and U. M. Moll (2010). "p63 and canonical Wnt signaling." Cell Cycle **9**(4): 648-649.

Tateishi, K., S. Motojima, A. Kushima, T. Fukuda and S. Makino (1996). "Comparison between allergen-induced and exercise-induced asthma with respect to the late asthmatic response, airway responsiveness, and Creola bodies in sputum." Ann Allergy Asthma Immunol **77**(3): 229-237.

Tepper, R. S., R. S. Wise, R. Covar, C. G. Irvin, C. M. Kercsmar, M. Kraft, M. C. Liu, G. T. O'Connor, S. P. Peters, R. Sorkness and A. Togias (2012). "Asthma outcomes: pulmonary physiology." J Allergy Clin Immunol **129**(3 Suppl): S65-87.

Testoni, B., S. Borrelli, E. Tenedini, D. Alotto, C. Castagnoli, S. Piccolo, E. Tagliafico, S. Ferrari, M. A. Vigano and R. Mantovani (2006). "Identification of new p63 targets in human keratinocytes." Cell Cycle **5**(23): 2805-2811.

Thiery, J. P. (2002). "Epithelial-mesenchymal transitions in tumour progression." Nat Rev Cancer **2**(6): 442-454.

Thomas, B., A. Rutman, R. A. Hirst, P. Haldar, A. J. Wardlaw, J. Bankart, C. E. Brightling and C. O'Callaghan (2010). "Ciliary dysfunction and ultrastructural abnormalities are features of severe asthma." J Allergy Clin Immunol **126**(4): 722-729 e722.

Toelle, B. G., J. K. Peat, C. M. Salome, J. Crane, D. McMillan, J. Dermand, W. D'Souza and A. J. Woolcock (1994). "Comparison of two epidemiological protocols for measuring airway responsiveness and allergic sensitivity in adults." Eur Respir J **7**(10): 1798-1804.

Tomita, K., G. Caramori, K. Ito, H. Sano, S. Lim, T. Oates, B. Cosio, K. F. Chung, Y. Tohda, P. J. Barnes and I. M. Adcock (2012). "STAT6 expression in T cells, alveolar macrophages and bronchial biopsies of normal and asthmatic subjects." J Inflamm (Lond) **9**: 5.

Trautmann, A., K. Kruger, M. Akdis, D. Muller-Wening, A. Akkaya, E. B. Brocker, K. Blaser and C. A. Akdis (2005). "Apoptosis and loss of adhesion of bronchial epithelial cells in asthma." Int Arch Allergy Immunol **138**(2): 142-150.

Tsao, P. N., M. Vasconcelos, K. I. Izvolsky, J. Qian, J. Lu and W. V. Cardoso (2009). "Notch signaling controls the balance of ciliated and secretory cell fates in developing airways." Development **136**(13): 2297-2307.

Tsuchiya, S., M. Oku, Y. Imanaka, R. Kunimoto, Y. Okuno, K. Terasawa, F. Sato, G. Tsujimoto and K. Shimizu (2009). "MicroRNA-338-3p and microRNA-451 contribute to the formation of basolateral polarity in epithelial cells." Nucleic Acids Res **37**(11): 3821-3827.

Tunggal, J. A., I. Helfrich, A. Schmitz, H. Schwarz, D. Gunzel, M. Fromm, R. Kemler, T. Krieg and C. M. Niessen (2005). "E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions." EMBO J **24**(6): 1146-1156.

Turato, G., A. Barbato, S. Baraldo, M. E. Zanin, E. Bazzan, K. Lokar-Oliani, F. Calabrese, C. Panizzolo, D. Snijders, P. Maestrelli, R. Zuin, L. M. Fabbri and M. Saetta (2008). "Nonatopic children with multitrigger wheezing have airway pathology comparable to atopic asthma." Am J Respir Crit Care Med **178**(5): 476-482.

Turley, E. A. (1992). "Hyaluronan and cell locomotion." Cancer Metastasis Rev **11**(1): 21-30.

Turner, J., J. Roger, J. Fitau, D. Combe, J. Giddings, G. V. Heeke and C. E. Jones (2011). "Goblet cells are derived from a FOXJ1-expressing progenitor in a human airway epithelium." Am J Respir Cell Mol Biol **44**(3): 276-284.

Tyner, J. W., E. Y. Kim, K. Ide, M. R. Pelletier, W. T. Roswit, J. D. Morton, J. T. Battaile, A. C. Patel, G. A. Patterson, M. Castro, M. S. Spoor, Y. You, S. L. Brody and M. J. Holtzman

(2006). "Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals." J Clin Invest **116**(2): 309-321.

Uddin, M., G. Seumois, L. C. Lau, P. Rytila, D. E. Davies and R. Djukanovic (2008). "Enhancement of neutrophil function by the bronchial epithelium stimulated by epidermal growth factor." Eur Respir J **31**(4): 714-724.

Valadi, H., K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee and J. O. Lotvall (2007). "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells." Nat Cell Biol **9**(6): 654-659.

Valcourt, U., M. Kowanetz, H. Niimi, C. H. Heldin and A. Moustakas (2005). "TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition." Mol Biol Cell **16**(4): 1987-2002.

Van Winkle, L. S., C. D. Brown, J. A. Shimizu, A. D. Gunderson, M. J. Evans and C. G. Plopper (2004). "Impaired recovery from naphthalene-induced bronchiolar epithelial injury in mice exposed to aged and diluted sidestream cigarette smoke." Toxicol Lett **154**(1-2): 1-9.

Van Winkle, L. S., M. V. Fanucchi, L. A. Miller, G. L. Baker, L. J. Gershwin, E. S. Schelegle, D. M. Hyde, M. J. Evans and C. G. Plopper (2004). "Epithelial cell distribution and abundance in rhesus monkey airways during postnatal lung growth and development." J Appl Physiol **97**(6): 2355-2363; discussion 2354.

Vander Heiden, M. G., L. C. Cantley and C. B. Thompson (2009). "Understanding the Warburg effect: the metabolic requirements of cell proliferation." Science **324**(5930): 1029-1033.

Vasudevan, S., Y. Tong and J. A. Steitz (2007). "Switching from repression to activation: microRNAs can up-regulate translation." Science **318**(5858): 1931-1934.

Venables, W. N. and B. D. Ripley (2002). Modern Applied Statistics with S. New York, Springer.

Wan, H., H. L. Winton, C. Soeller, G. W. Taylor, D. C. Gruenert, P. J. Thompson, M. B. Cannell, G. A. Stewart, D. R. Garrod and C. Robinson (2001). "The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus." Clin Exp Allergy **31**(2): 279-294.

Wang, Y. H., K. S. Voo, B. Liu, C. Y. Chen, B. Uygungil, W. Spoede, J. A. Bernstein, D. P. Huston and Y. J. Liu (2010). "A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma." J Exp Med **207**(11): 2479-2491.

Warburg, O. (1956). "On the origin of cancer cells." Science **123**(3191): 309-314.

Ward, C., I. A. Forrest, D. M. Murphy, G. E. Johnson, H. Robertson, T. E. Cawston, A. J. Fisher, J. H. Dark, J. L. Lordan, J. A. Kirby and P. A. Corris (2005). "Phenotype of airway epithelial cells suggests epithelial to mesenchymal cell transition in clinically stable lung transplant recipients." Thorax **60**(10): 865-871.

Ward, J. P. (2006). Respiratory System at a Glance, Wiley, John & Sons, Incorporated.

Warner, S. M., T. L. Hackett, F. Shaheen, T. S. Hallstrand, A. Kicic, S. M. Stick and D. A. Knight (2013). "Transcription Factor p63 Regulates Key Genes and Wound Repair in Human Airway Epithelial Basal Cells." Am J Respir Cell Mol Biol.

Wenzel, S. E. (2012). "Asthma phenotypes: the evolution from clinical to molecular approaches." Nat Med **18**(5): 716-725.

Westfall, M. D., D. J. Mays, J. C. Sniezek and J. A. Pietenpol (2003). "The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations." Mol Cell Biol **23**(7): 2264-2276.

Willart, M. A., K. Deswarte, P. Pouliot, H. Braun, R. Beyaert, B. N. Lambrecht and H. Hammad (2012). "Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33." J Exp Med **209**(8): 1505-1517.

Williams, A. E., H. Larner-Svensson, M. M. Perry, G. A. Campbell, S. E. Herrick, I. M. Adcock, J. S. Erjefalt, K. F. Chung and M. A. Lindsay (2009). "MicroRNA expression profiling in mild asthmatic human airways and effect of corticosteroid therapy." PLoS One **4**(6): e5889.

Willis, B. C. and Z. Borok (2007). "TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease." Am J Physiol Lung Cell Mol Physiol **293**(3): L525-534.

Wilson, J. W. and X. Li (1997). "The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway." Clin Exp Allergy **27**(4): 363-371.

Wong, A. P., A. Keating, W. Y. Lu, P. Duchesneau, X. Wang, A. Sacher, J. Hu and T. K. Waddell (2009). "Identification of a bone marrow-derived epithelial-like population capable of repopulating injured mouse airway epithelium." J Clin Invest **119**(2): 336-348.

Woodruff, P. G., H. A. Boushey, G. M. Dolganov, C. S. Barker, Y. H. Yang, S. Donnelly, A. Ellwanger, S. S. Sidhu, T. P. Dao-Pick, C. Pantoja, D. J. Erle, K. R. Yamamoto and J. V. Fahy (2007). "Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids." Proc Natl Acad Sci U S A **104**(40): 15858-15863.

Woods, A. J., D. P. White, P. T. Caswell and J. C. Norman (2004). "PKD1/PKCmu promotes alphavbeta3 integrin recycling and delivery to nascent focal adhesions." Embo J **23**(13): 2531-2543.

Wu, N., J. Rollin, I. Masse, J. Lamartine and X. Gidrol (2012). "p63 regulates human keratinocyte proliferation via MYC-regulated gene network and differentiation commitment through cell adhesion-related gene network." J Biol Chem **287**(8): 5627-5638.

Wu, Z., L. Yang, L. Cai, M. Zhang, X. Cheng, X. Yang and J. Xu (2007). "Detection of epithelial to mesenchymal transition in airways of a bleomycin induced pulmonary fibrosis model derived from an alpha-smooth muscle actin-Cre transgenic mouse." Respir Res **8**: 1.

Xiang, Y., Y. R. Tan, J. S. Zhang, X. Q. Qin, B. B. Hu, Y. Wang, F. Qu and H. J. Liu (2008). "Wound repair and proliferation of bronchial epithelial cells regulated by CTNNAL1." J Cell Biochem **103**(3): 920-930.

Xiao, C., S. M. Puddicombe, S. Field, J. Haywood, V. Broughton-Head, I. Puxeddu, H. M. Haitchi, E. Vernon-Wilson, D. Sammut, N. Bedke, C. Cremin, J. Sones, R. Djukanovic, P. H. Howarth, J. E. Collins, S. T. Holgate, P. Monk and D. E. Davies (2011). "Defective epithelial barrier function in asthma." J Allergy Clin Immunol **128**(3): 549-556 e541-512.

Xu, K. P., Y. Ding, J. Ling, Z. Dong and F. S. Yu (2004). "Wound-induced HB-EGF ectodomain shedding and EGFR activation in corneal epithelial cells." Invest Ophthalmol Vis Sci **45**(3): 813-820.

Xu, W., A. J. Janocha, R. A. Leahy, R. Klatte, D. Dudzinski, L. A. Mavrakis, S. A. Comhair, M. E. Lauer, C. U. Cotton and S. C. Erzurum (2014). "A novel method for pulmonary research: Assessment of bioenergetic function at the air-liquid interface." Redox Biol **2**: 513-519.

Xu, W. C., S.A.; Janocha, A.J.; Mavrakis, L.A.; Erzurum, S.C. (2010). "Alteration of nitric oxide synthesis related to abnormal cellular bioenergetics in asthmatic airway epithelium." Am J Respir Crit Care Med **181**: A1436.

Yalcin-Ozuysal, O., M. Fiche, M. Guitierrez, K. U. Wagner, W. Raffoul and C. Brisken (2010). "Antagonistic roles of Notch and p63 in controlling mammary epithelial cell fates." Cell Death Differ **17**(10): 1600-1612.

Yamaguchi, K., L. Wu, O. L. Caballero, K. Hibi, B. Trink, V. Resto, P. Cairns, K. Okami, W. M. Koch, D. Sidransky and J. Jen (2000). "Frequent gain of the p40/p51/p63 gene locus in primary head and neck squamous cell carcinoma." Int J Cancer **86**(5): 684-689.

Yan, X., Y. Liu, Q. Han, M. Jia, L. Liao, M. Qi and R. C. Zhao (2007). "Injured microenvironment directly guides the differentiation of engrafted Flk-1(+) mesenchymal stem cell in lung." Exp Hematol **35**(9): 1466-1475.

Yang, A., M. Kaghad, Y. Wang, E. Gillett, M. D. Fleming, V. Dotsch, N. C. Andrews, D. Caput and F. McKeon (1998). "p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities." Mol Cell **2**(3): 305-316.

Yang, A., R. Schweitzer, D. Sun, M. Kaghad, N. Walker, R. T. Bronson, C. Tabin, A. Sharpe, D. Caput, C. Crum and F. McKeon (1999). "p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development." Nature **398**(6729): 714-718.

Yang, J. S., M. D. Phillips, D. Betel, P. Mu, A. Ventura, A. C. Siepel, K. C. Chen and E. C. Lai (2011). "Widespread regulatory activity of vertebrate microRNA* species." RNA **17**(2): 312-326.

Yi, R., M. N. Poy, M. Stoffel and E. Fuchs (2008). "A skin microRNA promotes differentiation by repressing 'stemness'." Nature **452**(7184): 225-229.

Yick, C. Y., A. H. Zwinderman, P. W. Kunst, K. Grunberg, T. Mauad, A. Dijkhuis, E. H. Bel, F. Baas, R. Lutter and P. J. Sterk (2013). "Transcriptome sequencing (RNA-Seq) of human endobronchial biopsies: asthma versus controls." Eur Respir J **42**(3): 662-670.

Ying, S., Q. Meng, C. J. Corrigan and T. H. Lee (2006). "Lack of filaggrin expression in the human bronchial mucosa." J Allergy Clin Immunol **118**(6): 1386-1388.

Yu, L., M. C. Hebert and Y. E. Zhang (2002). "TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses." Embo J **21**(14): 3749-3759.

Zabner, J., M. Winter, K. J. Excoffon, D. Stoltz, D. Ries, S. Shasby and M. Shasby (2003). "Histamine alters E-cadherin cell adhesion to increase human airway epithelial permeability." J Appl Physiol (1985) **95**(1): 394-401.

Zavadil, J., M. Bitzer, D. Liang, Y. C. Yang, A. Massimi, S. Kneitz, E. Piek and E. P. Bottinger (2001). "Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta." Proc Natl Acad Sci U S A **98**(12): 6686-6691.

Zavadil, J., L. Cermak, N. Soto-Nieves and E. P. Bottinger (2004). "Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition." Embo J **23**(5): 1155-1165.

Zavadil, J., M. Narasimhan, M. Blumenberg and R. J. Schneider (2007). "Transforming growth factor-beta and microRNA:mRNA regulatory networks in epithelial plasticity." Cells Tissues Organs **185**(1-3): 157-161.

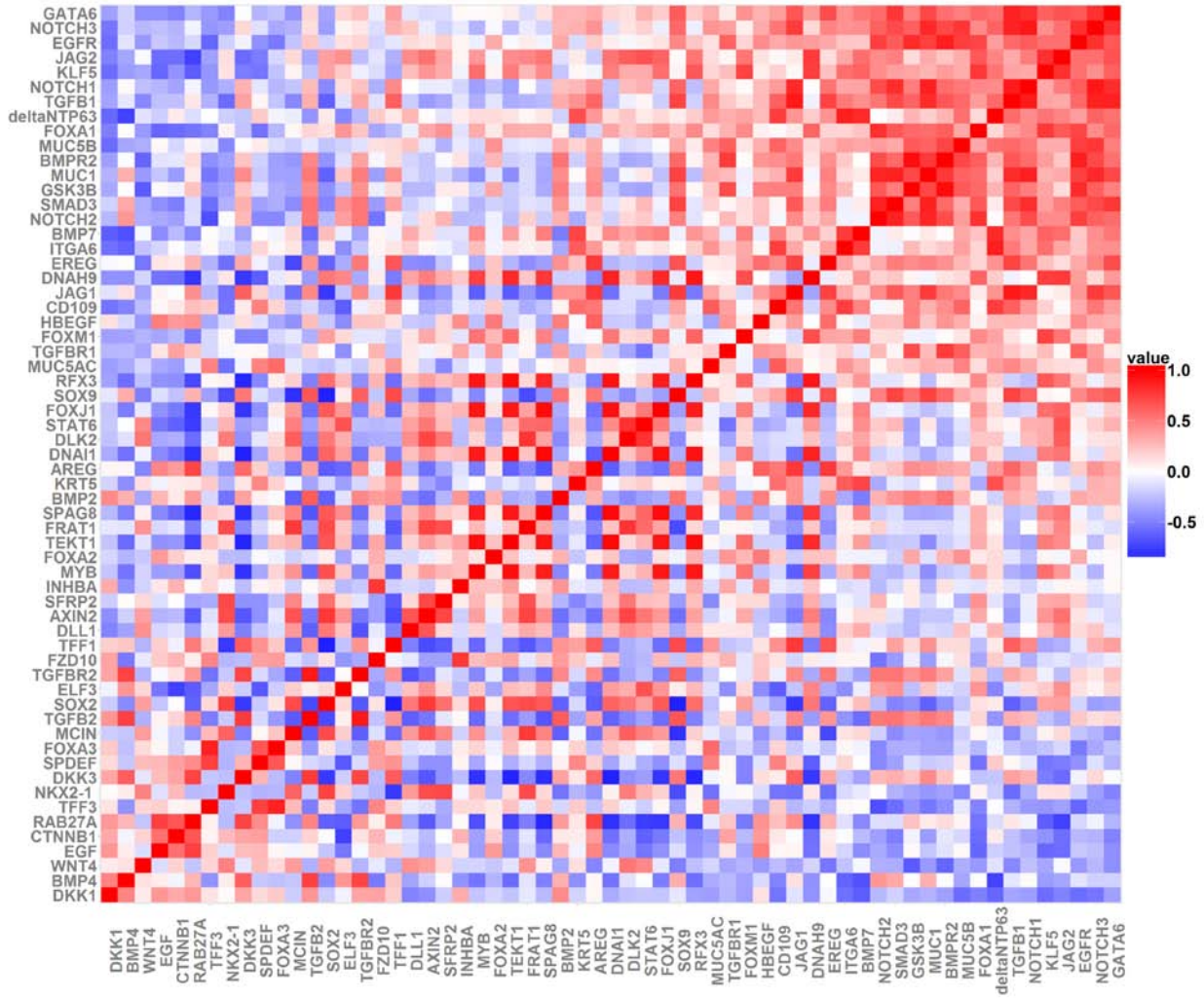
Zemke, A. C., R. M. Teisanu, A. Giangreco, J. A. Drake, B. L. Brockway, S. D. Reynolds and B. R. Stripp (2009). "beta-Catenin is not necessary for maintenance or repair of the bronchiolar epithelium." Am J Respir Cell Mol Biol **41**(5): 535-543.

Zhang, D. and L. Aravind (2010). "Identification of novel families and classification of the C2 domain superfamily elucidate the origin and evolution of membrane targeting activities in eukaryotes." Gene **469**(1-2): 18-30.

Zhang, Y., A. M. Goss, E. D. Cohen, R. Kadzik, J. J. Lepore, K. Muthukumaraswamy, J. Yang, F. J. DeMayo, J. A. Whitsett, M. S. Parmacek and E. E. Morrisey (2008). "A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration." Nat Genet **40**(7): 862-870.

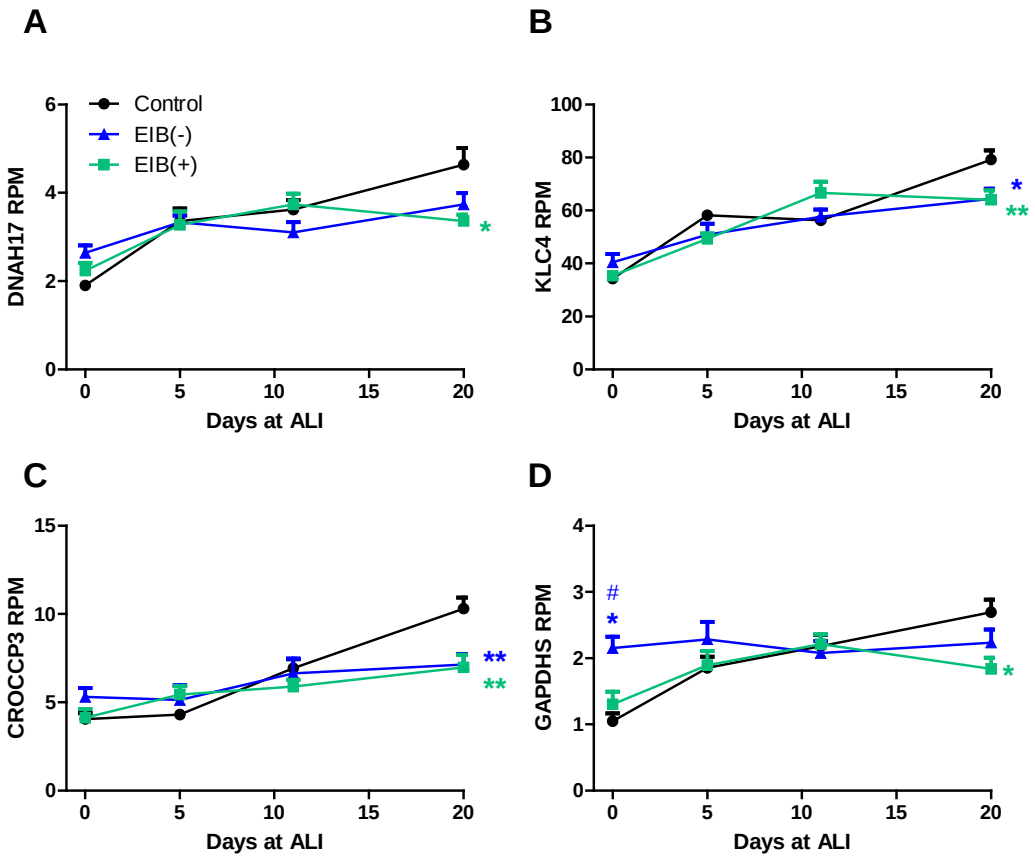
Appendices

Appendix A Supplemental data for chapter 6: Distinct mRNA and miRNA profiles in EIB(-) and EIB(+) asthma during mucociliary differentiation

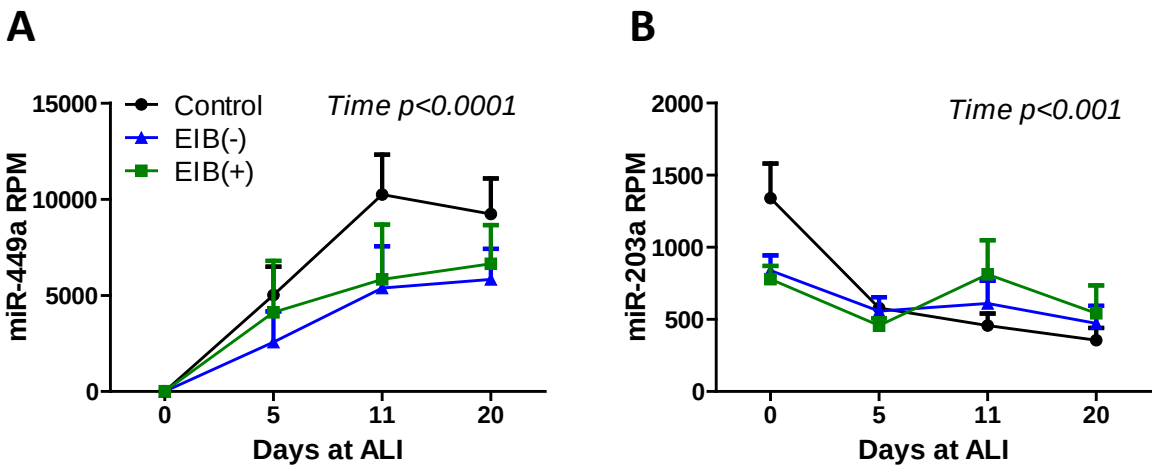


A.1 Co-expression plot of 61 candidate genes.

The 61 candidate genes described in **Chapter 6.3.1** are listed on both axes. Red indicates positive correlation and blue indicates negative correlation of expression between genes across all samples.



A.2 Ciliogenesis-related genes in cluster 4 are significantly decreased in asthma.
(A) DNAH17 (dynein, axonemal, heavy chain 17), **(B)** KLC4 (kinesin light chain 4), **(C)** CROCCP3 (ciliary rootlet coiled-coil, rootletin pseudogene 3) and **(D)** GAPDHS (GAPDH, spermatogenic). Values are shown as mean ± SEM reads per million reads (RPM) for each gene. Statistical significance was assessed by two-way ANOVA with Bonferroni post-test (* $p<0.05$, ** $p<0.01$ vs. control; # $p<0.05$ vs. EIB(+)).



A.3 Candidate miRNAs do not differ in asthma.

Expression of **(A)** miR-449a and **(B)** miR-203a are shown as the mean ± SEM reads per million reads (RPM). Expression of both miR-449a and miR-203a changed significantly over time as determined by two-way ANOVA, with p-values provided in the top right corner of each graph.

mirConnX hub	Mature miRNA(s)[#]	Log_2 (day20/day0)[$]			Two-way ANOVA FDR q-value†		
		Control	EIB(-)	EIB(+)	group	time	group:time
miR-375	hsa-miR-375	**6.10**	**6.83**	**6.78**	**3.6E-02**	**0.0E+00**	1.0E+00
miR-190	hsa-miR-190b	**5.22**	**4.44**	**4.79**	2.0E-01	**0.0E+00**	1.0E+00
miR-187	hsa-miR-187-3p	**4.01**	**3.80**	**3.97**	**3.1E-02**	**0.0E+00**	1.0E+00
miR-223	hsa-miR-223-3p	**2.76**	**2.03**	**2.55**	3.7E-01	**6.1E-13**	1.0E+00
	hsa-miR-223-5p	0.83	0.64	0.78	1.5E-01	**6.6E-07**	1.0E+00
miR-200a	hsa-miR-200a-3p	**2.11**	**2.05**	**2.16**	1.0E+00	**0.0E+00**	1.0E+00
	hsa-miR-200a-5p	0.23	0.36	-0.04	2.7E-01	**3.5E-02**	1.0E+00
miR-141	hsa-miR-141-3p	**2.07**	**1.76**	**2.60**	6.1E-01	**0.0E+00**	1.0E+00
	hsa-miR-141-5p	-0.24	-0.12	-0.45	4.6E-01	**8.4E-03**	1.0E+00
miR-32	hsa-miR-32-5p	**1.37**	**1.02**	**1.57**	6.0E-01	**6.4E-14**	1.0E+00
	hsa-miR-32-3p	0.07	0.25	-0.27	6.0E-01	1.2E-01	1.0E+00
miR-328	hsa-miR-328	**1.34**	**1.00**	**1.21**	8.5E-02	0.0E+00	1.0E+00
miR-152	hsa-miR-152	0.66	0.89	0.90	3.7E-01	**2.5E-13**	1.0E+00
miR-22	hsa-miR-22-5p	0.50	0.78	0.82	**2.2E-02**	**1.6E-06**	1.0E+00
	hsa-miR-22-3p	-0.26	-0.13	-0.32	**4.5E-02**	1.4E-01	1.0E+00
miR-96	hsa-miR-96-5p	0.38	0.39	0.54	2.9E-01	**9.7E-05**	1.0E+00
miR-182	hsa-miR-182-3p	0.26	0.23	-0.07	9.4E-01	4.7E-01	1.0E+00
	hsa-miR-182-5p	0.02	0.17	0.14	1.3E-01	**1.4E-02**	1.0E+00
miR-192	hsa-miR-192-5p	0.13	0.38	0.27	9.7E-01	**7.6E-03**	1.0E+00
miR-107	hsa-miR-107	0.09	-0.11	0.08	9.7E-01	1.3E-01	1.0E+00
miR-93	hsa-miR-93-5p	0.07	0.11	-0.09	9.2E-01	**1.7E-04**	1.0E+00
	has-miR-93-3p	-0.74	-0.29	-0.71	1.5E-01	**2.8E-04**	1.0E+00
miR-195	hsa-miR-195-5p	-0.04	0.26	0.35	7.9E-01	4.9E-01	1.0E+00
miR-186	hsa-miR-186-5p	-0.07	-0.20	-0.13	9.4E-01	**5.0E-02**	1.0E+00
miR-149	hsa-miR-149-5p	-0.34	-0.07	-0.61	4.7E-01	**4.2E-03**	1.0E+00
miR-132	hsa-miR-132-5p	-0.26	-0.04	-0.78	**1.2E-02**	8.8E-02	1.0E+00
	hsa-miR-132-3p	-0.38	-0.04	-0.17	**3.3E-02**	4.1E-01	1.0E+00
miR-183	hsa-miR-183-5p	-0.30	-0.31	-0.11	3.4E-01	6.2E-02	1.0E+00
	hsa-miR-183-3p	-0.68	-0.51	-0.85	3.7E-01	**3.6E-09**	1.0E+00
miR-185	hsa-miR-185-5p	-0.43	-0.22	-0.49	**1.3E-02**	**3.1E-04**	1.0E+00
	hsa-miR-185-3p	-0.91	-0.43	-0.87	2.2E-01	**1.4E-03**	1.0E+00
miR-31	hsa-miR-31-3p	-0.84	-0.75	-0.91	4.0E-01	**1.4E-07**	1.0E+00
	hsa-miR-31-5p	-0.92	-0.88	-1.05	4.6E-01	**2.6E-12**	1.0E+00
miR-210	hsa-miR-210	**-1.07**	**-1.10**	**-1.17**	1.0E-01	**3.5E-15**	1.0E+00
miR-21	hsa-miR-21-3p	**-1.12**	-0.85	**-1.07**	6.4E-02	**1.4E-15**	1.0E+00
	hsa-miR-21-5p	-0.57	-0.26	-0.47	1.5E-01	**2.1E-05**	1.0E+00
miR-212	hsa-miR-212-3p	**-1.18**	-0.53	-0.84	**1.9E-03**	**4.3E-04**	1.0E+00
	hsa-miR-212-5p	**-1.02**	-0.38	-0.86	**2.4E-02**	**1.4E-03**	1.0E+00

mirConnX hub	Mature miRNA(s)[#]	Log_2 (day20/day0)[$]			Two-way ANOVA FDR q-value†		
		Control	EIB(-)	EIB(+)	group	time	group:time
miR-25	hsa-miR-25-5p	**-1.64**	**-1.53**	**-1.50**	6.2E-01	**6.0E-15**	1.0E+00
	hsa-miR-25-3p	-0.30	-0.16	-0.31	2.3E-01	**5.1E-04**	1.0E+00
miR-205	hsa-miR-205-3p	**-1.78**	**-2.03**	**-2.25**	8.0E-02	**9.2E-16**	1.0E+00
miR-203	hsa-miR-203a	**-1.91**	-0.83	-0.52	6.3E-01	**1.3E-03**	1.0E+00
miR-221	hsa-miR-221-5p	**-1.92**	**-1.50**	**-1.88**	6.0E-01	**0.0E+00**	1.0E+00
	hsa-miR-221-3p	-0.67	-0.68	-0.75	3.2E-01	**1.1E-06**	1.0E+00
miR-335	hsa-miR-335-3p	**-2.18**	**-1.86**	**-2.27**	**2.9E-04**	**0.0E+00**	1.0E+00
	hsa-miR-335-5p	-0.04	-0.28	-0.38	**1.9E-02**	6.1E-01	1.0E+00
miR-126	hsa-miR-126-5p	**-2.33**	**-2.19**	**-2.51**	7.0E-02	**0.0E+00**	1.0E+00
	hsa-miR-126-3p	**-1.88**	**-2.00**	**-2.28**	2.1E-01	**9.2E-16**	1.0E+00
miR-222	hsa-miR-222-3p	**-1.60**	**-1.42**	**-1.65**	5.3E-01	**0.0E+00**	1.0E+00
	hsa-miR-222-5p	**-3.42**	**-3.10**	**-3.32**	6.5E-01	**0.0E+00**	1.0E+00
miR-155	hsa-miR-155-5p	**-3.89**	**-3.72**	**-4.23**	4.3E-01	**0.0E+00**	1.0E+00
miR-100	hsa-miR-100-5p	**-4.06**	**-4.46**	**-4.61**	9.0E-01	**0.0E+00**	1.0E+00
	hsa-miR-100-3p	**-4.37**	**-4.38**	**-5.02**	8.6E-01	**0.0E+00**	1.0E+00

A.4 MiRNA hubs identified by mirConnX analysis.

Mature miRNAs that correspond to a given mirConnX hub.

$ Fold change in expression at day 20 vs. day 0 for each of the three donor groups are given as log_2 values. Red indicates upregulation by ≥2-fold, while blue indicates downregulation by ≥2-fold.

† FDR q-values are provided for the overall analysis of differential expression for each mature miRNA. **Bold** face indicates miRNAs with q<0.05.

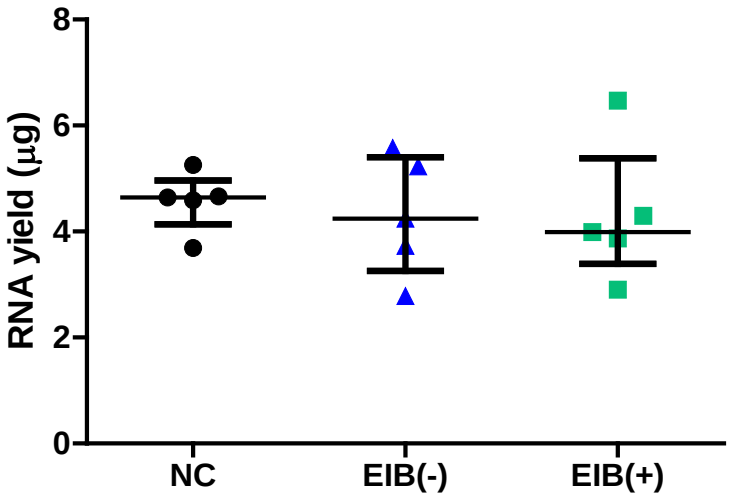
mirConnX hub	Activated			Suppressed		
	# targets	Gene Ontology	Genes in pathway	# targets	Gene Ontology	Genes in pathway
miR-100	4	None		8	GO:0044238 [4]: primary metabolism	FGFR3 HOXA1 HS3ST3B1 ICMT MTMR3 SMARCA5 TRIB2
miR-126	1	None		0	None	
miR-155	30	GO:0006355 [7]: regulation of transcription, DNA-dependent	BACH1 BRD1 CHD7 ELL2 ETS1 HIF1A HIVEP2 SMARCA4	37	GO:0050789 [2]: regulation of biological process	CEBPB FGF7 HBP1 HDAC4 MECP2 MYB NFAT5 NR4A3 PKIA SATB1 SOCS1 SOCS6 SOX6 SP3 TLE4 TRPS1 ZNF238
miR-203	37	None		58	GO:0006139 [5]: nucleobase, nucleoside, nucleotide and nucleic acid metabolism	AHR ARNTL CITED2 FMR1 HOXA1 ISL1 MECP2 MEF2C MORF4L1 NLK NMNAT2 NR4A3 PDE4D PRPS2 RARB SFRS1 TARDBP XRN2 ZMYND11 ZNF265
miR-205	24	None		40	None	
miR-21	24	GO:0050879 [4]: organismal movement	RP2 TGFBI TIMP3	22	None	
miR-210	2	None		0	None	
miR-212	15	GO:0042981 [6]: regulation of apoptosis	DEDD PEA15 YWHAG	40	GO:0006139 [5]: nucleobase, nucleoside, nucleotide and nucleic acid metabolism	ARHGEF11 BRCA1 CBFA2T2 DPYSL3 FMR1 ISL1 MECP2 NLK NMNAT2 NR4A2 PAPOLA PHF20L1 PTBP2 SIRT1 SOX5 WHSC1L1 ZNF238 ZNF644

mirConnX hub	Activated			Suppressed		
	# targets	Gene Ontology	Genes in pathway	# targets	Gene Ontology	Genes in pathway
miR-221	17	GO:0050789 [2]: regulation of biological process	ARID1A CDKN1C ETS1 GNAI2 HTLF MAP3K10 PAIP1 PBX3 TIMP3 YWHAG	39	GO:0007156 [6]: homophilic cell adhesion	PCDHA1 PCDHA10 PCDHA12 PCDHA13 PCDHA4 PCDHA5 PCDHA6 PCDHA7 PCDHAC1 PCDHAC2
miR-222	20	GO:0050789 [2]: regulation of biological process	ARID1A CDKN1C ETS1 GNAI2 HTLF MAP3K10 PAIP1 PBX3 TIMP3 YWHAG	42	GO:0007156 [6]: homophilic cell adhesion	PCDHA1 PCDHA10 PCDHA12 PCDHA13 PCDHA4 PCDHA5 PCDHA6 PCDHA7 PCDHAC1 PCDHAC2
miR-25	31	GO:0000278 [6]: mitotic cell cycle	CCNE2 LATS2 POLS RAD21	15	None	
miR-335	18	None		26	None	

A.5 Gene ontologies targeted by miRNAs that decrease during differentiation.

mirConnX hub	Activated			Suppressed		
	# targets	Gene Ontology	Genes in pathway	# targets	Gene Ontology	Genes in pathway
miR-141	53	GO:0006355 [7]: regulation of transcription, DNA-dependent	ARNTL ELF2 ESRRG HDAC4 HMG20A HTATSF1 LHX6 RARB RUNX1 SIM2 SOX5 STAT4 STAT5A ZNF238	64	GO:0008283 [4]: cell proliferation	ATF5 CCNE2 CDC25A E2F3 IRS2 MTSS1 MYH10 NRP1 PBEF1 PPP2CA TOP1 YWHAG
miR-187	2	None		0	None	
miR-190	20	GO:0000375 [8]: RNA splicing, via transesterification reactions	MYEF2 SFRS1 TNRC6A	13	None	
miR-200a	2	None		4	GO:0050791 [3]: regulation of physiological process	PBEF1 TCERG1 WHSC1 YY1
miR-223	29	None		18	None	
miR-32	61	GO:0006355 [7]: regulation of transcription, DNA-dependent	ARID1B BAZ2B CEBPA CREM EGR2 ESRRG KLF2 NFIA NLK NR4A3 NRF1 PER2 SIM2 SMAD6 TFDP2	59	GO:0000278 [6]: mitotic cell cycle	CCNE2 CDK6 CDKN1C LATS2 POLS RAD21 STAG2
miR-328	12	None		12	None	
miR-375	2	None		2	None	

A.6 Gene ontologies targeted by miRNAs that increase during differentiation.



A.7 Total RNA yield per cell does not differ between donor groups.

After trypsinization of airway epithelial cells from 25cm^2 flasks for seeding in ALI inserts, total RNA was isolated from 2x10^5 cells and quantified using the Nanodrop spectophotometer. The RNA yield was calculated based on volume of eluate and was not found to differ between donor groups by Kruskal-Wallis with Dunn's post-test. Data are shown as median RNA yield in µg with interquartile range.