The Identification of Airway Squamous Metaplasia Inhibitors: Modulating Cell Fate as a Therapeutic Strategy

The Identification of Airway Squamous Metaplasia Inhibitors:

Modulating Cell Fate as a Therapeutic Strategy

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Abstract

In humans, the conducting airway consists of a pseudostratified epithelium with three main cell types: secretory, ciliated and basal cells. Airway basal cells act as multipotent stem cells, which proliferate and differentiate into luminal secretory cells (goblet and club cells) as well as ciliated cells. The proper balance between basal cell proliferation and differentiation into the appropriate numbers and types of specialized cells of the epithelium is essential for defending against airborne pathogens and other insults as well as for tissue homeostasis. Pathological remodeling of these cell types occurs in conditions such as chronic obstructive pulmonary disease (COPD). Epithelial phenotypes in COPD include basal cell hyperplasia, goblet cell hyperplasia and squamous metaplasia, resulting in impaired mucociliary clearance, an increase in bacterially-colonized airways, and pulmonary exacerbations.

Here, I describe a 384-well, three-dimensional (3-D) organoid squamous metaplasia model used to screen for modulators of airway squamous metaplasia (ASM), and the identification of a small molecule inhibitor of ASM, ASMi-1 (airway squamous metaplasia inhibitor-1). Real-time quantitative RT-PCR and immunohistochemistry were used to examine markers of normal differentiated cells types, squamous epithelial markers and tight junction protein Occludin. ASMi-1 resulted in the restoration of the mucociliary and squamous markers. First-line therapies for COPD focus on symptomatic management and not on treating the underlying cause of the disease. Addressing airway remodeling as a strategy to restore homeostasis in an airway that has

undergone squamous metaplasia may result in sustained, improved lung function. This work may provide a rationale for development of a new class of therapies to treat pulmonary diseases such as COPD.

Dedication

To my family: mom (Angeles), Tio Lucho (Luis), Aracelis, Mohammed, Alana, Miguel Torres, and Gibo (Emilio Giboyeaux) always providing inspiration to keep going. To Francesca and Peter Pessotti whom I love dearly. To Lauren, Steve and Chase McCabe for being a center part of my village. My husband Andrew for being my rock, thank you for your unconditional love and support. And to our sweet son Antonio Rafael.

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Chapter I.

Introduction

The conducting airway contains a pseudostratified epithelium, which serves to protect the body from environmental injury and promote tissue homeostasis. The epithelium contains basal progenitor cells (BCs), so called because of their close proximity to the basement membrane, which can self-renew and differentiate into several cell types including secretory cells (such as goblet and club cells) and ciliated cells (Rock, Randell, & Hogan, 2010). Goblet cells secrete mucins to trap airborne particles and pathogens, while ciliated cells transport mucous out of the airway through ciliary beating (Fahy & Dickey, 2010). Disruption of the composition and organization of the airway epithelium is a hallmark of various lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Fahy & Dickey, 2010; Hogan et al., 2014).

COPD is a major global health problem and is the third-leading cause of death in the world (WHO, 2018). Smoking, which is the leading cause of COPD (Barnes, 2018) has important pathological consequences, resulting in many COPD and non-COPD comorbidities. The airways of patients with COPD display regions of basal and goblet cell hyperplasia as well as squamous metaplasia. Squamous metaplasia is characterized by the transformation of the normal pseudostratified epithelium to a stratified epithelium consisting of flattened squamous cells devoid of the normal differentiated cell populations (Auerbach, Stout, Hammond, & Garfinkel, 1961; Rock et al., 2010). This airway remodeling prevents proper mucociliary clearance leading to infection, plugging of airways, and impaired respiration.

COPD studies have failed to provide statistically meaningful data due to the heterogeneity in disease pathology, practical challenges in sample collection, analysis (Rock et al., 2010) and poor patient recruitment (Markun, Rosemann, Dalla-Lana, & Steurer-Stey, 2016). Currently there is no clinical approach targeting squamous metaplasia as a strategy for COPD therapy. The signaling pathways regulating the progenitor lineage choice to a squamous epithelium are largely unknown (Li et al., 2008; Rock et al., 2010; Wistuba, & Gazdar, 2006). With the exception of Involucrin and several cytokeratins, few molecular markers that characterize the squamous phenotype have been identified. Due to the phenotypic heterogeneity throughout the conducting airway epithelium, methods such as bronchial brushing are potentially unable to specifically sample between normal and squamous regions (Rock et al., 2010). Furthermore, characterization of regional patterns of gene expression in basal cells and other airway epithelial cell types in normal and diseased tissue is still under investigation. These challenges make this complex multifactorial disease difficult to model *in vitro* and *in vivo*, hampering research efforts.

Our therapeutic hypothesis is that restoring the proper number and distribution of cell types in an airway that has undergone squamous metaplasia will restore tissue homeostasis and improve lung function in patients with COPD. This work outlines a research program intended to identify stimuli and pathways that promote squamous metaplasia and to subsequently identify inhibitors of this phenotype.

Although squamous metaplasia correlates with severity of COPD and is negatively correlated with lung function (Cosio et al., 1978; Lapperre et al., 2007; Rigden et al., 2016), and COPD is currently one of the leading causes of death in the world

(Quaderi SA., & Hurst JR., 2018) there are no known active clinical trials that are directly modulating squamous metaplasia (clinicaltrials.gov). Therefore, the discovery and development of therapeutics that reverse squamous metaplasia and restore airway homeostasis would be a novel approach to addressing this unmet medical need.

Defining the lesion

COPD is a complex disease comprised of chronic bronchitis and emphysema caused by irritant exposure such as air pollution, respiratory infections (Petty, 2006), and most commonly exposure to cigarette smoke (CDC, 2018). Although, not explicitly defined as chronic obstructive pulmonary disease (COPD) until 1965 (Briscoe & Nash, 1965; Fishman, 2005), phenotypes now recognized as associated with COPD were noted as far back as 1679, when Theophile Bonet described "voluminous lungs" (Bonet, 1679). By the 1800's, COPD had been recognized as a debilitating disease that was characterized by chronic airway obstruction and limited airflow (Petty, 2006). COPD is currently the third leading cause of death globally, claiming 3 million lives in 2016 (WHO, 2018), and it is projected to be the leading cause of death in the next 15 years (Quaderi & Hurst, 2018).

The proper balance between proliferation and differentiation of airway epithelial cells is disrupted in pathological conditions such as COPD, resulting in impaired mucociliary clearance, an increase in colonized airways, and exacerbations. For

example, goblet cell hyperplasia and squamous metaplasia are observed in patients with COPD, resulting in an increased number of mucous producing goblet cells and the transformation of a normal pseudostratified epithelium to a stratified, squamous epithelium devoid of mucociliary cell types, respectively (Crystal, 2014; Decramer, Janssens, & Miravitlles, 2012; Hogan et al., 2014).

Squamous metaplasia severity correlates with COPD progression and is negatively correlated with lung function (Araya et al., 2007; Cosio et al., 1978; Lapperre et al., 2007; Rigden et al., 2016). Squamous metaplasia is also an early smoking-induced lesion, linked to the onset of lung carcinogenesis (Auerbach et al., 1961; Wistuba & Gazdar, 2006), and patients with COPD are at an increased risk to develop lung cancer (Rigden et al., 2016).

The sequence of molecular events leading to squamous metaplasia *in vivo* is unclear. Studies have demonstrated that smoking-induced squamous metaplasia is a multistep process most likely of basal cell origin (Jetten, 1987; Jetten, 1989). These data suggest that in response to tobacco smoke, there is an induction of basal cell proliferation (hyperplasia). The amplifying cells of the now stratified epithelium express different keratins associated with the squamous phenotype. Finally, superficial, flat, squamous cells replace normal differentiated cell types (squamous metaplasia) (Crystal, 2014; Dye, 1994; Jetten, 1989; Leube & Rustad, 1991; Rigden et al., 2016), expressing markers of terminally-differentiated squamous epithelial cells such as involucrin (IVL) and small proline rich proteins (SPR) (Deng, Chen, & Wu., 2000; Jetten, 1987; Koo et al., 1999; Rigden et al., 2016).

Therapeutic targeting of COPD

COPD is a heterogeneous disease characterized by progressive airflow limitation and chronic inflammation. It is associated with structural changes of the airway epithelium and breakdown of the parenchyma (Ejiofor & Turner, 2013; Lakshmi, Reddy, & Reddy, 2017; Watz et al., 2013). Several therapeutic approaches are currently being investigated in the clinic to target various aspects of the disease. Strategies for the treatment of COPD generally target epithelial cells, smooth muscle, immune defenses or the alveolar epithelial cells (which make up the gas-exchange units in the lungs). Each of these areas are discussed below, highlighting the current status of a number of clinical trials that have been conducted to test a variety of therapeutic hypotheses in patients.

Smooth muscle

There is no cure for COPD and current standard of care focuses on addressing symptoms. First-line therapy is inhaled bronchodilators, which include short-acting β2-agonists (SABA) and long-acting β2-agonists (LABA). β2 adrenergic receptor agonists act on smooth muscle cell to stimulate adenylyl cyclase activity, resulting in an intracellular increase in cyclic adenosine monophosphate (cAMP). cAMP binds and

activates protein kinase A (PKA), leading to phosphorylation of myosin light chain kinase, resulting in decreased affinity for the calcium/calmodulin complex, finally leading to smooth muscle relaxation (Alvarado, 2017). This reduces air trapping and facilitates lung emptying during expiration, thereby rescuing symptoms when they occur. Bronchodilators are prescribed alone or in combination with glucocorticoids. Glucocorticoids bind the cytoplasmic glucocorticoid receptor (GR), which subsequently translocates to the nucleus and modulates expression of anti- and pro-inflammatory cytokines. Due to their side effects and modest efficacy, glucocorticoids are recommended only for severe COPD patients or those experiencing frequent exacerbations. However, they remain one of the main therapies prescribed for COPD (Ejiofor & Turner, 2013; Montuschi, 2006). While current therapies provide improved pulmonary function, neither of these first-line therapies prevent the long-term decline in lung function characteristically associated with COPD.

Immune system

Exposure to irritants activate inflammatory cells that release cytokines and chemoattractants in the lung, inducing chronic inflammation and contributing to the pathogenesis of COPD. CXCR2 is a chemoattractant for neutrophils upregulated in patients with COPD. An inhibitor to CXCR2 was found to improve lung function, as measured by FEV1 (forced expiratory volume in 1 second), only at the highest dose

tested of 50 mg (Rennard et al., 2015). This effect was only observed in the current smokers subpopulation and not in ex-smokers. Unfortunately, there were dose-dependent adverse events including significant absolute neutrophil count decrease, leading to dose-related discontinuation of treatment (Rennard et al., 2015). GlaxoSmithKline has also stopped development of danirixin, a CXCR2 antagonist as an anti-inflammatory agent for COPD after not achieving their primary endpoint in their recent Phase 2B trial (NCT03034967).

Several cytokines have been shown to be elevated in the plasma of COPD patients, making anti-cytokine antibodies an attractive therapeutic proposition. IL-1, IL-5, TNF, and IL17A are among these and are thought to augment inflammation. However, there are no positive trials that have been reported (Eich et al., 2017; Lakshmi et al., 2017). Due to their immuno-modulating properties, which have the potential to impact tissue repair and regeneration, cytokine-specific inhibitors are still of great interest. Notably dupilumab, a dual inhibitor of IL-4 and IL13 (NCT03930732) and tezepelumab (NCT04039113), an anti-TSLP antibody, are currently recruiting for Phase 2 and Phase 3 trials, respectively. Investigations for REGN3500, an anti-IL-33 monoclonal antibody is also currently ongoing as an anti-inflammatory target for the treatment for COPD (NCT03546907).

Pro-inflammatory signaling pathway inhibitors

Numerous pro-inflammatory signaling pathway inhibitors are also under investigation as COPD therapeutics. Considerable effort has been focused on p38-mitogen-activated protein kinase (p38 MAPK), which activates a pro-inflammatory response in lymphocytes and is increased in patients with COPD. Of eight p38 inhibitors that have been evaluated for COPD, only one remains in active development; Acumapimod, a Novartis oral treatment, reported clinical efficacy in their Phase II trial (Strambu et al., 2019). Other p38 inhibitors: RV-568 (Robinson et al., 2016), AZD-7624 (Patel et al., 2018) and losmapimod (Pascoe et al., 2017) did not demonstrate clinical benefits. PF-03715455 (NCT02366637) resulted in early study termination due to insufficient patient recruitment. Development of GSK-610677 was terminated following an unreported Phase I trial (Carroll, 2016). Despite PH-797804 demonstrating improved lung function, development was also terminated (Xing et al., 2012). Dilmapimod was also terminated with little characterization (Lakshmi et al., 2017).

Prostoglandin D2 (PTG2) and selectins are all elevated in patients with COPD and are currently under investigation. These mediate the pro-inflammatory chemotaxis of immune cells such as eosinophils and basophils. Selectins represent a family of cell adhesion molecules. Antagonists of selectin have demonstrated favorable results for the primary outcomes of their Phase II trials: reduction in macrophages and CXCL8 in the sputum of patients. However, there was no significant improvement in lung function (Watz et al., 2013). KBP-7026, a DP2 antagonist is on track to initiate Phase 1 trials (Kbp admin, 2015).

Stimulation of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear hormone receptor, has been shown to provide anti-inflammatory effects by inhibiting the expression of inflammatory cytokines such as TNFα, IL-6 and IL-1β (Lakshmi et al., 2017), which are highly expressed in patients with COPD. Targeting a factor that regulates the levels of multiple cytokines, rather than treating with cytokine-specific inhibitors, may be a more beneficial way to dampen the inflammatory response in COPD. However, 16% of patients with COPD (and a coexisting diagnosis for diabetes), that had been prescribed Thiazolidinediones (TZD), an anti-hyperglycemic medication and a selective PPAR agonist, experienced exacerbations. Eighteen percent of patients prescribed an alternative anti-hyperglycemic medication experienced exacerbations (Rinne et al., 2015). Although results from the TZD group was reported as a statistically significant reduction in exacerbations compared to an alternative anti-hyperglycemic medication, the improvement observed in the TZD prescribed group was small. Therefore, there remains a need to further explore PPARγ agonists as a therapeutic for COPD.

Phosphoinositide 3-kinase δ (PI3Kδ) is a lipid kinase that participates in multiple effector functions of leukocytes. Increased activation of the PI3Kδ pathway has been demonstrated in lung samples from COPD patients and has been shown to correlate with disease severity (To et al., 2010). GSK2269557, a PI3Kδ inhibitor, has been shown to reduce neutrophil migration and inhibit inflammation with an acceptable safety profile (Cahn et al., 2017).

cAMP and cGMP phosphodiesterase inhibitors: Targeting inflammation and bronchoconstriction

Due to their anti-inflammatory and smooth muscle bronchodilation effects, phosphodiesterase enzyme family (PDE) inhibitors are being investigated as COPD therapeutics (Fan Chung, 2006). PDEs hydrolyze and thereby inactivate cAMP and cGMP. Early efforts to develop PDE inhibitors for COPD failed due to a lack of efficacy and dose-limiting side effects, which was thought to be a consequence of pan-PDE inhibition (Barnes, 2003). The oral selective phosphodiesterase-4 inhibitor roflumilast suppresses COPD-associated inflammation by inhibiting cAMP degradation, exerting a broad range of anti-inflammatory effects (Wedzicha, Calverley, & Rabe, 2016), and received regulatory approval for the treatment of COPD (U.S. Food and Drug Administration, 2012). Roflumilast resulted in a modest but significant improvement in lung function in severe, stable COPD (Giembycz & Field, 2010). A decrease in exacerbation episodes was also found in a subset of patients with severe COPD (Calverley et al., 2007). However, the drug is frequently associated with dose limiting adverse events, namely gastrointestinal effects (Giembycz & Field, 2010, Watz et al., 2018). To improve upon the effects of roflumilast, Verona Pharma is investigating the inhalation delivery route for ensifentrine, a dual PDE3 and 4 inhibitor, to minimize adverse effects that have been associated with oral administration of PDE4 inhibitors. Ensifentrine acts both as a bronchodilator by inhibiting hydrolysis of cGMP and as an

anti-inflammatory agent (Smith, 2019). In a recent Phase IIb study, ensifentrine administration resulted in improved lung function (Singh et al., 2020).

Dual anti-inflammatory and antioxidant agents

Targeting both inflammation and oxidative stress is another therapeutic approach that is of interest. Reactive oxygen species (ROS) production as a result of oxidants present in cigarette smoke enhances inflammation by activating proinflammatory transcription factors such as activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This response is accompanied by a downregulation of the antioxidant transcription factor nuclear factor erythroid-2-related factor 2 (Nrf2). The imbalance of oxidants/antioxidants coupled with the inflammatory response contributes to the pathogenesis of COPD. *In vitro* and *in vivo* studies have shown that excess ROS contributes to alveolar airspace epithelial injury, while activation of Nrf2 has been shown to promote bacterial clearance and reduce tissue damage and oxidative stress in *in vivo* studies (Lakshmi et al., 2017; Wise et al., 2016). A Phase 2 study stimulating Nrf2 with sulforaphane demonstrated no improvement in pulmonary function, and no difference in inflammation or antioxidant markers (Wise et al., 2016). Interestingly, although sulforaphane stimulated Nrf2 activity *in vitro*, Nrf2 target genes in this study were not affected (Wise et al., 2016), making the study uninterpretable. Therefore, this could potentially still be a viable option for investigation.

Erdosteine, a mucolytic with anti-inflammatory effects mediated by antioxidant properties, has shown improvement in several clinical parameters. These include reduced frequency and severity of exacerbations, reduced recovery time after exacerbations, and improved mucociliary clearance (Moretti, 2009). Erdosteine has produced better clinical outcomes than agents that are either exclusively mucolytic or exclusively anti-inflammatory, suggesting that a multifactorial approach may be necessary for beneficial clinical outcome.

Peripheral airway

With the field's increasing understanding of re-alveolarization, the parenchyma has become a focus area for regenerative therapy approaches. Targets involved in the destruction of the extracellular matrix and lung parenchyma include matrix metalloproteinases (MMPs) and the neutrophil product elastase, both implicated in COPD.

AZD9669, a neutrophil elastase inhibitor, did not improve lung function or other primary endpoints in a short-term proof-of-concept study (Kuna A., Jenkins M., O'Brien CD., & Fahy WA, 2010). AZD1236, an MMP-9 and -12 inhibitor, also did not achieve a positive clinical outcome in a Phase 2 study (Dahl et al., 2012). Gilead has completed a safety and tolerability study for GS-5745, an anti-MMP-9 antibody, in COPD patients (NCT02077465), but have not reported the results.

Retinoic acid receptor (RAR) signaling is required for proper tissue development and tissue maintenance, and to promote alveolar septation (Roth et al., 2006; Stolk et al., 2012). A study performed by Massaro and Massaro (1997) demonstrated that treatment with all-trans retinoic acid (ATRA) increased alveolar numbers in rats with emphysema. This and other studies motivated the investigation of retinoic acid in the treatment of COPD. A 6-month feasibility study in patients with moderate-to-severe COPD, treated with either a high or low dose of ATRA or 13-cis retinoic acid determined that there were minimal side effects but no improvement in lung function or quality of life (Roth et al., 2006). Concerns about complications with RA metabolism prompted other investigators to look at a similar compound with different metabolic properties, palovarotene, an oral γ-selective RAR agonist. Although palovarotene treatment increased alveolar area and oxygen in arterial blood in rats, patients with moderate-to-severe emphysema failed to demonstrate any significant changes to lung function by multiple measurements, including gas-transfer and CT analysis of lung volume (Stolk et al., 2012).

Epithelial Cells: Mucous Hypersecretion

The airway epithelium contributes to lung defense in various ways including acting as a physical barrier, regulating various immunological processes, and by performing mucociliary clearance. Strategies for therapeutic intervention in COPD patients include improving mucociliary clearance by limiting mucus synthesis and

secretion. Mucus hypersecretion in the airway is a clinical feature of patients with COPD, leading to airway obstruction. Additionally, goblet cell abundance is inversely correlated with lung function (Hogg et al., 2004).

The epidermal growth factor receptor (EGFR) signaling cascade can result in goblet cell hyperplasia and mucin production (Nadel, 2001). The expression of EGFR has been found to be elevated in COPD patients (Lakshmi et al., 2017). The EGFR signaling cascade can be induced by cigarette smoke, upregulating mucin production through signal transducer and activation of transcription 6 (STAT6) (Ha & Rogers, 2016). An investigation of BIBW2948, an inhaled EGFR antagonist, demonstrated that treatment had no effect on mucosal gene expression or goblet cell numbers, no impact on neutrophilic inflammation, and a dose-related decline in lung function. (Woodruff et al., 2010).

Several groups have demonstrated interleukin-13 (IL-13) increases differentiation of goblet cells and mucus production (Laoukili et al., 2001). IL-13 is detected in the plasma of COPD patients and is inversely correlated with lung function. (Lee et al., 2007). The safety and efficacy of lebrikizumab, an anti-IL-13 monoclonal antibody, has been investigated in a Phase 2 study in patients with COPD, but the results have not yet been reported (NCT02546700).

In vitro and *in vivo* studies demonstrated myristoylated alanine-rich C kinase substrate (MARCKS) plays a critical role in mucin secretion by mediating movement of intracellular mucin granules to the apical membrane of goblet cells, and ultimately exocytosis (Green, Crews, Park , Fang, & Adler, 2011; Lakshmi et al., 2017). BioMarck Phamaceuticals has reported that a MARCKS inhibitor, Bio-11006, reduced mucus

hypersecretion and inflammation, and improved lung function in a Phase 2, 21-day trial (Bio-11006) (Van, Kraft, Hanania, Parikh, & Murphy, 2011). It should be noted that this was a small sample size (n=172, distributed amongst five treatment cohorts) and short administration period, and may not fully provide sufficient data on the long-term effects on the disease in the broader population. Furthermore, there is a concern that blocking mucus secretion would lead to intracellular mucin accumulation and an unexpected release of the mucin and flooding of the airways (Ha & Rogers, 2016).

Epithelial Cells: Ion Channels

Due to its critical role in the hydration of airway mucous, airway epithelial ion transport has attracted considerable attention as an emerging target for several airway diseases including COPD. Cystic fibrosis transmembrane conductance regulator (CFTR) is an ion channel expressed in several tissues including the airway. CFTR conducts chloride ions apically across epithelial cell membranes contributing to airway hydration. Cigarette smoke has been shown to drive CFTR dysfunction, reducing airway surface liquid and increasing mucus thickening, resulting in impaired mucociliary clearance and ultimately leading to infection and respiratory failure. QBW251, a CFTR potentiator, is currently under investigation for COPD, and has shown favorable results in multiple endpoints including increased FEV1 relative to placebo and improved bacterial colonization and inflammation (Rowe et al., 2018). TMEM16A and epithelial Na^+

channel (ENaC), are additional ion channels that play critical roles in fluid homeostasis (Caputo et al., 2008; Matalon, Bartoszewski, & Collawn, 2015). Therapies targeting ENaC and TMEM16A are currently being investigated in preclinical models of COPD (Danahay et al., 2020), and are expected to enter human studies in 2020 (Enterprise Therapeutics).

COPD is a progressive disease in which standard of care focuses on symptomatic improvement and not on modifying long-term progression of disease. Only smoking cessation and long-term oxygen therapy have provided patients with prolonged life (Petty, 2006). As the pathogenesis of COPD is complex, numerous strategies have been investigated, as summarized above. Most of these investigations have shown minimal clinical benefit and have not improved survival. Therefore, novel therapeutic strategies need to be investigated.

Chapter II.

Materials and Methods

Culturing, differentiating and treatment of primary human bronchial epithelial cells

Primary human bronchial epithelial cells (HBECs) purchased from Lonza (CC-4175) were characterized for basal cell marker expression as previously described (Danahay et al., 2015). Passage 1 (P1) HBECs were expanded in BEGM (CC-3171; Lonza) media. HBECs were trypsinized and seeded into assay plates in 3D generating 'bronchospheres' as previously described (Danahay et al., 2015). Briefly, 10 µl of differentiation media containing 25% Matrigel® (354230; BD Biosciences) was plated onto 384-well plates (781091; Greiner). P1 HBECs were resuspended in differentiation media containing 5% Matrigel® solution at a density of 30,000 cells/ml. Twenty microliters of suspension was plated in each well of the pre-coated plate. Bronchospheres were treated at day 2 and day 8 of culture by adding 30 µl of media containing the indicated treatment.

To drive normal differentiation of basal cells, standard growth conditions include all-trans retinoic acid (0.25% DMSO final concentration). To assess the capacity of airway basal cells to generate a squamous phenotype, bronchospheres were cultured in media lacking all-trans retinoic acid (i.e., 0.25% DMSO final concentration, 'vehicle' control). Scale bar for phase images represents 100 µm. To validate other potential stimuli that induce squamous metaplasia, bronchospheres were treated with increasing concentrations of EGF, HBEGF or AREG (236-EG, 259-HE, 262-AR; R&D Systems) in

the presence of all-trans retinoic acid. Novartis internal compounds that inhibit LATS-1 and LATS-2 were tested in the bronchophere assay in a similar fashion.

The Mechanism of Action (MoA) Box, a Novartis set of 1609 tool compounds (Liu et al., 2018), was screened in the bronchosphere assay described above, in the absence of all-trans retinoic acid at a dose of 10 μM (data not shown), and validated in dose response (0.25% DMSO, final concentration).

Air-liquid interface (ALI) cultures were performed as previously described (Danahay et al., 2002). On day 1 of ALI, the basolateral compartment was replenished with differentiation media lacking all-trans retinoic acid, or complete differentiation media as control. To test the ability of a compound to suppress squamous metaplasia, ASMi-1 was added to differentiation media lacking all-trans retinoic acid during the 14 days of ALI. To examine the effect of ASMi-1 treatment on an established squamous phenotype over time, HBECs were grown on transwell filters in RA-free medium for 21 days. On day 21 (day 14 of ALI) cultures were treated with ASMi-1 at a concentration of 1 μM. At either Day 23, 26, 28, 30 and 33 cells were harvested and gene expression analysis was performed for the indicated cell types. Control treatments were cultured in RA-containing media from day 0 to day 33.

Gene expression analysis

To quantify transcript levels gene expression analysis of bronchosphere cultures, qRT-PCR was performed. First, bronchospheres were prepared for lysis by removing culture medium from assay plate. Cultures were subsequently washed with 110 μl PBS and aspirated to 4 μl. 15 μl of RealTime ready Cell Lysis Buffer (07248431001; Roche)

containing 1:80 protector RNase Inhibitor was transferred to each well. After mixing, lysates were incubated at room temperature for 15 minutes. qRT-PCR was performed using the Cells-to-CT Kit (4391852C; Ambion). Reverse transcription (RT) master mix was assembled as follows:

Component	Each per well (µl)
2X RT buffer	7.5
20X RT Enzyme mix	0.75
Water	4.5
Lysate	2.3
Total	15.05

RT thermal cycler program was run with the following settings: 1 hour at 37 °C, 5 minutes at 95 °C. qRT-PCR was performed on a ViiA7 Real-Time PCR System (Applied Biosystems)with the following TaqMan (ThermoFisher) gene expression assays: *Actin*, Hs01060665_g1; *GAPDH*, Hs99999905_m1; *MUC5AC*, Hs01365601_m1; *MUC5B*, Hs00861588_m1; *FOXA3*, Hs00270130_m1; *FOXJ1*, Hs00230964_m1; *DNAI2*, Hs01001544_m1; *SCGB1A1*, Hs00171092_m1; TP*63*, Hs00978340_m1; *ITGA6*, Hs01041011_m1; *IVL*, Hs00902520_m1; *SPRR1A*, Hs00954595_s1; *KRT6B*, Hs00749101_s1; *KRT14*, Hs00265033_m1; *CTGF*, Hs00170014_m1; *CYR61*, Hs00155479_m1. Viia7 program was run with the following settings: Comparative CT, hold stage: 20 sec. at 95 °C, PCR stage: 1 sec. at 95 °C, 20 sec. at 60 °C.

Immunofluorescence staining and imaging

On day 14 of ALI (day 21 of the assay) the ALI cultures were fixed with 4% paraformaldehyde (15713; Electron Microscopy Sciences) for 30 minutes at room

temperature. Filters were washed three times with IF wash buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, and 0.05% Tween- 20), for 10 minutes each. Cultures were then blocked with IF buffer containing 10% normal goat serum (NGS) for 1 hour, and subsequently incubated with primary antibodies diluted in IF buffer containing 10% NGS overnight at 4 °C. For detection of goblet cells, cultures were incubated with primary antibody mouse anti-MUC5AC (RRID: AB_2314822; MS-145-P0; ThermoScientific), at a dilution of 1:400. For the detection of squamous cells, cells were incubated with rabbit anti-SPRR1A (RRID: AB_11130171; ab125374; Abcam) at a dilution of 1:100. To examine tight junctions, cells were incubated with mouse anti-Occludin (RRID: AB_2533101; 33-1500; ThermoScientific), at a dilution of 1:200. The following day, filters were washed three times with IF wash buffer, for 20 minutes each, and counterstained with AlexaFluor 488-conjugated anti-mouse (A21121; ThermoScientific), AlexaFluor 568-conjugated anti-rabbit (A11011; ThermoScientific) secondary antibodies plus Hoechst 33342 (H3570; ThermoScientific) at a dilution of 1:200 and 1:5,000 respectively for 1 hour. After washing, filters were punched out with 8-mm biopsy punches (33-37; Integra Miltex) and mounted onto a CytoSpin slide (5991057; ThermoScientific) in Prolong gold antifade containing DAPI for nuclear staining (P36935; Thermo Fisher Scientific).

Fluorescent images were collected on a confocal microscope (Axiovert 200; Zeiss), including a Yokogawa CSU-X1 spinning disc head, and an electron-multiplying charge-coupled device camera (Evolve 512; Photometrics) with a 10x objective (EC Plan-Neofluar 10x/0.30 Ph 1; Zeiss). Images were processed using Zen Blue software (Zeiss). The analysis pipeline was first optimized by measuring the cells of interest of the

positive and negative controls, and the parameters were to the entire dataset. Quantification of the cell types was determined using CellProfiler and is expressed as percent positive area by calculating the ratio of $MUC5AC^+$ and $SPRR1A^+$ over $DAPI^+$. Scale bar represents 1,000 μm. Scale bar for images for Occludin analysis represent 20 μm.

RXR/RAR Reporter Gene Assays

To determine whether ASMi-1 was acting by modulating the activity of RAR or the related retinoid-x-receptor (RXR), the compound was tested in a panel of RARα, β, γ (IB02201, IB02101, IB02001; Indigo Biosciences) and RXRα, β, γ reporter gene assays (IB00801, IB00811, IB00821; Indigo Biosciences) according to the manufacturer's specifications. The RXR/RAR reporter gene assays are comprised of a luciferase reporter gene linked to RAR/RXR-responsive promoters. Luciferase expression is induced if the test sample agonizes RAR or RXR.

Chapter III

Results

This study outlines a research program designed to identify stimuli and pathways that promote squamous metaplasia, and small molecule inhibitors that restore normal epithelial lineages in a squamous metaplasia airway *in vitro*.

Development of an *in vitro* model of airway squamous metaplasia

Previous studies have implicated a number of signaling pathways in the normal differentiation of airway basal cells into a mucociliary epithelium (Rock et al., 2010). Deregulation of some of these pathways, including retinoic acid (RA), epidermal growth factor receptor (EGFR), and the Hippo-Yap pathways, can result in airway squamous metaplasia *in vitro* and *in vivo* (Shaykhiev, 2013; Koo et al., 1999; Zhao et al., 2014), as reflected by morphological and gene expression changes. To develop an unbiased screening approach for inhibitors of squamous metaplasia, I validated the effect of stimuli, which modulate each of these pathways, for their effect on the expression of molecular markers of the mucociliary epithelium and squamous epithelium utilizing 'bronchospheres', three-dimensional (3-D) organoids derived from primary human airway basal cells that allow for high throughput screening (Hild & Jaffe, 2016) (Appendix, Figure 1a-i). Through gene expression analysis of cell type-specific markers

by qRT-PCR, I found that treating bronchospheres with three different EGFR ligands, epidermal growth factor (EGF), Heparin-binding EGF-like growth factor (HB-EGF), or amphiregulin (AREG), failed to elicit a phenotype consistent with squamous metaplasia. Although treatment with each EGFR-activating ligand resulted in a decrease of multiple goblet cell-specific genes (*MUC5AC, MUC5B, and FOXA3*), ciliated cell-specific genes (*FOXJ1* and *DNAI2*), and basal cell-specific genes (*TP63 and ITGA6*), relative to control cultures, they also reduced markers of squamous epithelia (*KRT6B, KRT14 and IVL*) (Appendix, Figure 1a-d). Similarly, treatment with the Novartis LATS inhibitors LATSi-1 and LATSi-2 resulted in a dose dependent decrease in goblet, ciliated and squamous markers, inconsistent with a role for the Hippo-Yap pathway in squamous metaplasia (Appendix, Figure 1e-h). LATSi-1 and LATSi-2 induced expression of the Yap pathway target genes connective tissue growth factor (*CTGF*) and cysteine-rich angiogenic inducer 61 (*CYR61*) (Zhao et al., 2008) in a dose dependent manner confirming target engagement and pathway modulation (Appendix, Figure 1i). These data indicate that perturbing the EGFR or Hippo-Yap pathway does not bias the differentiation of airway basal cells towards a squamous lineage.

Retinoic acid, a metabolite of vitamin A, is essential for normal development. Retinoic acid exerts its biological effects by binding to retinoic acid receptors and retinoid X receptors (RAR/RXR), inducing transcription of target genes involved in the maintenance and differentiation of stem and progenitor cells in developing and adult organisms (Gudas & Wagner, 2011). In the upper respiratory tract, retinoids are required for normal mucociliary differentiation. Pioneering work nearly 100 years ago demonstrated that vitamin A deficiency leads to squamous metaplasia in multiple tissues

in vivo, including in the airway (Mori, 1922; Wolbach & Howe, 1925). This effect of vitamin A deficiency has also been demonstrated in an *in vitro* model system of human airway epithelial cell differentiation (Koo et al., 1999; Cozens et al., 2018). In this *in vitro* model, airway epithelial cells are normally cultured at air-liquid interface (ALI) in media containing a number of soluble factors, including all-trans retinoic acid (ATRA), resulting in the formation of a mucociliary epithelium resembling the *in vivo* airway (Gray, Guzman, Davis, Abdullah, & Nettesheim, 1996). Culturing airway epithelial cells in media lacking retinoic acid leads to an increase in squamous differentiation and a decrease in the differentiation of goblet and ciliated cells.

I confirmed these previously published observations utilizing the bronchosphere assay (Appendix, Figure 1j). Consistent with a squamous phenotype, culturing HBECs in media lacking retinoic acid resulted in reduced expression of goblet cell and ciliated cell markers relative to control cultures, with a concomitant increase of squamous epithelial markers, suggesting a biasing of basal cell differentiation towards a squamous lineage at the expense of normal, mucociliary differentiation. Under normal differentiation conditions, phase contrast images revealed bronchospheres contained a central lumen similar to the morphology reported in Danahay et al., 2015 (Appendix, Figure 1k). Culturing in media lacking ATRA resulted in bronchospheres with less-pronounced lumens relative to bronchospheres in ATRA-containing media, a phenotype similar to the 'squamous' spheres reported in Kumar et al., 2011 (Appendix, Figure 1k). The development of this 'squamous' bronchosphere assay, which is performed in 384-well plates, enables unbiased, high-throughput screening of compounds to identify inhibitors of ASM using qPCR as a readout.

LMW compound identified from ASM *in vitro* phenotypic screen

After establishing a screening platform to identify factors that inhibit a squamous metaplasia phenotype *in vitro*, we screened a Novartis set of tool compounds called the 'mechanism of action box' (MoA box) (Liu et al., 2018), using cell type-specific marker genes as endpoints. The library consists of 1609 well-annotated compounds covering a broad range of biological activities. We identified a single compound, ASMi-1. which markedly increased mucociliary markers and reduced basal and squamous epithelial markers in a dose dependent manner, relative to DMSO-treated controls lacking retinoic acid.

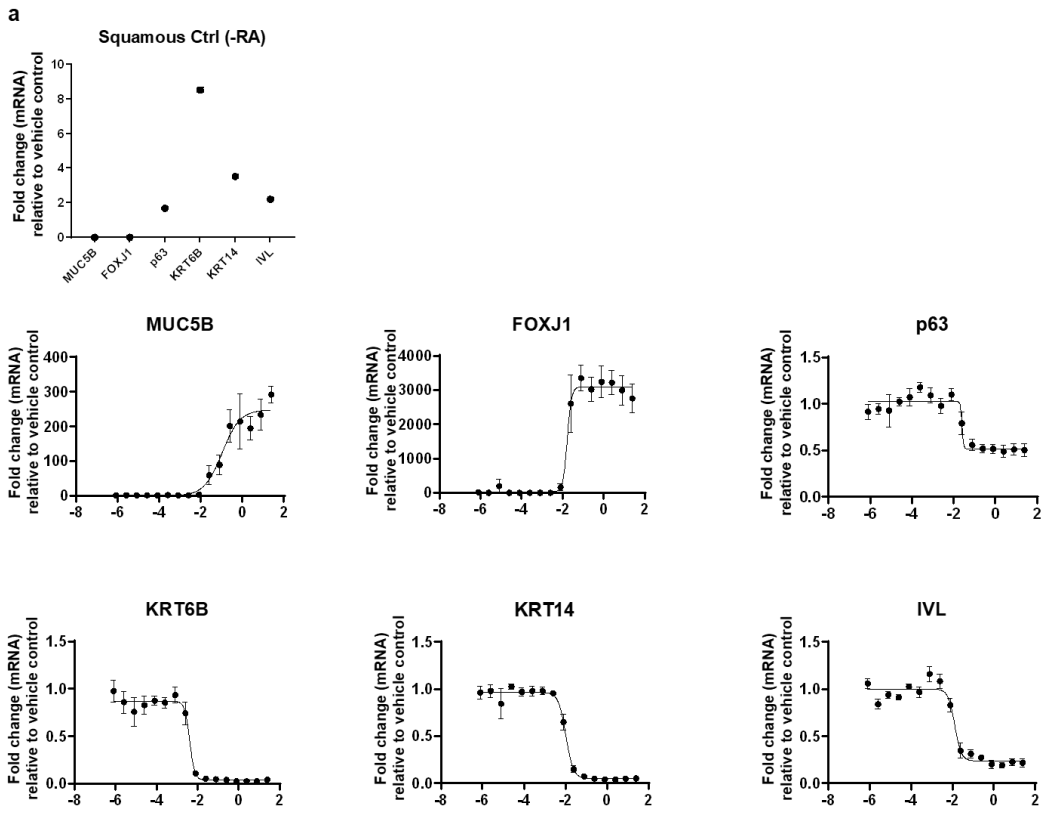


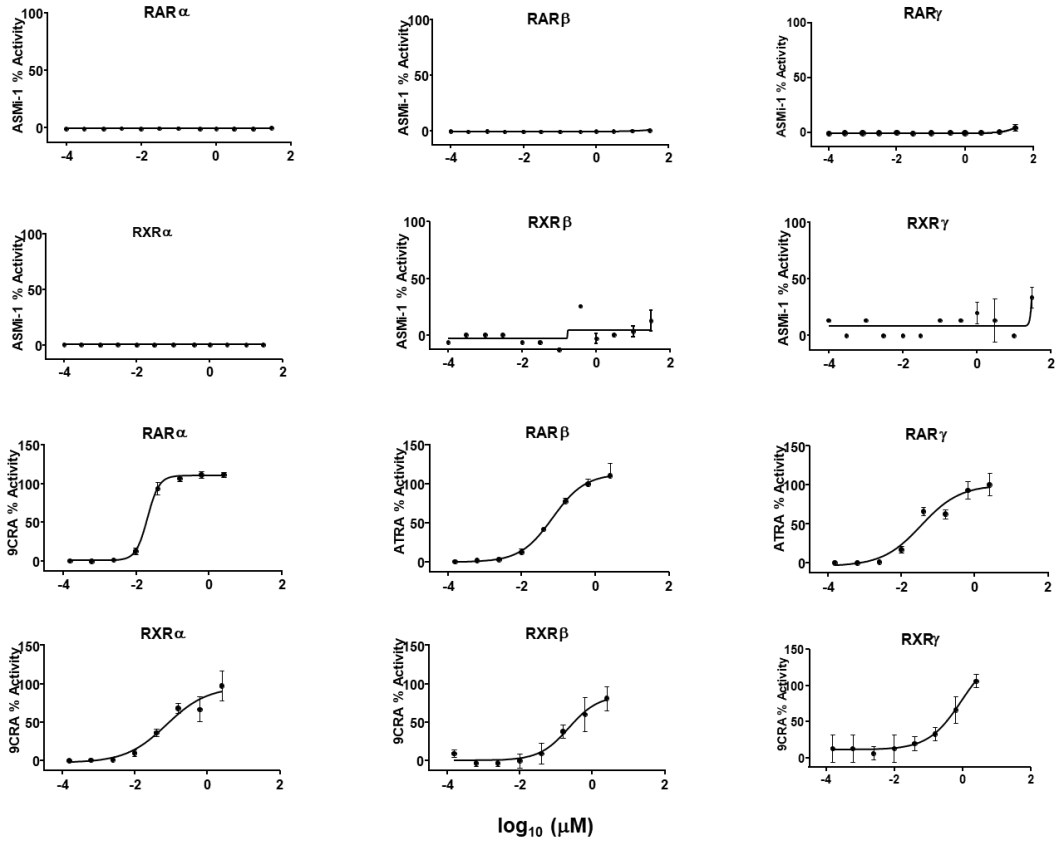
Figure 2: Identification of an inhibitor of squamous metaplasia in vitro. *Bronchospheres cultured in the absence of retinoic acid were subjected to treatment with the MoA box library. The resulting organoids were analyzed for expression levels of the indicated goblet (MUC5B), ciliated (FOXJ1), basal (TP63) and squamous cell (KRT6B, KRT14, IVL) markers. Treatment with ASMi-1 leads to a reduction in squamous and basal cell markers, while increasing goblet and ciliated markers in a dose-dependent manner, relative to DMSO-treated control lacking retinoic acid. Data is presented as the average ± SEM of biological triplicates (n=3).*

Validation of Hit

Although retinoic acid withdrawal has been shown to share phenotypic characteristics of airway squamous metaplasia (Koo et al., 1999; Sachs, Finkbeiner & Widdicombe, 2003; Cozens et al., 2018), retinoid treatment in COPD patients does not

lead to improved lung function (Roth et al., 2006). Retinoids exert their effects via the retinoic acid receptor (RAR) and retinoid X receptor (RXR), each consisting of three isoforms, RARα, β, γ and RXRα, β, γ (Ghyselinck & Duester, 2019). To determine whether ASMi-1 suppressed squamous metaplasia by agonizing retinoic acid receptors, we tested its activity in a panel of isoform-specific RAR/RXR reporter gene assays (Indigo Biosciences). Activation of RAR or RXR isoforms in this cell-based system is achieved by adding pathway specific ligands to induce expression of luciferase, which is then measured using a luminescence plate reader. Compounds identified in the MoA box screen that activated one or more RAR or RXR reporter gene assays were presumed to prevent squamous metaplasia via a mechanism similar to ATRA, and were therefore deprioritized. While induction of luciferase was observed in the presence of the control RAR/RXR agonists ATRA and 9-cis-RA, no measurable RAR/RXR activation was detected in response to treatment with ASMi-1, strongly suggesting that the effect of ASMi-1 on the airway epithelium is through a retinoic acid receptor-independent mechanism (Figure 3).

a



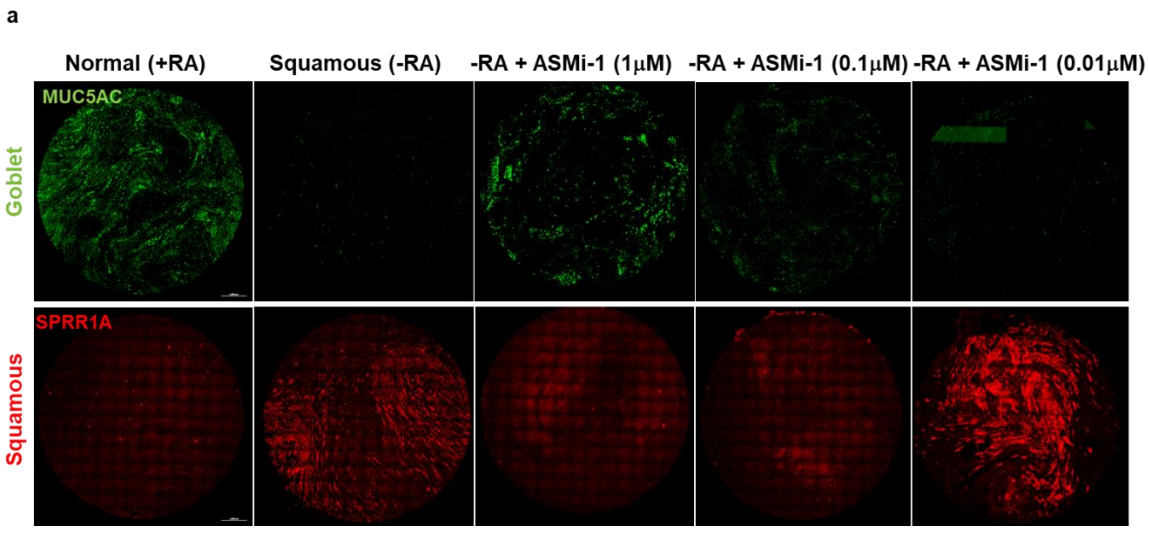
b

Substance ID	RARα EC_{50} (μM)	RARβ EC_{50} (μM)	RARγ EC_{50} (μM)	RXRα EC_{50} (μM)	RXRβ EC_{50} (μM)	RXRγ EC_{50} (μM)
ASMi-1	>30	>30	>30	>30	>30	>30
9-cis-RA	0.022			0.048	0.17	0.45
All-trans-RA		0.0044	0.0018			

Figure 3: ASMi-1 functions through a retinoic acid receptor-independent mechanism
(a) RAR/RXR luciferase reporter gene assays were treated with test compound ASMi-1 or with RAR/RXR agonists 9-cis-RA (9CRA) or ATRA. Treatment with ATRA and 9CRA, but not ASMi-1, results in an increase in luciferase expression in a dose-dependent manner. Data points represent the average +/- SD of technical duplicates. (*b) EC_{50} for reference compounds and ASMi-1 was calculated.*

To determine whether ASMi-1 can inhibit squamous metaplasia markers at the protein level, ASMi-1 was evaluated utilizing the ALI model with immunofluorescence readouts (Figure 4a). HBECs were seeded on transwell filters submerged in

differentiation medium. On day 7, prior to the initiation of differentiation, the apical surface was exposed to air (Day 0 of ALI). Cells were cultured for an additional 14 days in differentiation media with or without retinoic acid, as positive and negative controls, respectively. While the negative control group (-RA) resulted in increased SPRR1A staining and decreased MUC5AC staining relative to the positive control group (+RA), reflecting a squamous metaplasia phenotype, treatment with ASMi-1 resulted in near normal levels of MUC5AC staining, with a concomitant suppression of the squamous epithelial marker SPRR1A in a dose-dependent manner. Quantification of immuno-positive cells was performed on four independent HBEC donors (Figure 4b) confirming these observations. Together, these data indicate that ASMi-1 is sufficient to suppress the squamous metaplasia induced by retinoic acid withdrawal *in vitro*.



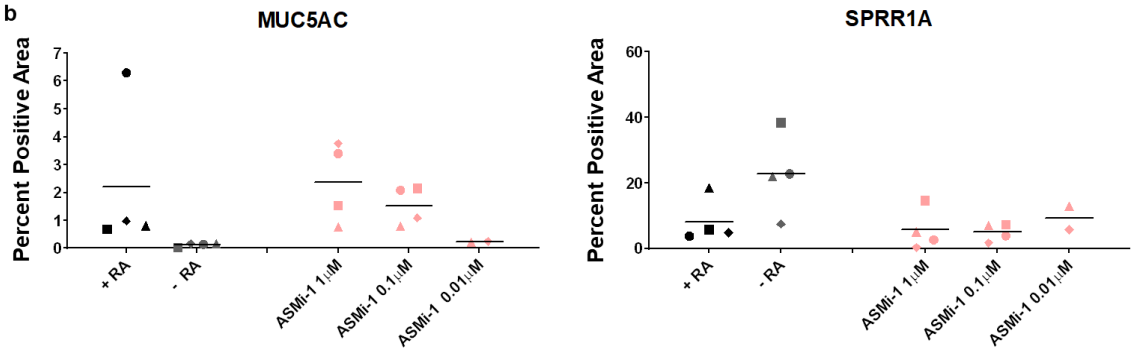


Figure 4: ASMi-1 inhibits airway squamous metaplasia in vitro *(a) Human airway basal cells were grown on transwell filters at air-liquid interface with or without retinoic acid. Cells were treated with the indicated concentrations of ASMi-1 by supplementing differentiation media lacking retinoic acid from day 7. At day 21 (14 days at ALI), cells were fixed and stained for the goblet cell marker MUC5AC and the squamous marker SPRR1A. (b) Quantification of immunofluorescence staining using CellProfiler image analysis. Data is presented as the percent positive staining area. Each donor data point is represented by a unique shape.*

Characterization of ASMi-1 in preexisting squamous metaplastic condition

The data above indicates that ASMi-1 prevents squamous metaplasia *in vitro* by promoting normal epithelial differentiation. However, COPD is typically a disease of the ageing population. Furthermore, COPD is usually not diagnosed until it is clinically apparent and moderately advanced (U.S. Public Health Service, 2005). Therefore, I sought to characterize ASMi-1 in a setting that more closely resembles a therapeutic scenario. To examine the reversibility of a preexisting squamous phenotype by ASMi-1, cells were cultured in ALI for 21 days in RA-free medium. Cells were then treated with ASMi-1 from day 21 through day 33. A time course was performed by collecting samples on day 23, 26, 28, 30 and 33. To monitor changes in epithelial cell

differentiation, I measured mRNA for goblet cell-specific genes *MUC5AC* and *MUC5B*, ciliated cell-specific genes *FOXJ1* and *DNAI2*, the club cell-specific gene *SCGB1A1*, basal cell markers *TP63 and ITGA6*, and four squamous epithelial cell markers *KRT6B, KRT14, SPRR1A* and *IVL* (Figure 5). Control cultures were treated with media containing RA for the entirety of the experiment and samples were collected at the same time points as the experimental arm. As in the normal differentiation conditions, ASMi-1 treatment resulted in an increase of goblet, club and ciliated markers, with a concomitant reduction of the basal and squamous epithelial markers in a time dependent manner, relative to DMSO-treated controls lacking retinoic acid (dotted line). Together, these data demonstrate ASMi-1 both prevents and restores a squamous epithelium to a normal mucociliary epithelium.

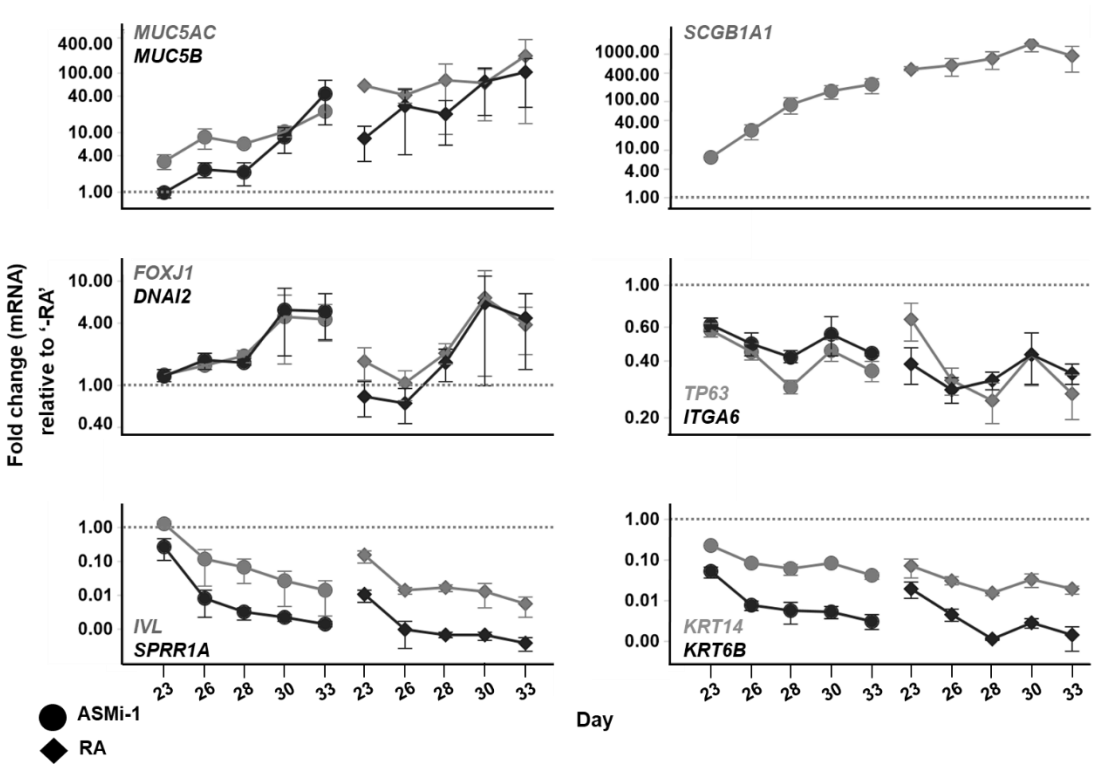


Figure 5: ASMi-1 restores a preestablished squamous epithelium to a normal mucociliary epithelium. *Human airway basal cells were grown on transwell filters at ALI with or without retinoic acid for 21 days. At day 2, cultures lacking retinoic acid were treated with ASMi-1 diluted in RA-free media. Control cultures were treated with RA for from day 0 to day 33. A time course was performed by harvesting cells at the indicated day. Cells were analyzed for expression levels of markers of goblet cells (MUC5AC, MUC5B), club cells (SCGB1A1), ciliated cells (FOXJ1, DNAI2), basal cells (ITGA6, TP63), and squamous epithelial cells (IVL, SPRR1A, KRT6B, KRT14) normalized to controls lacking retinoic acid (dotted line). Data points represents the average ± SEM of biological triplicates, n=3.*

Evaluation of ASM preventative effect of ASMi-1 on tight junction protein Occludin

In addition to proper cellular composition, homeostasis of the epithelial lining of the airway is maintained by the apical junctional complexes (AJC), which include tight and adherens junctions. Tight junctions, which are comprised of the transmembrane proteins Occludin and Claudin, and the cytoplasmic scaffolding proteins Zonula

occludens-1 (ZO-1), -2, and -3, regulate apical junctional barrier function by establishing cell-cell contacts, apicobasal cell polarity and restricting paracellular permeability between adjacent cells (Georas & Rezaee, 2014; Hartsock & Nelson, 2008). *In vitro* airway squamous metaplasia studies have reported decreased integrity of epithelial tight junctions resulting in impaired barrier function (Shaykhiev, 2013; Heijink, Brandenburg, Postma, van Oosterhout, 2012; Schamberger, Staab-Weijnitz, Mise-Racek, & Oliver Eickelberg, 2015).

To examine the effect of ASMi-1 on tight junctions, I analyzed the pattern of Occludin staining in cells cultured at ALI under ASM conditions, with or without the addition of 1 μM ASMi-1 (Figure 6). While the pattern of labeling for Occludin was interrupted (arrows) in the ASM group (-RA), treatment with ASMi-1 resulted in a more uniform and continuous immunostaining pattern, similar to the normal condition (+RA). Together, these data indicate ASMi-1 driven differentiation of specific epithelial cell populations and maintenance of tight junction integrity may restore epithelial homeostasis in airway squamous metaplasia.

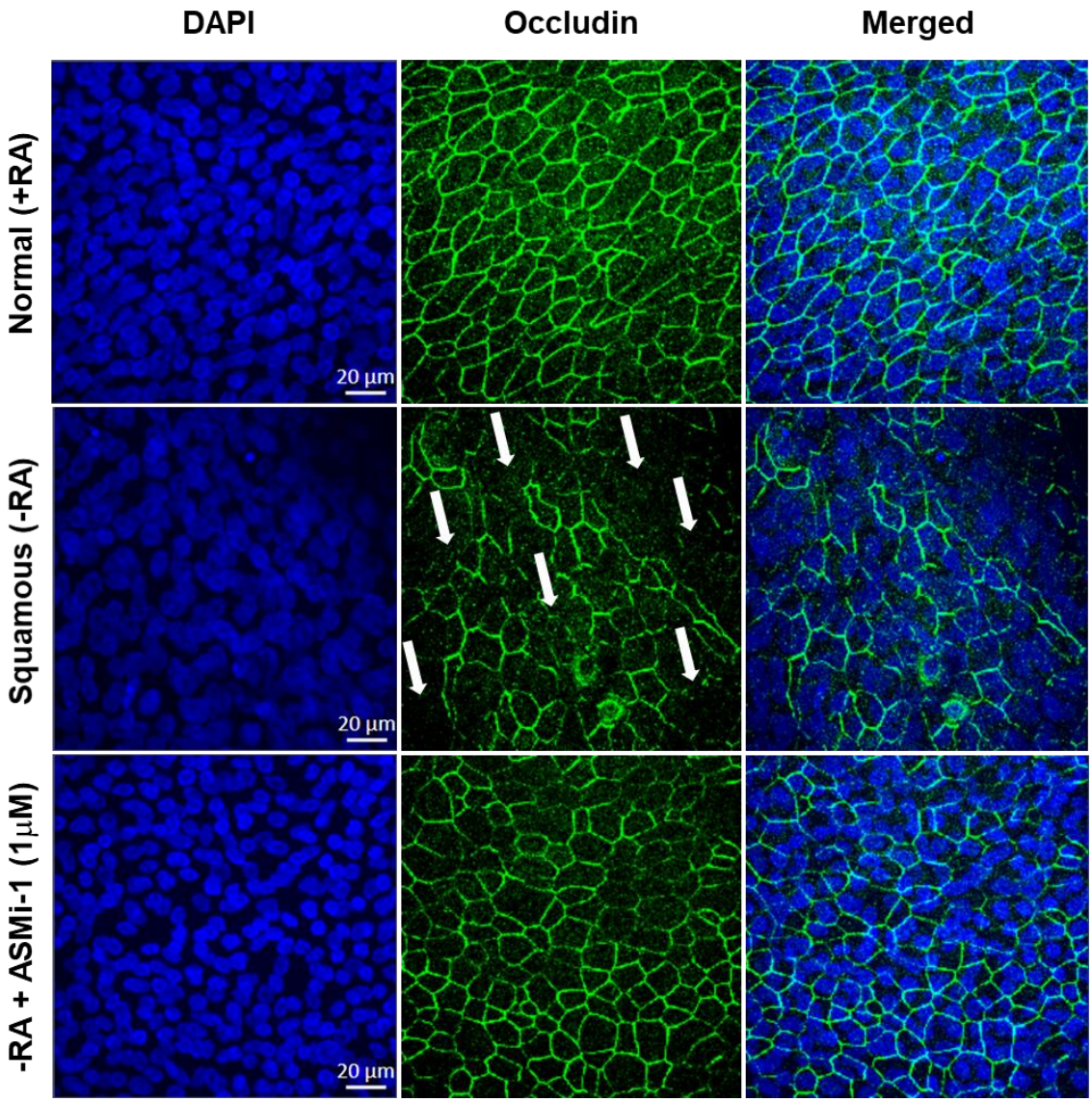


Figure 6: Inhibition of ASM by ASMi-1 maintains apical Occludin localization. *Human airway basal cells were grown at ALI with or without retinoic acid. Cells were treated with 1 μM ASMi-1 by supplementing differentiation media lacking retinoic acid. Representative staining for the tight junction protein Occludin (green) and nuclei (blue). Scale bar = 20 μm.*

Chapter IV.

Discussion

Chronic obstructive pulmonary disease, due primarily to cigarette smoking, is a leading cause of mortality worldwide. COPD is defined by chronic inflammation, airflow limitation, and destruction of the parenchyma leading to lung function decline (Ejiofor & Turner, 2013; Lakshmi et al., 2017; Watz et al., 2013). Development efforts for therapies targeting these characteristics of COPD are underway. However, the effectiveness of current first-line therapies such as LABAs is limited, providing only symptomatic relief and reduction in exacerbations without treating the underlying cause of disease (Alvarado, 2017). This unmet medical need provides a strong rationale for novel approaches to be investigated.

To date, therapeutic strategies for the treatment of COPD have focused on reducing hypersecretion of mucous by epithelial cells, smooth muscle relaxation, broad-spectrum anti-inflammatory drugs, or by targeting the alveolar cells to address destruction of the parenchyma. However, smoking-associated changes observed in COPD patients also include squamous metaplasia of the epithelium (Crystal, 2014; Dye, 1994; Hogan et al., 2014). Clinical evidence suggests lung function inversely correlates with the degree of squamous metaplasia. Furthermore, squamous metaplasia correlates with severity of COPD (Cosio et al., 1978; Lapperre et al., 2007; Rigden et al., 2016). Targeting squamous metaplasia provides a novel approach to treat COPD, by focusing on restoring the epithelium from squamous metaplasia phenotype to a homeostatic, pseudostratified epithelium. The aim of this thesis is to identify compounds that inhibit airway squamous metaplasia, with the therapeutic hypothesis that restoring normal

epithelial lineages will restore tissue homeostasis, improve mucociliary clearance (MCC) and improve lung function in patients.

The present study utilized a 3D culture system to study epithelial morphogenesis and identify inhibitors of squamous metaplasia. Few examples exist for the manipulation of human cultures to study airway squamous metaplasia in a 3D organoid culture system. Kumar et al. (2011) described generating a gene expression profile using human tracheal airway stem cells (hTASC). Following 21 days in culture, hTASC cultured in Matrigel® in chambered glass slides were positive for the squamous marker keratin 10. While the authors demonstrated normal differentiation capacity in ALI, it was unreported whether the squamous spheres also expressed mucociliary or club cell markers.

In order to identify inhibitors of squamous metaplasia, I developed a robust, high throughput assay to model squamous metaplasia *in vitro*. Under standard differentiation conditions, the 3D cultures contain the normal cell types; $TRP63^{+}$ basal cells, functional ciliated cells and secretory goblet cells (Danahay et al., 2015; Hild & Jaffe, 2016). However, culturing in media lacking retinoic acid leads to spheres devoid of the normal differentiated cells types: goblet, ciliated and club cells, with a robust increase in multiple markers of squamous epithelium, which can be analyzed by qRT-PCR. This is the first study to describe a 3D organotypic culture that displays multiple features characteristic of airway squamous metaplasia. In this 14-day assay, a direct comparison may be made to normal conditions, as opposed to the publication by Kumar et al., (2011), which only demonstrated squamous marker expression in their 21-day tracheal epithelial 3D organoid culture with no report on secretory or ciliated expression.

In vivo and *in vitro* COPD studies have demonstrated an increased expression of proinflammatory mediators (Eich et al., 2017; Lakshmi et al., 2017). Studies have reported decreased integrity of the epithelial tight junction during lung inflammation resulting in structural changes and impaired barrier function (Shaykhiev, 2013; Heijink et al., 2012; Schamberger et al., 2015; Wittekindt, 2017). Evaluation by confocal immunofluorescence microscopy for the tight junction protein Occludin demonstrated an apparent disruption of Occludin localization in the squamous condition. ASMi-1 treatment resulted in maintenance of localization of the tight junction protein similar to the normal condition. ASMi-1-induced preservation of the tight junction protein may provide an additional layer of epithelial homeostasis by restoring physiological function of the mucosal barrier in airway diseases.

The findings from the current study demonstrate that the compound ASMi-1 prevents airway squamous metaplasia *in vitro.* First, ASMi-1 prevented the gene expression changes, at both the mRNA and protein level, induced by culturing in media lacking retinoic acid (which induces squamous metaplasia). Second, ASMi-1 prevented the disruption of tight junctional integrity associated with squamous metaplasia, as indicated by the maintenance of the apical localization of Occludin. Furthermore, ASMi-1 treatment led to the restoration of a pre-established squamous phenotype. Importantly, ASMi-1 restores SCGB1A1-expressing club cells. Secretory club cells possess diverse host defense functions. Protective characteristics include xenobiotic metabolism, immune system regulation, a progenitor for ciliated cell population and a source of antimicrobial peptides (Reynolds & Malkinson, 2010).

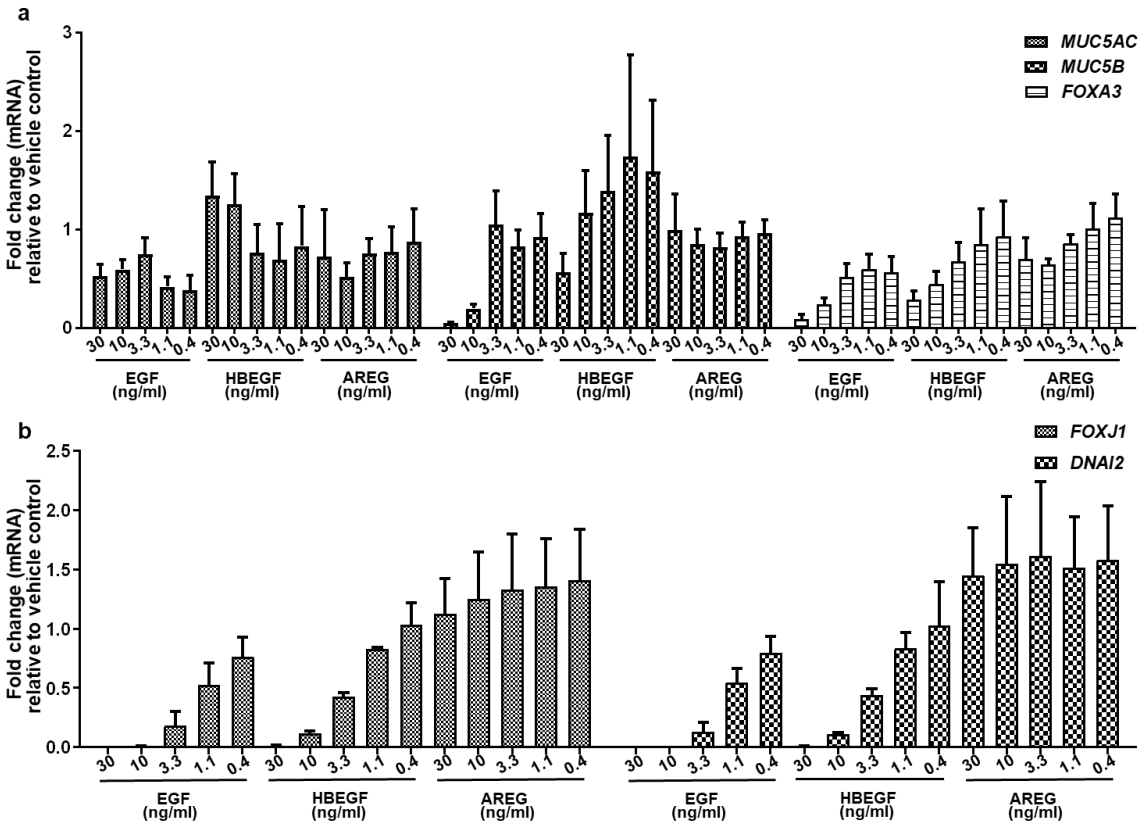
While our *in vitro* model of squamous metaplasia is driven by the lack of RA in the culture media, ASMi-1 exerts its activity through a retinoid-independent mechanism as evidenced by its lack of activity in RAR/RXR reporter gene assays. Since RA has been reported to be ineffective at improving the decline in lung function associated with COPD (Roth et al., 2006), ASMi-1 may represent a new, therapeutically viable mechanism for restoring a normal airway epithelium worthy of further investigation.

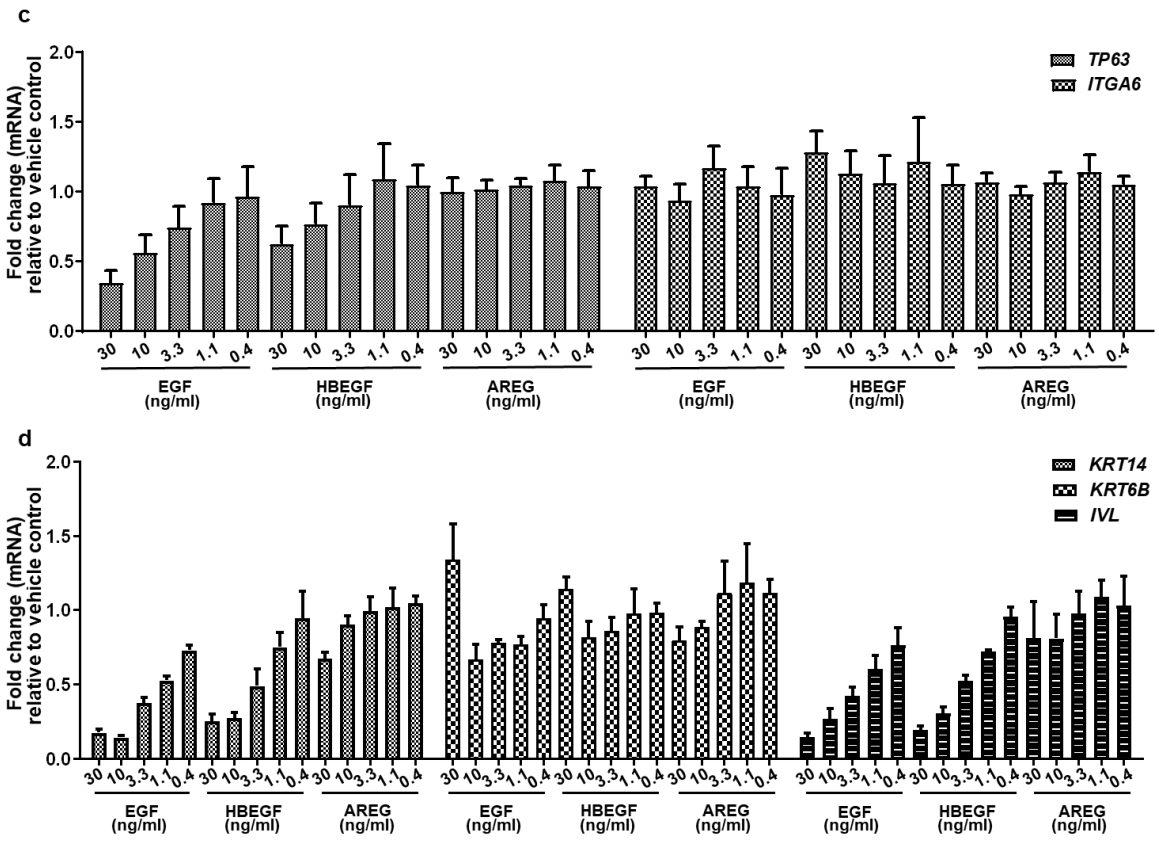
In vitro COPD studies have reported epithelial tight junction barrier dysfunction (Shaykhiev, 2013; Heijink et al., 2012; Schamberger et al., 2015). Moreover, with the loss of mucociliary differentiation observed in squamous metaplasia (Auerbach et al., 1961; Rock et al., 2010), the impairment of barrier function may increase susceptibility to infection and promote a proinflammatory environment or further exacerbate an existing one, contributing to disease pathogenesis in COPD. Future studies investigating whether the reversal of squamous metaplasia improves barrier integrity, reduces microbial colonization and airway inflammation, and decreases the frequency of exacerbations, are warranted. Addressing these key pathologies observed in COPD by focusing on airway remodeling rather than symptomatic treatment could result in sustained, improved lung function and may be able to modify the progression of disease.

Squamous metaplasia is a pathology observed in several lung diseases such as idiopathic pulmonary fibrosis (IPF) (Shaykhiev, 2019), cystic fibrosis (CF) (Rock et al., 2010) and asthma (Préfontaine & Hamid, 2007). Moreover, squamous metaplasia is an early lesion observed in the pathogenesis of squamous cell lung carcinoma (Wistuba & Gazdar, 2006), providing opportunities for indication expansion and potentially decreasing the frequency of airway squamous cell carcinoma.

Appendix

Additional Figures

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Fold change (mRNA) relative to vehicle control
2.0
1.5
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TP63
ITGA6
30 10 3.3 1.1 0.4
EGF (ng/ml)
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d
KRT14
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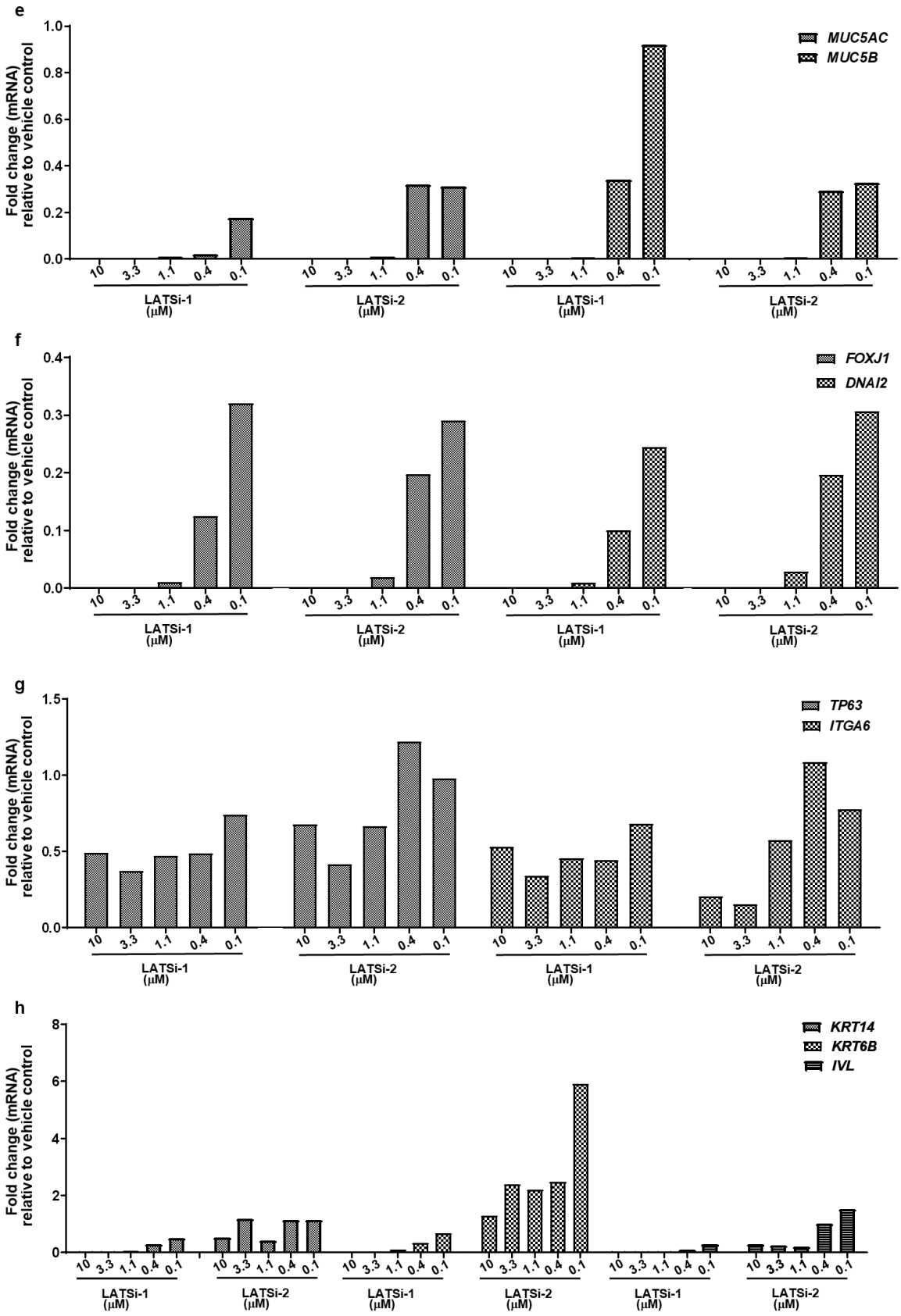
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Fold change (mRNA) relative to vehicle control
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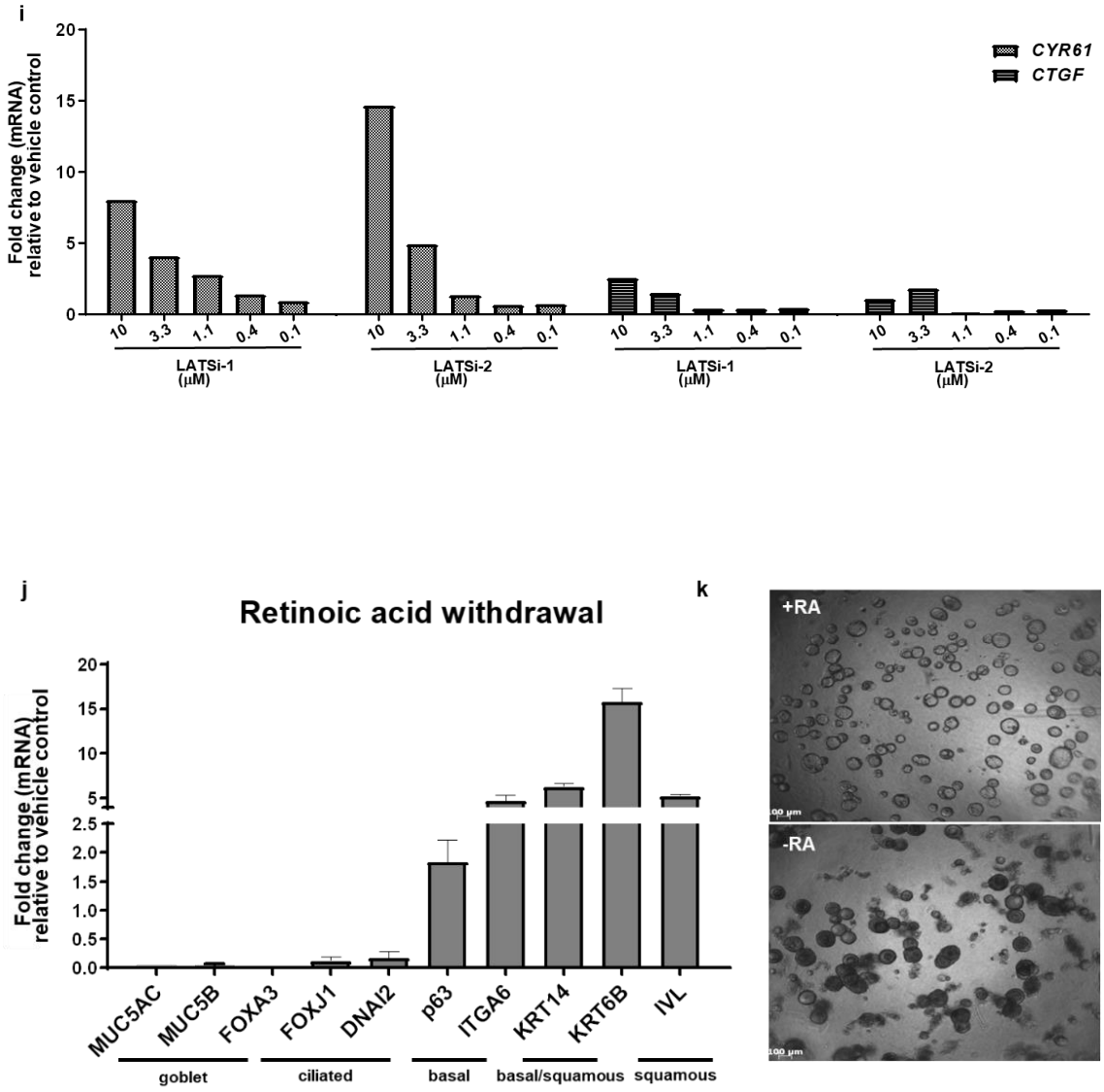

Figure 1: Validation of previously reported squamous metaplasia stimuli. *(a–k) Human airway basal cells were grown in Matrigel to produce bronchospheres. qRT-PCR analysis of transcript levels of goblet cell markers, ciliated cell markers, basal cell markers, and squamous epithelial markers were measured. Bronchospheres were cultured in normal differentiation media (control) or: (a-d) increasing concentrations of EGF, HBEGF or AREG, n=3; (e-i) LATS inhibitors 1 and 2, n=1; (j) differentiation media lacking ATRA. Data are depicted as mean fold change ± SEM relative to control (n=1-3). (k) Phase contrast image of bronchospheres in the presence or absence of retinoic acid. The scale bar represents 100 μm.*

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