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VEGF receptor 2 (KDR) protects airways from mucus metaplasia through a Sox9 dependent pathway

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Author Contributions

M.J. and J.Q. designed experiments, analyzed data, and wrote the manuscript. M.J. performed immunostaining, imaging, flow cytometry, ALI culture and mouse genetics. Y.F. performed mouse H1N1 viral infection. Y.L. performed isolation of lung cells for single-cell RNA sequencing. H.H. and X.G. performed quantitative PCR and immunostaining. Z.W. and J.R. assisted with mouse genetics. L.Q. and Q.C. assisted with data analysis. H.S. and J.H. provided the *Vegf-hyper, Vegf-hypo and Vegf*$^{loxp/loxp}$ mouse line and reagents. D.J. and J.W. provided human asthma and cystic fibrosis samples and help manuscript writing. X.A. provided HDM mouse model of allergic asthma and participated in data analysis and manuscript writing.

Declaration of Interests

A patent application (CU21016, "SUPPRESSION OF KDR, VEGF OR THE DOWNSTREAM MEK/ERK KINASE PATHWAY") related to this work has been filed by Columbia Technology Ventures. M.J. and J.Q. are listed as co-inventors on this application.

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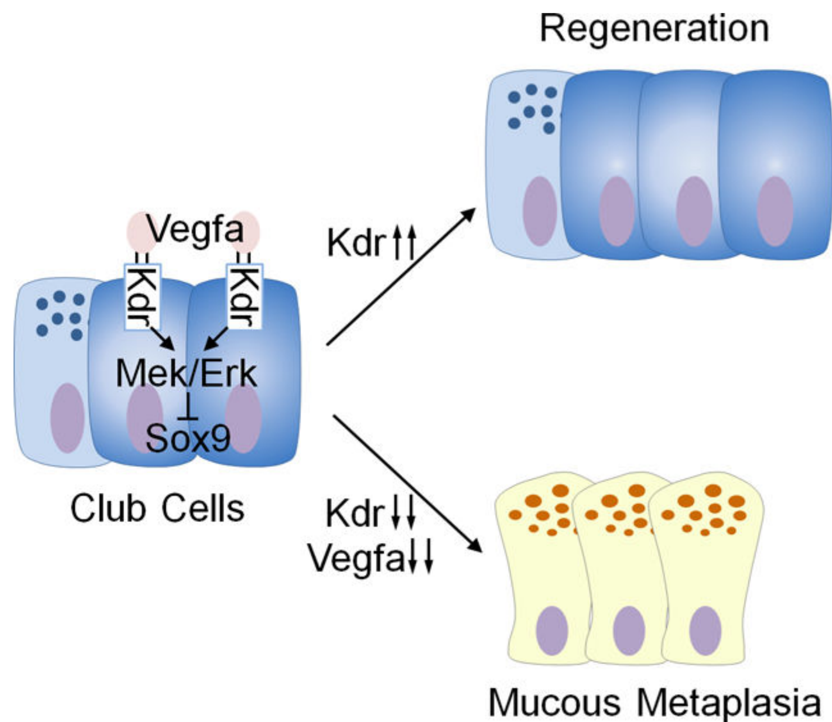
Summary

Mucus-secreting goblet cells are the dominant cell type in pulmonary diseases e.g. asthma and cystic fibrosis (CF), leading to pathologic mucus metaplasia and airway obstruction. Cytokines including IL-13 are the major players in the transdifferentiation of club cells into goblet cells. Unexpectedly, we have uncovered a previously undescribed pathway promoting mucous metaplasia that involves VEGFa and its receptor KDR. Single cell RNA sequencing analysis coupled with genetic mouse modeling demonstrates that loss of epithelial VEGFa, KDR or MEK/ERK kinase, promotes excessive club-to-goblet transdifferentiation during development and regeneration. Sox9 is required for goblet cell differentiation following Kdr inhibition in both mouse and human club cells. Significantly, airway mucous metaplasia in asthmatic and CF patients is also associated with reduced KDR signaling and increased SOX9 expression. Together, these findings reveal an unexpected role for VEGFa/KDR signaling in the defense against mucous metaplasia, offering a potential therapeutic target for this common airway pathology.

eTOC blurb:

Jiang et al. demonstrate that the VEGFa/KDR pathway protects the airway epithelium against mucous metaplasia during regeneration. Suppressed VEGF signaling leads to increased levels of SOX9 which promotes goblet cell differentiation. These findings introduce critical players in airway mucous metaplasia that is commonly seen in asthma and cystic fibrosis.



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Introduction

In the conducting airways of the lung, controlled mucus production and effective clearance are dependent on a balanced composition of mucus producing and multiciliated cells in the luminal epithelium. The differentiation of mucus cells occurs towards the end of gestation and, following a "burst" of production during the early neonatal period, the population is maintained as a minor proportion of the total in the normal airways. However, in pulmonary diseases, including allergic asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), the numbers of mucous cells significantly expand, resulting in mucous metaplasia which leads to airway obstruction, infection and even mortality (Boucher, 2019; Curran and Cohn, 2010; Fahy and Dickey, 2010).

Past research into mucous metaplasia has focused on immune cells, revealing the importance of Th1 and Th2 inflammatory cytokines such as IL-1, IL-4 and IL-13 in driving the appearance of goblet cells (Gour and Wills-Karp, 2015; Li et al., 2019; Whitsett, 2018; Wills-Karp et al., 1998; Zhou-Suckow et al., 2017). Investigation of asthmatic animal models indicates that group 2 innate lymphoid cells (ILC2s) secret IL-5 and IL-13 which promote goblet cell hyperplasia following allergen challenge (Klein Wolterink et al., 2012; Kuperman et al., 2002). Moreover, transgenic overexpression of IL-5, IL-13 or its downstream player *SAM pointed domain ETS factor* (SPDEF) leads to extensive mucous metaplasia in mouse airways (Lee et al., 1997; Park et al., 2007; Rajavelu et al., 2015; Schmid-Grendelmeier et al., 2002; Zhu et al., 1999). Interestingly, recent research implicates the critical roles of non-hematopoietic cells/tissues in mucous metaplasia (Branchfield et al., 2016; Sui et al., 2018). For example, rare neuroendocrine cells can modulate mucous cell differentiation through neuropeptides and neurotransmitters, including CGRP and GABA (Barrios et al., 2017; Sui et al., 2018). That being said, the molecular mechanism driving the differentiation of airway progenitor cells towards mucous cell lineage remains largely unknown.

Both ciliated cells and club cells were initially thought to generate mucous cells depending on the models used (Chen et al., 2009; Evans et al., 2004; Reader et al., 2003; Tyner et al., 2006). However, lineage-tracing experiment suggested that ciliated cells do not transdifferentiate into mucous cells in the airways in an asthmatic mouse model (Pardo-Saganta et al., 2013). Instead, a subset of club cells express mucin proteins following ovalbumin (OVA) challenge (Evans et al., 2004). Electron microscopy analysis further revealed that the metaplastic mucous cells contain many ultrastructural characteristics of club cells (Hayashi et al., 2004), suggesting that club cells are the progenitors for the metaplastic mucous cells. Consistently, the newly formed mucous cells are lineage-labeled in the airways of *Scgb1a1-rtTA; Otet*$_7$*-CMV*$_{min}$*Cre (Otet-Cre); R26*^{lacZ} mice following OVA challenge (Chen et al., 2009). These studies support the hypothesis that club cells serve as a cell of origin for mucous cells in the intrapulmonary airways. However, club cells are heterogeneous and contain multiple subpopulations including variant club cells and newly identified MHC class I marker H2-K1high progenitor cells (Guha et al., 2017; Hong et al., 2001; Kathiriya et al., 2020; Kim et al., 2005; Reynolds et al., 2000; Yuan et al., 2019). Therefore, it remains an open question which subpopulation(s) generate mucous/goblet cells.

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In this study, our single cell RNA sequencing analysis surprisingly revealed that VEGFR2 (also known as FLK1 or KDR) is expressed in club cell subpopulations. Loss of KDR, its ligand VEGFa, or downstream MEK/ERK causes excessive differentiation of club cells into mucous cells (mainly goblet cells) during development and regeneration following Naphthalene (NAPH) challenge. We further show that deletion of the transcription factor Sox9 attenuates mucous metaplasia caused by KDR loss. KDR inhibition also promotes goblet cell differentiation of human club cells that form in air-liquid interface (ALI) culture, and this is accompanied by increased levels of SOX9. Consistently, airway mucous metaplasia is associated with reduced KDR signaling and increased SOX9 expression in patients with asthma and CF. Together, these findings identify an unexpected role for VEGFa/KDR signaling in the defense against mucous metaplasia.

Results

Single cell analysis identified Kdr expression in club cell subpopulations.

To gain initial insights into club cell heterogeneity under normal and repair conditions, we performed single-cell RNA sequencing of EPCAM+ intrapulmonary cells of the adult mouse before and after treatment with a single dose of NAPH (Figure 1A and S1A). Previous studies have shown that NAPH selectively ablates the majority of club cells, and that the residual, NAPH resistant club cells can fully regenerate the epithelium (Guha et al., 2017; Hong et al., 2001; Kotton and Morrisey, 2014; Rawlins et al., 2009; Reynolds et al., 2000; Van Winkle et al., 1995; Volckaert et al., 2011). Cells were isolated 14 days after treatment when the epithelial cells are proliferating and differentiating extensively (Van Winkle et al., 1995). A total of 3298 and 3685 cells were analyzed from control and NAPH-injured mice, respectively, and cell types assigned based on the expression of specific lineage markers (Figure 1B, 1C and S1B). The major cell types included club, ciliated, neuroendocrine and alveolar type 2 (AT2) cells (Figure 1B and S1B). A minor population of immune cells also expressed EPCAM in both control and NAPH-injured lungs, which is consistent with previous findings (Figure 1B and 1C) (Gautier et al., 2012). Notably, NAPH challenge also led to the presence of a minor population of basal cells (Trp63+) (Figure 1B and S1B).

Cluster analysis revealed that club cells (Scgb1a1+, Scgb3a2+) were divided into three major subpopulations, Sub1/2/3 (Figure 1D and S1C). Gene enrichment analysis suggests that Sub1 and 2 share similar gene expression, with relatively higher expression of *Igfbp6* and *Prdx6* in Sub1 (Figure 1E and Table S1). By contrast, Sub3 expresses transcripts for the mucous cell markers *Muc5B* and *Tff2* (Figure 1F), although the proteins are undetectable in the cytoplasm (data not shown). Surprisingly, Sub1/2 are characterized by the expression of transcripts of *Kdr*, which has previously only been found in mesoderm-derived cells (e.g., endothelial cells) and a rare population of hepatocytes (Figure 1E, 1F and S1D) (Ema et al., 2006; Goldman et al., 2013; Holmes et al., 2007). We used a *Kdr-GFP* knock-in mouse line to localize cells transcribing the gene under normal and repair conditions. At steady-state, GFP expression was low or undetected. However, there was clear expression in the airways during repair and this was confirmed by quantitative PCR (Figure 1G and S3B).

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Transient Kdr expression prevents mucous metaplasia in the developing airways

Kdr-Cre activity was barely detected in the airway epithelium at E16.5, but became prominent at P0 (Figure S2A). Consistently, *Kdr-GFP* expression was limited to club cells and the levels increased from E16.5 to P0 (Figure S2B–E), correlating with the timing of club cell maturation (Reynolds et al., 2008). While Kdr-driven GFP expression in the airways progressively declined from P5 to P60, the levels of expression in the blood vessels remained unaltered (Figure S2B and S2C). Notably, the dynamic expression of Kdr in the epithelium is inversely correlated with the transient "burst" of mucous cell appearance that normally occurs in the distal trachea and intrapulmonary airways during the early postnatal period (Roy et al., 2011). When Kdr expression peaked at around P0, the number of mucous cells was minimal but increased from P0 to P10 (Figure S2B and S2C), suggesting that Kdr suppresses the differentiation of club cells towards mucous cell lineage. To test whether Kdr is indeed required for suppressing mucous cell differentiation, we generated *Shh-Cre; Kdr*$^{loxp/loxp}$ mutants in which *Kdr* is conditionally deleted in the lung epithelium as early as E9.0 (Harris-Johnson et al., 2009). Significantly, loss of Kdr led to an increased number of mucous (goblet) cells (Alcian blue+ Muc5AC+ Clca3+) in the airways at P10 (24.3±4.6% vs 41.4%±4.2% , $p<0.05$) (Figure S2F–H). However, the number of mucous cells reverted back to normal at P60 in both controls and mutants (Figure S2F–H), suggesting that Kdr is transiently required for suppressing mucous cell fate during the early neonatal stage.

Transient Kdr expression is required to block mucous metaplasia during regeneration

A characteristic feature of injury-repair mechanisms is the redeployment of development signaling pathways (Barker et al., 2010; Lynch et al., 2018; Mou et al., 2016; Vaughan et al., 2015; Zacharias et al., 2018). Following NAPH challenge the levels of Kdr-driven GFP transiently increased from day7 and peaked at approximately day14–21 in the distal trachea, main bronchi and intrapulmonary airways (Figure S3A, S3B and data not shown). Kdr-GFP expression reverted back to normal when the repair was accomplished (Figure S3A and S3B). To test whether re-expression of Kdr is critical for airway epithelial regeneration, we subjected *Shh-Cre;Kdr*$^{loxp/loxp}$ mutants to NAPH challenge. As described above, the airways of these mice appeared normal. However, when the airway epithelium lacking Kdr was examined 14 days after NAPH injury there was evidence for severe mucous metaplasia and the airways were filled with mucus (8.8%±2.6% vs 32.8%±6.1% , $p<0.001$) (Figure 2A and 2B). This result suggests that Kdr is essential for inhibiting mucous differentiation of the regenerated epithelium. Consistently, loss of Kdr in club cells led to similar extensive mucous metaplasia in the regenerated airways of *Scgb1a1-CreER;Kdr*$^{loxp/loxp}$ mutants following NAPH treatments (5.6%±2.6% vs 29.3%±4.8% $p<0.001$) (Figure 2C and 2D). Increased numbers of mucous cells could be detected 60 days after injury (Figure 2E). Furthermore, treatment with the selective Kdr inhibitor Semaxanib (SU5416) (Fong et al., 1999) also induced similar mucous metaplasia following NAPH challenge (4.2%±1.7% vs 24.4%±2.6% , $p<0.001$) (Figure S3C and S3D). Together these results confirm that transient re-expression of Kdr is required for normal airway epithelial regeneration and that loss of Kdr during the process of restoration leads to mucous metaplasia.

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The Kdr ligand VEGFa is required for suppressing mucous metaplasia during airway regeneration

KDR has two major ligands, VEGFa and VEGFc (Hamada et al., 2000; Mustonen and Alitalo, 1995). While VEGFa/KDR signaling is critical for vascular development (Carmeliet et al., 1996; Shalaby et al., 1995), and VEGFc/KDR plays an essential role in the formation of lymphatic vessels (Makinen et al., 2001), no evidence has been described for function in airway epithelial tissues. Single-cell RNA sequencing data showed that in the adult lung epithelium VEGFa transcripts were enriched in club cells and AT2 cells, whereas VEGFc was not detected in any populations present in t-SNE plot (Figure 3A and data not shown). In addition to KDR, VEGFa also binds VEGF receptor 1 (also known as FLT1 (Ferrara et al., 2003)) which was enriched in a subpopulation of immune cells (Figure 3A). We confirmed that VEGFa expression was limited to club cells (Scgb1a1+) in both control and NAPH-injured airway epithelium as evidenced by a *Vegfa-lacZ* reporter mouse line (Figure 3B) (Miquerol et al., 1999). Notably, mucous metaplasia occurred in the airways of *Vegfa* hypomorphic mutants (*Vegf-hypo*) (Damert et al., 2002), in which Vegfa levels were dramatically reduced (Figure 3C and 3D). Conversely, increased expression of Vegfa protected the regenerated airway epithelium from mucous metaplasia in *Vegfa* hypermorphic mutants (*Vegf-hyper*) (Miquerol et al., 1999) (Figure 3C and 3D). To further test whether club cell-derived VEGFa is required for airway regeneration, we deleted *Vegfa* using *Scgb1a1-CreER* and subjected the mutants to NAPH challenge. *Vegfa* deletion resulted in extensive mucous differentiation of the regenerated epithelium in *Scgb1a1-CreER*;*Vegfa*$^{loxp/loxp}$ mutants (3.7%±1.5% vs 23.3%±7.7%, $p<0.001$) (Figure 3E and 3F). Notably, mucous metaplasia was less severe in the terminal airways, presumably due to compensatory VEGFa secretion by the neighboring alveolar epithelial cells (Figure 3A and 3E) (Vila Ellis et al., 2020). Together these results support a model in which club cell-produced VEGFa activates KDR to block mucous cell differentiation of the airway epithelium during regeneration.

Kdr suppresses mucous metaplasia of the regenerated epithelium via MEK/ERK signaling

VEGFa/KDR signaling activates multiple downstream pathways, including the p38 MAPK, JNK and MEK/ERK pathways (Carmeliet and Jain, 2011; Koch and Claesson-Welsh, 2012). We found that p-MEK1/2 and p-ERK1/2 but not p-p38 or p-JNK were prominently expressed in the airway epithelium during regeneration (Figure 4A, S4A and S4B). Consistently, p-MEK1/2 and p-ERK1/2 were lost upon *Kdr* deletion in *Shh-Cre;Kdr*$^{loxp/loxp}$ mutants (Figure 4B), suggesting that VEGFa/KDR activates the MEK/ERK pathway during airway regeneration. In line with this finding, treatment with the MEK1/2 inhibitor PD98059 or the ERK inhibitor FR180204 but not the p38 MAPK inhibitor SB203580 or JNK inhibitor SP600125 led to severe mucous metaplasia in the airways of wildtype mice following NAPH challenge (Figure S4C and S4D). PD98059 has also been shown to block the NF-κB pathway in addition to MEK1/2 (Di Paola et al., 2010). We therefore genetically deleted *Mek1/2* with the *Scgb1a1-CreER* mouse line and observed mucous metaplasia in the regenerated airway epithelium following NAPH challenge (4.1%±2.2% vs 27.8%±5.3%, $p<0.001$) (Figure 4C and 4D). Together these data support the conclusion that KDR activates MEK/ERK signaling to block mucous cell differentiation during airway regeneration.

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Sox9 is critical for mucous metaplasia induced by Kdr loss during airway epithelial regeneration

Sox9 is critical for goblet cell differentiation in the intestine (Bastide et al., 2007), raising the possibility that the transcription factor also plays a role in the adult lung, in addition to its well-studied role in distal epithelial progenitors during lung development (Chang et al., 2013; Rockich et al., 2013). Our single cell RNA sequencing data revealed that *Sox9* was expressed in the Sub3 population of club cells in the adult lung (Figure S5A and S5B). Lineage labeling with a knockin *Sox9-CreER* mouse line confirmed that SOX9 was expressed in a minor population of club cells in the proximal airways. Moreover, lineage labeled cells also included a minor population of ciliated cells in the distal airways (Figure 5A and S5C). These lineage-labeled cells remained solitary throughout a chase period of three months, suggesting that they are quiescent (Figure S5D). Interestingly, SOX9 expression was seen in the abundant mucous cells present in the regenerated airways of both *Shh-Cre;Kdr*$^{loxp/loxp}$ (0.3%±0.2% vs 23.1%±3.9% , $p<0.001$) and *Scgb1a1-CreER; Kdr*$^{loxp/loxp}$ (0.2%±0.1% vs 9.8%±2.5% , $p<0.001$) mutants following NAPH challenge (Figure 5B–E). SOX9 was also enriched in the nuclei of mucous cells following challenge with PD98059 (0.1%±0.2% vs 5.1%±1.6% , $p<0.001$) (Figure S5E and S5F). Notably, a subpopulation of Sox9+ cells did not express Mu5AC, suggesting that these cells are club cells that are going to undergo mucous differentiation (Figure 5B–E, S5E and S5F). To test whether activation of SOX9 is critical for mucous metaplasia, we generated *Scgb1a1-CreER; Kdr*$^{loxp/loxp}$*; Sox9*$^{loxp/loxp}$ mutants. Loss of Sox9 blocked mucous cell differentiation following NAPH challenge (28.6%±8.1% vs 9.5%±3.9% , $p<0.01$) (Figure 5F and 5G). We further found that *Sox9* deletion reduced the expression of Spdef and Foxa3 in *Scgb1a1-CreER;Kdr*$^{loxp/loxp}$*;Sox9*$^{loxp/loxp}$ mutants (Figure S5G and S5H). Thus, these findings suggest that SOX9 expression is critical for mucous cell differentiation upon *Kdr* deficiency.

Reduced VEGFa/Kdr levels are associated with mucous metaplasia in influenza and asthmatic mouse models

Infection of the lung by viruses, including influenza and SARS-Cov-2, leads to severely disrupted airway epithelium and excessive mucous secretion (Fang et al., 2020; Rane et al., 2019; Wang et al., 2020). We used H1N1 PR8 influenza virus to address whether KDR is involved in these pathological changes. Excessive mucus production in the dysplastic regions of the injured lungs was observed after viral infection (Figure S6A). Notably, mucous cells were present in the regions where Kdr-GFP was not expressed. By contrast, the adjacent epithelial cells expressing Kdr-GFP contained minimal mucous cells (Figure S6B). Consistently, the transcript levels of *Vegfa* and *Kdr* were reduced in the metaplastic mucous epithelium (H score of *Vegfa*: 99.4±9.6 vs 70.1±5.7, $p<0.05$; H score of *Kdr*: 90.4±10.4 vs 21.5±6.6, $p<0.001$) (Figure S6C and S6D). Meanwhile, SOX9 was also enriched in the nuclei of mucous cells upon H1N1 infection (0.2%±0.2% vs 7.3%±1.1% , $p<0.001$) (Figure S6E and S6F).

Mucous metaplasia is one of the most common phenotypes in the airways of patients with allergic Th2 dependent asthma (Busse et al., 1999; Tanizaki et al., 1993). To test whether abnormal VEGFa/KDR levels are associated with asthmatic mucous metaplasia, we employed an established murine allergic asthma model in which house dust mite (HDM)

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antigen is used to sensitize and re-challenge the mice (Figure 6A) (Wang et al., 2019). HDM treatment led to mucous metaplasia in the airways (1.2%±0.6% vs 32.4%±6.7% , $p<0.001$) (Figure 6B and 6C). Notably, mucous metaplasia was associated with the reduced transcript levels of *Vegfa* and *Kdr* (H score of *Vegfa*: 80.9±7.3 vs 17.3±7.3, $p<0.001$; H score of *Kdr*: 98.1±12.6 vs 18.3±4.9, $p<0.001$) in contrast to the unchanged levels in the blood vessels and the accompanying increase in SOX9 levels (0.1%±0.04% vs 8.6%±3.1% , $p<0.001$) (Figure 6D–G). Together these data support a model in which reduced Vegfa/Kdr signaling is associated with mucous metaplasia during both regeneration and asthmatic pathogenesis.

Kdr signaling suppression is associated with mucous metaplasia of human airways

We next asked whether VEGF/KDR signaling activities are associated with mucous metaplasia in the human lung related to pulmonary diseases (asthma and CF). RNAScope analysis revealed relatively high levels of *VEGFA* and *KDR* transcripts in the conducting airway epithelium of young adolescents compared to adults (H score of *VEGFA*: 176.1±30.5 vs 103.3±20.8, $p<0.05$; H score of *KDR*: 147.7±28.3 vs 90.2±22.9, $p<0.05$) (Figure 7A, 7C and 7D). Positive staining of p-MEK1/2 and p-ERK1/2 was also observed in the epithelium of healthy controls (Figure 7B and S7A). However, *KDR* transcripts were reduced, although not the levels of *VEGFA,* in the airways of asthma (H score of *KDR*: 90.2±22.9 vs 36.7±10.4, $p<0.05$) and cystic fibrosis patients (H score of *KDR*: 90.2±22.9 vs 24.3±18.3, $p<0.05$). Consistently, the epithelium exhibited decreased p-MEK1/2 and p-ERK1/2 staining (Figure 7A–D and S7A). These results are consistent with our findings in mouse models that suppressed VEGF/KDR signaling contributes to mucous metaplasia of the airway epithelium.

To further test whether VEGF/KDR signaling blocks the differentiation of the human airway epithelium towards mucous cells, we cultured human small airway epithelial cells (HSAECs) in an air-liquid interface (ALI) culture system (Whitcutt et al., 1988). Under these conditions KDR was present in a subpopulation of club but not basal or ciliated cells, similar to the mouse airway epithelium (Figure S7B and data not shown). Treatment with the KDR inhibitor Semaxanib or the MEK inhibitor PD98059 from the start of the culture reduced the numbers of club and ciliated cells while promoting mucous cells concomitant with increased SOX9 expression (Figure 7E, S7C and S7D). Previous studies have shown that basal cells give rise to mucous cells in murine airways (Hong et al., 2004; Rock et al., 2011). We therefore used FACS to exclude basal cells (p75+) and reseeded the differentiated cells into ALI culture (Figure S7E). Remarkably, a significant number of club cells differentiated into mucous cells upon treatment with Semaxinib or PD98059 (Figure 7F and S7F), confirming that VEGF/KDR signaling plays a conserved role in suppressing mucous cell differentiation of club cells.

Discussion

Previous studies have demonstrated the important role for inflammatory cytokines in the pathogenesis of airway mucous metaplasia (Gour and Wills-Karp, 2015; Klein Wolterink et al., 2012; Kuperman et al., 2002; Lee et al., 1997; Li et al., 2019; Schmid-Grendelmeier et al., 2002; Whitsett, 2018; Wills-Karp et al., 1998; Zhou-Suckow et al., 2017; Zhu et al.,

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1999). Here, we describe the unexpected yet essential function for VEGF/KDR signaling in achieving normal airway epithelial regeneration and mucous cell differentiation. Our single-cell RNA analysis identified that club cell subpopulations transiently increase KDR expression during airway regeneration. Genetic or pharmacological inhibition of VEGFa/KDR or its downstream MEK/ERK signaling disrupts normal epithelial differentiation program, leading to accumulated mucous cells. Further genetic analysis suggests that SOX9 is an important mediator of mucous metaplasia following KDR loss. ALI culture confirmed that KDR inhibition also causes mucous differentiation of human club cells, accompanied by increased SOX9 protein levels. In asthmatic and CF patients airway mucous metaplasia is associated with decreased KDR and increased SOX9 protein levels (Figure 7G).

Club cells constitute a heterogeneous population with stem/progenitor cell function, contributing to the regeneration of the epithelium of the airways and alveoli following injury (Guha et al., 2017; Hong et al., 2001; Kathiriya et al., 2020; Kim et al., 2005; Rawlins et al., 2009; Reynolds et al., 2000; Van Winkle et al., 1995; Yuan et al., 2019). More recently, single cell RNA sequencing has revealed that MHC class I marker H2-K1high progenitor cells are critical for alveolar repair (Kathiriya et al., 2020). Our single cell analysis identified three subpopulations of club cells, which are associated with the distinct expression of KDR and mucin-related genes. Numerous studies demonstrate that KDR is critical for vasculature and hematopoiesis development (Carmeliet et al., 1996; Olsson et al., 2006; Shalaby et al., 1995; Yeh et al., 2003). Here, we uncovered a critical role for KDR in the regulation of epithelial differentiation. Our in vivo and in vitro studies support the hypothesis that VEGF/KDR is a guardian against mucous cell differentiation of the regenerated airway epithelium. Interestingly, reduced levels of VEGF and KDR have been found in the bronchoalveolar lavage fluid and lungs of smokers and patients with COPD which is characterized by small airway mucous metaplasia (Kim et al., 2008; Koyama et al., 2002; Marwick et al., 2006). We found that VEGFa and KDR levels are also reduced in asthmatic and CF patients, suggesting a common role for suppressed VEGF/KDR signaling in the pathogenesis of mucous metaplasia in these pulmonary diseases.

In the developing lung the Notch signaling pathway participates in the regulation of goblet cell differentiation (Guseh et al., 2009; Lafkas et al., 2015; Tsao et al., 2011). Transgenic overexpression of the active intracellular domain of the mouse Notch1 receptor (NotchIC) using *Sftpc-Cre* results in increased numbers of mucous cells in the airways (Guseh et al., 2009). However, Notch signaling was also shown to prevent mucous metaplasia of the airway epithelium during postnatal development. Conditional deletion of the essential Notch pathway components *protein O-fucosyltransferase1* (*Pofut1*) or *RbpjK* with *Tgfb3-Cre* which is activated postnatally causes goblet cell metaplasia concomitant with decreased club cell numbers. In the same study lineage tracing also suggests mucous cells are derived from club cell subpopulations (Tsao et al., 2011). In addition, inhibition of Notch signaling with an antibody against Jag1/2 attenuates IL-13-induced mucous metaplasia (Lafkas et al., 2015). We also examined the potential involvement of Notch1 signaling by evaluating the Notch1 intracellular domain (NICD1) and Jag1/2 expression in *Kdr* deletion mutants. No apparent changes were observed in the metaplastic airway epithelium during regeneration

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(unpublished data), suggesting that Notch1 signaling unlikely mediates Kdr function in the regulation of mucous cell differentiation.

Previous studies have shown that HDM-induced mucous metaplasia involves the activation of other signaling molecules such as Toll-like receptor 4 (TLR4) and EGF (Hammad et al., 2009; Jacquet and Robinson, 2020; Le Cras et al., 2011; Yasuda et al., 2020). Of note is that both EGF and TLR4 can directly or indirectly regulate MEK/ERK activities (Aomatsu et al., 2008; Athari et al., 2017; Avraham and Yarden, 2011; Le Cras et al., 2011). It is possible that reduced KDR also impacts the expression of Egf and/or TLR4 which contribute to the reduced MEK/ERK signaling. Our genetic study further indicates that Sox9 acts downstream of VEGF/KDR signaling. Deletion of *Sox9* attenuates mucous metaplasia of the regenerated airway epithelium following KDR loss. Sox9 has been shown to play essential roles in the development of multiple organs (Bastide et al., 2007; Chang et al., 2013; Rockich et al., 2013). In the developing lung, Sox9 is enriched in the distal tips of the branching epithelium. *Sox9* deletion impacts both lung branching and alveolar differentiation (Chang et al., 2013; Rockich et al., 2013), leading to small lungs with dilated airway branches and premature differentiation of alveolar cells (Chang et al., 2013). Further study demonstrates that FGF/Kras regulates Sox9 during lung branching morphogenesis (Chang et al., 2013). The role of Sox9 in adult lung remains largely unexplored. In the tracheal submucosal gland, Sox9 is required for the proliferation and migration of myoepithelial cells to the airways during regeneration (Tata et al., 2018). Our lineage labeling with a knockin *Sox9-CreER* allele suggests that the *Sox9* promoter is active in a limited number of club cells in the upper airways. However, during metaplastic changes, extensive mucous cells express Sox9 following *Kdr* deletion, suggesting that Sox9 is required for the mucous differentiation of club cells during regeneration. Consistently, *Sox9* deletion attenuated mucous metaplasia caused by *Kdr* deletion. Our findings are consistent with the role played by Sox9 in the development of secretory cell lineages in the intestine where Sox9 inactivation results in the loss of Paneth cells and decreased numbers of goblet cells (Bastide et al., 2007).

Taken together our findings now introduce new and unexpected players into the field of lung pathogenesis, namely Kdr, Vegf and Sox9. In the future, it will be important to understand the extent to which these factors are independent of and/or integral to cytokine signaling pathways at the cellular and tissue level. Our findings also raise the exciting possibility that new druggable pathways can be identified to inhibit mucus metaplasia in respiratory diseases.

Limitations of study

This study revealed inverse correlation of VEGF/Kdr signaling activities and mucous metaplasia. We further used the MEK1/2 inhibitor PD98059 and genetic ablation of *MEK1/2* with the *Scgb1a1-CreER* mouse line to demonstrate that MEK1/2 act downstream of VEGF/Kdr signaling to guard against mucous metaplasia of the regenerated airway epithelium following NAPH challenge. However, we do not know whether MEK1 or MEK2 is equally important for blocking mucous differentiation since both our pharmaceutical and genetic approaches target MEK1 and 2 simultaneously. In addition, it remains unknown

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whether MAPK signaling is also involved in blocking premature mucous differentiation of airway progenitors during development.

STAR Methods

Resource availability

Lead contact—Further information and requests for reagents may be directed to and will be fulfilled by the lead contact, Dr. Jianwen Que (jq2240@cumc.columbia.edu)

Materials availability—This study did not generate new unique reagents.

Data and code availability—The single cell RNA-sequencing datasets of airway epithelium isolated from NAPH- or vehicle-treated mice has been deposited to the Expression Omnibus: GSE171571.

Experimental model and subject details

Mice: *Shh-Cre* (Harfe et al., 2004), *Scgb1a1-CreER* (Rawlins et al., 2009), *Sox9-CreER* (Soeda et al., 2010), *Mek1$^{loxp/loxp}$* (Bissonauth et al., 2006) , *Mek2$^{-/-}$* (Belanger et al., 2003) mice were previously described. *Kdr-GFP, Kdr-Cre*, *Kdr$^{loxp/loxp}$*, *Sox9$^{loxp/loxp}$*, *R26^{tdTomato}*, *R26^{LacZ}* mice were purchased from the Jackson Laboratory. *Vegfa-hyper, Vegfa-hypo and Vegfa$^{loxp/loxp}$* mice were kindly provided by Dr. Hoon-Ki Sung of The Hospital for Sick Children. To lineage trace cells derived from Kdr+ progenitors, *Kdr-Cre* mice were crossed with *Rosa^{lacZ}* mice to generate *Kdr-Cre;Rosa^{lacZ}* compounds. To lineage trace cells derived from Sox9+ epithelium, Tamoxifen (Tmx, T5648, Sigma-Aldrich) was injected intraperitoneally into *Sox9-CreER;Rosa^{tdTomato}* mice (200 mg/kg body weight). Mice were sacrificed at defined time points as indicated in the text. *Shh-Cre;Kdr$^{loxp/loxp}$* mice were generated to conditionally delete *Kdr* in the foregut epithelium. To delete genes in club cells, *Scgb1a1-CreER;Kdr$^{loxp/loxp}$*, *Scgb1a1-CreER;Vegfa$^{loxp/loxp}$* or *Scgb1a1-CreER; Mek1$^{loxp/loxp}$;Mek2$^{-/-}$* mice were administrated with four doses of Tmx prior to naphthalene (NAPH) injection. In addition, mice were exposed to NAPH and then treated with 10 mg/kg PD98059 (Mek inhibitor, S1177, Selleckchem), 20 mg/kg Semaxanib (Kdr inhibitor, S2845, Selleckchem), 20 mg/kg SP600125 (JNK inhibitor, S1460, Selleckchem), 10 mg/kg SB203580 (p38 inhibitor, S1076, Selleckchem) or 50 mg/kg FR180204 (Erk inhibitor, S7524, Selleckchem) at day 8–14 following NAPH challenge. Lungs were collected for analysis at various time points as indicated in the text. All mice were maintained in animal facilities of Columbia University or Zhejiang University according to institutional guidelines. All mouse experiments were conducted in accordance with procedures approved by the Institutional Animal Care and Use Committee. All relevant ethical regulations were followed to the text.

Method details

NAPH treatment to induce airway epithelial injury—NAPH solution (25 mg/ml) was prepared immediately before use by dissolving NAPH (84679, Sigma-Aldrich) in corn oil. A single dose of NAPH was administrated intraperitoneally into mice at least 8 weeks of age (275 mg/kg body weight). NAPH was injected at least 4 days after the final dose of Tmx to

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allow mice to recover. Approximately 90% of club cells are ablated three days after NAPH administration as measured by immunostaining or quantitative PCR of *Scgb1a1*. Control mice were injected with the same amount of corn oil.

H1N1 PR8 influenza viral infection—Mice at 8–12 weeks of age were anesthetized with isoflurane and infected intranasally with 250 plaque forming units (pfu) of A/Puerto Rico/8/1934 H1N1 (PR8) virus in 40 μl DMEM medium. Control mice were administrated intranasally with equal volume of DMEM medium. Successful infection was confirmed by weight loss and lung sections.

Mouse model of Asthma—House dust mite (HDM)-induced asthma model was performed as previously described with a minor modification (Wang et al., 2019). Briefly, 8-week-old mice were sensitized intra-nasally with 5 μg of HDM in PBS (XPB70D3A25, Greer Laboratories) on day 0 and day 5. Mice were then intra-nasally challenged with 25 μg HDM for five consecutive days (day 11–15) and were sacrificed on day 18 for analysis.

Lung epithelium isolation and Fluorescence-Activated Cell Sorting (FACS)— Mice were perfused with PBS from right ventricles to remove blood in the lungs. Trachea was infused with 1.5ml digested solution containing 450 U/ml Collagenase Type I (17100–017, Gibco), 4 U/ml Elastase (LS002279, Worthington Biochemical Corporation), 5 U/ml Dispase II (354235, BD Biosciences) and 0.5 U/ml Dnase I (10104159001, Roche). Lungs were removed from the chest and further incubated in the digested solution for 45 mins at room temperature with gentle shaking. After digestion lung tissues were cut into small pieces and washed with DMEM buffer (10% FBS and 1% Penicillin-Streptomycin in DMEM medium). Single-cell suspension was obtained after passing through a 40 μm cell strainer and centrifuged at 1200rpm for 5 minutes. Cell pellet was resuspended in a red blood cell (RBC) lysis buffer for 1 minute to remove RBCs. Cells were centrifuged and resuspended in 100 μl sorting buffer (5% FBS, 0.2 mM EDTA, and 1% PS in PBS buffer). Cell suspension was incubated with an APC-conjugated EPCAM primary antibody for one hour at 4°C and washed for two times with cell sorting buffer. Dead cells were excluded by LIVE/DEAD Fixable Violet Dead Cell Stain Kit (L34963, Thermo Scientific). Cells were sorted into the sorting buffer. Analysis was performed using FlowJo software.

Air-liquid interface (ALI) culture of human small airway epithelial cells— Primary human small airway epithelial cells (PCS-301–010) were purchased from ATCC (Walkersville, MD). Cells were cultured in PneumaCult-Ex Plus (PnC-Ex-PLUS) media (05008, StemCell Technologies) for expansion. Once cells reached 80–90% confluency, they were digested and reseeded in 6.5 mm Transwells (353095, Corning) coated with 0.3 mg/mL Collagen type IV (234154, Sigma-Aldrich) with a density of 50,000 cells/well. Cells were firstly expanded in the PneumaCult-Ex PLUS media to reach confluency. Medium was then removed and replaced with the PneumaCult-ALI medium (05001, StemCell Technologies) to induce differentiation in the presence/absence of various inhibitors. Medium was changed every two days, and the transwell surface was also washed with PBS every two days. All cells were grown for four weeks airlifted (37°C, 5% CO_2) and physiological and characterization tests were subsequently performed. Additionally, the airway epithelial cells

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were initially induced to differentiate with ALI culture, and then FACS sorting was performed to exclude basal cells using the basal cell surface marker p75. The remaining cells were then re-plated in the transwells for further ALI culture as described above.

Tissue preparation, histology and immunostaining—Lungs were inflated and fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C and processed as previously described (Jiang et al., 2017). In brief, lungs were washed with PBS after fixation and dehydrated with ethanol prior to embedding in paraffin. 6μm sections were then prepared from the paraffin blocks. To prepare cryosections, fixed lung tissues were placed in 30% sucrose and embedded in OCT. 10μm sections were cut from the embedded lung tissues. Primary antibodies used for immunostaining analysis include rabbit anti-p63 (1:200, sc-8343, Santa Cruz Biotechnology); mouse anti-p63 (1:500, CM163, Biocare); chicken anti-GFP (1:1000, GFP-1020, Aves Labs); rabbit anti-Sox9 (1:1000, AB5535, Millipore); goat anti-tdTomato (1:1000, orb182397, Biorbyt); mouse anti-Foxj1 (1:100, 14–9965-82, eBioscience); rabbit anti-pMek1/2 (1:200, 2338S, Cell Signaling Technology); rabbit anti-pErk1/2 (1:200, 4370S, Cell Signaling Technology); rabbit anti-pP38 (1:200, 9215S, Cell Signaling Technology); rabbit anti-pJNK (1:200, 9251S, Cell Signaling Technology); rabbit anti-Kdr (1:200, 9698S, Cell Signaling Technology); mouse anti-Muc5AC (1:500, MS-145-P0, Lab Vision); rabbit anti-Clca3 (1:2000, ab46512, Abcam); rabbit anti-Scgb1a1 (1:500, 07–623, Millipore); rat anti-human SCGB1A1 (1:1000, MAB4218, R&D Systems); mouse anti-acetylated-tubulin (1:2000, T7451, Sigma). Biotinylated or fluorescent secondary antibodies were used for detection and visualization. Immunohistochemistry images were obtained using Nikon SMZ1500 Inverted microscope (Nikon). Confocal images were obtained with a Zeiss LSM T-PMT confocal laser-scanning microscope (Carl Zeiss).

RNAscope *in situ* hybridization—RNAscope *in situ* hybridizations was performed using the RNAscope 2.5 HD Assay-RED kit (322360, Advanced Cell Diagnostics), according to the manufacture instructions. The following probes were used in these analyses: *mm-Kdr* (414811, ACD), *mm-Vegfa* (405131, ACD), *hs-KDR* (312121, ACD) and *hs-VEGFA* (423161, ACD). Sections were baked for one hour at 60°C before use. After deparaffinization and hydration, sections were air-dried, treated with a hydrogen peroxidase solution and heated in a target retrieval solution for 20 min at 95–100°C. Sections were then incubated in proteinase solution at 40°C for 30 min. Target probes were hybridized for two hours at 40°C, followed by a series of amplification steps using Amp1–6 in the kit. ISH signals were detected with a Fast Red substrate reaction for 10 min at room temperature. Sections were counterstained with hematoxylin. After washing in PBS, sections were completely dried on a hot plate set at 60°C and cover-slipped with Mounting medium. Positive or negative controls were performed by hybridizing with PPIB (Cat. No. 313901, ACD) or DapB (Cat. No. 310043, ACD), respectively. Sections were examined by Nikon SMZ1500 Inverted microscope (Nikon). At least five random fields (20x magnified images) were captured for each section. Semi-quantitative histological scoring (H-score) of target genes was analyzed using ACD semi-quantitative scoring system, calculated as follows: H-score = (0 × % score0+ cells) + (1 × % score1+ Cells) + (2 × % score2+ Cells) + (3 × % score3+ Cells) +(4 × % score4+ Cells) (Jolly et al., 2019).

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Alcian blue and X-gal staining—Alcian blue and X-gal staining were performed as previously described (Hou et al., 2019). Briefly, for Alcian blue staining, sections were treated with 3% acetic acid solution for three minutes, then stained in Alcian blue solution (A3157, Sigma) for five minutes and counterstained with Nuclear Fast Red (N8002, Sigma). For X-gal staining, whole lungs were fixed in 4% paraformaldehyde for 30 minutes at room temperature, followed by X-gal (R0404, Thermo Scientific) staining overnight at 37°C. X-gal–stained samples were then dehydrated with isopropanol and embedded in paraffin for sectioning.

Reverse transcription and quantitative Real-Time PCR—Tissues and Cells were lysed with TRIzol reagent (15596026, Thermo Scientific), and RNA was purified using the RNeasy Mini Kit (74104, QIAGEN). RNA reverse transcription was performed using the Super-Script III First-Strand SuperMix (18080400, Invitrogen) according to the manufacturer's instructions. cDNA was quantified by real-time PCR using the iTaq Universal SYBR Green Supermix (1725122, Bio-Rad) and the StepOnePlus Real-Time PCR Detection System (Applied Biosystems). The transcript levels of genes were normalized to Actin expression. All real-time quantitative PCR experiments were performed at least triplicate. PCR primers were designed using the Lasergene Core Suite (DNASTAR Inc.), and the sequences of primers are listed in the Key Resources Table.

Quantification and statistical analysis—For cell counting in the slides at least five random fields (20x magnified images) were captured for each section. Statistical analysis was done using unpaired two-tailed student's t-test. Results were presented as mean ± s.e.m.; p values < 0.05 were considered statistically significant. *P < 0.05; **P < 0.01; ***P < 0.005. All statistical analyses were performed using GraphPad Software Prism 6.

Single-cell RNA sequencing analysis—EPCAM+ lung cells from control or NAPH-treated mice were purified by FACS and then processed following the 10x Genomics protocol. Cell Ranger pipeline was used to process raw sequencing data, and Cell Ranger R package and Seurat v2.0 pipeline were used to perform downstream scRNA-seq analysis. Differentially expressed genes in the heatmap were identified with the expression levels > 1.5 fold differences between the club cell subpopulations.

Data availability statement

The authors declare that the main data supporting the findings of this study are available within the paper and its supplementary information. Any additional data are available from the corresponding authors upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. VEGFa/KDR suppresses mucous differentiation of club cells during regeneration.
2. VEGFa/KDR signaling executes its epithelial function through MEK/ERK kinases.
3. Sox9 mediates mucous metaplasia of the airway epithelium upon VEGFa/KDR inhibition.
4. VEGFa/KDR/MEK blockage is linked to mucous metaplasia in asthma and cystic fibrosis.

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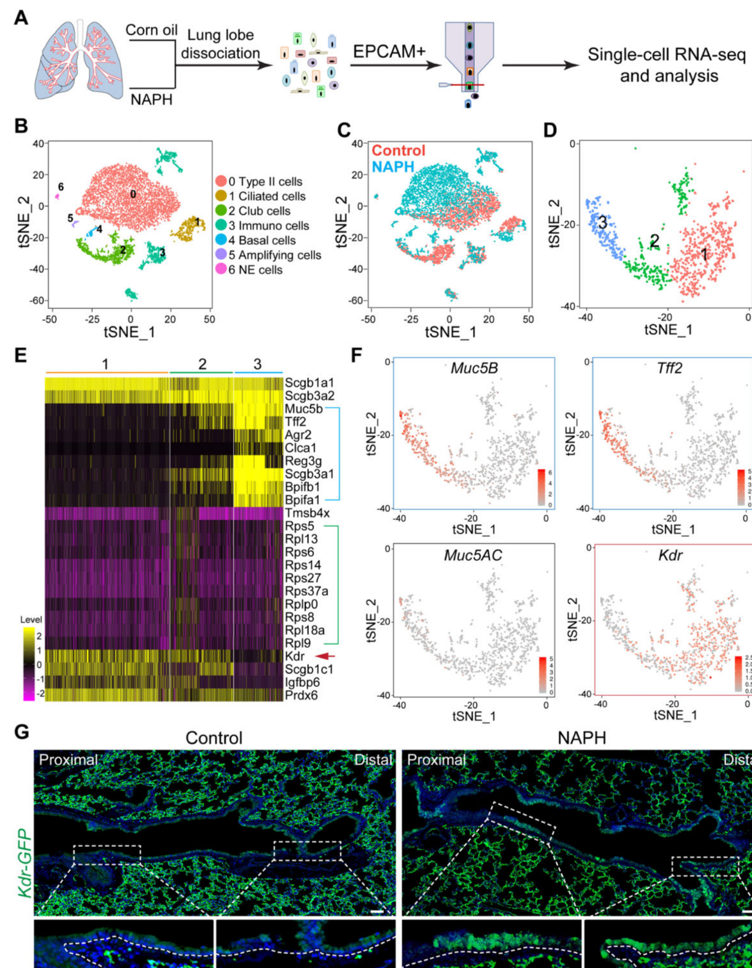

Figure 1: Kdr is expressed in subpopulations of intrapulmonary airway club cells.

(**A**) Schematics for single cell RNA sequencing of airway EPCAM+ cells isolated from mice treated with corn oil or Naphthalene (NAPH). (**B and C**) t-Distributed Stochastic Neighbor Embedding (t-SNE) plot indicative of cell population changes upon NAPH treatment. (**D**) Three subpopulations of club cells are present in the airways. (**E**) Differential gene expression in the three subpopulations of club cells. Note distinct gene expression enriched in Sub2 (green bracket) and Sub3 (blue bracket). Also note that Kdr is enriched in Sub1 and 2 (red arrow). (**F**) Enrichment of *Kdr* transcripts in Sub1/2 in contrast to expression of mucous cell-related genes *Muc5B*, *Tff2*, and *Muc5AC* in Sub3. (**G**) Increased expression of Kdr-GFP in the regenerated airway epithelium 14 days following NAPH challenge. Scale bar: 50 μm. See also Figure S1 and Table S1.

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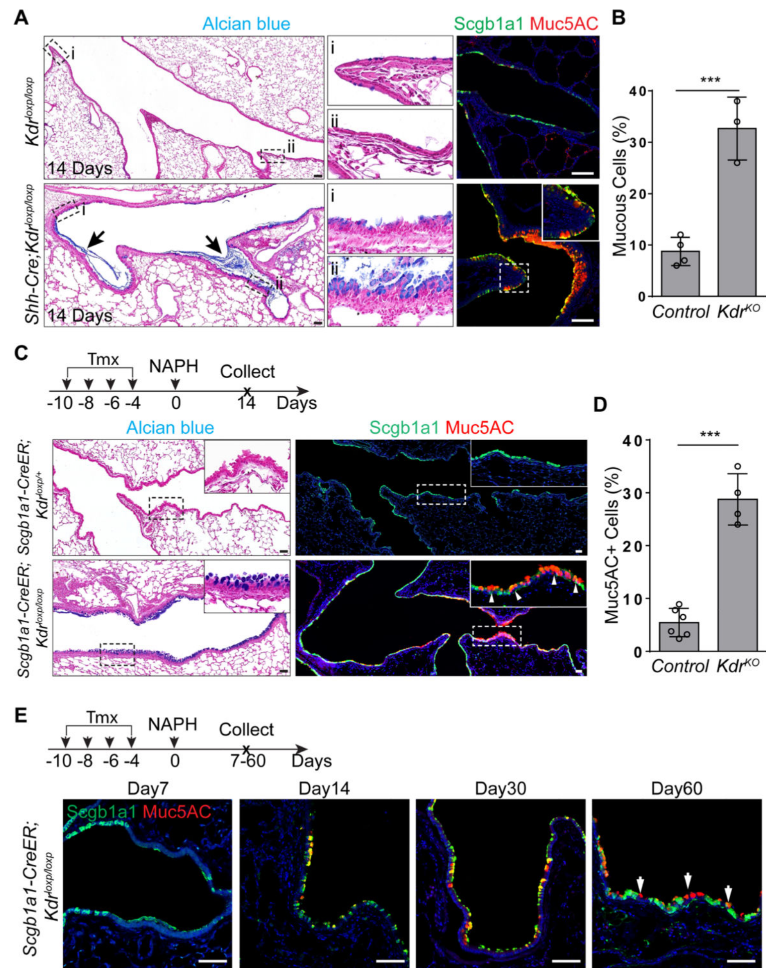

Figure 2: Kdr signaling is required for airway epithelial regeneration.

(**A and B**) Extensive mucous metaplasia in the regenerated airways of *Shh-Cre;Kdr*$^{loxp/loxp}$ mutants following NAPH challenge (n=3). (**C and D**) Loss of *Kdr* in *Scgb1a1-CreER;Kdr*$^{loxp/loxp}$ mutants promotes mucous metaplasia of regenerated club cells following NAPH challenge (n=4). (**E**) Mucous cells remain in the airways 60 days following NAPH challenge. Note that mucous cells co-express Muc5AC and Scgb1a1 at day 14 and day 30, but lose Scgb1a1 expression at day 60. Data represent mean ± s.e.m. ***p<0.001; statistical analysis by unpaired two-tailed Student's *t*-test. Scale bar: 50 μm. See also Figure S2 and S3.

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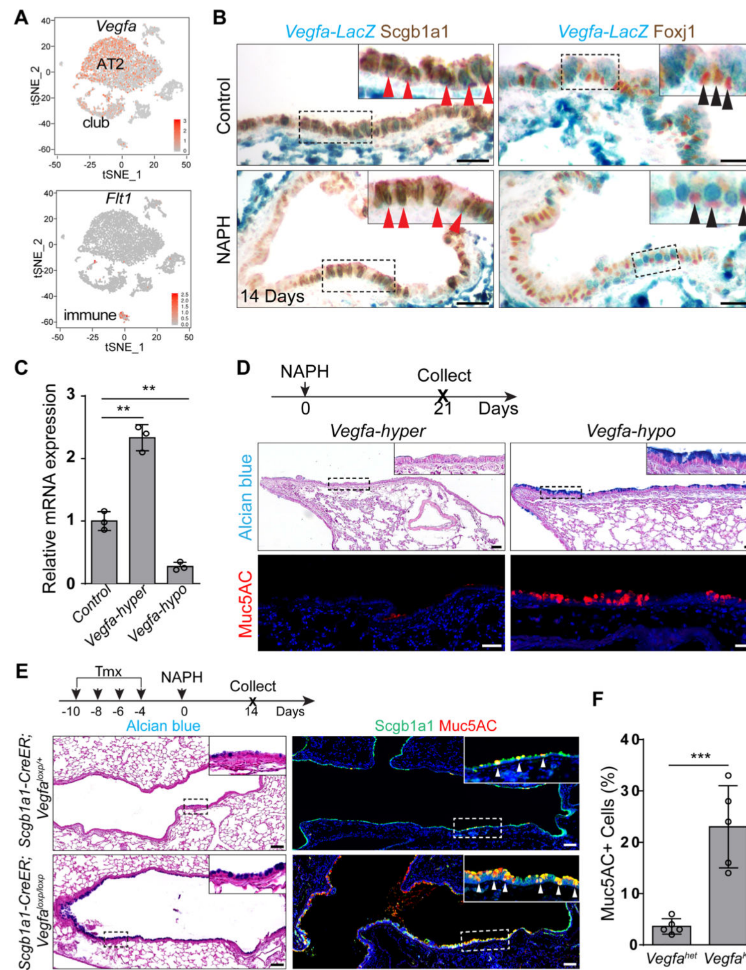

Figure 3: Club cell-derived Vegfa is required for airway epithelial regeneration.

(**A**) *Vegfa* transcripts are expressed in club cells. Note Vegfa is also enriched in AT2 cells. Also note that *Flt1* is not detected in the epithelium but expressed in immune cells. (**B**) *Vegfa-lacZ* is expressed in club cells but not ciliated cells of the airway epithelium as shown by X-gal staining. (**C**) The transcript levels of *Vegfa* are increased and decreased in the isolated EPCAM+ cells of *Vegf-hyper* and *Vegf-hypo* mice, respectively (n=3). (**D**) Mucous metaplasia occurs in the airways of *Vegf-hypo* mice following NAPH challenge. (**E and F**) Loss of *Vegfa* promotes mucous metaplasia of regenerated club cells following NAPH challenge (n=5). Data represent mean ± s.e.m. $**p<0.01$, $***p<0.001$; statistical analysis by unpaired two-tailed Student's *t*-test. Scale bar: 100 μm.

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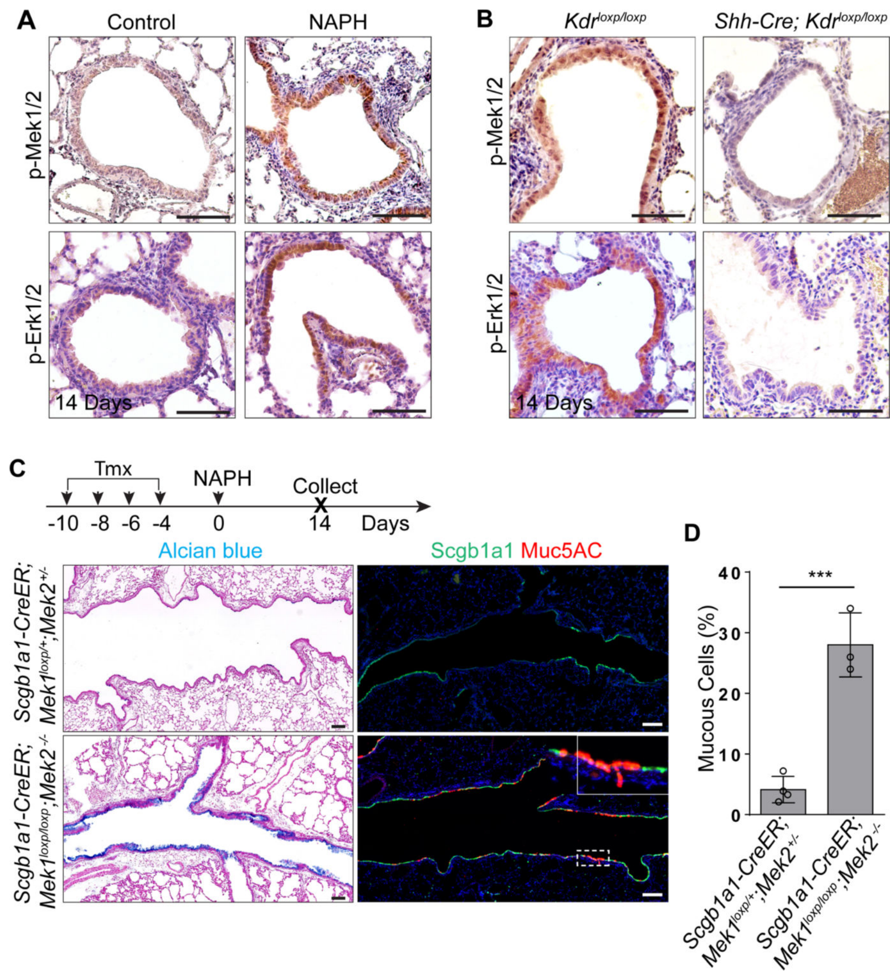

Figure 4: Inhibition of Mek/Erk signaling promotes mucous metaplasia of the regenerated airway epithelium.

(A) Increased expression of p-Mek1/2 and p-Erk1/2 in the airway epithelium following NAPH challenge. (**B**) *Kdr* deletion reduces the expression of p-Mek1/2 and p-Erk1/2 in *Shh-Cre;Kdr$^{loxp/loxp}$* mutants following NAPH challenge. (**C and D**) Genetic inhibition of Mek signaling promotes airway mucous metaplasia in *Scgb1a1-CreER;Mek1$^{loxp/loxp}$;Mek2$^{-/-}$* mutants following NAPH treatment (n=3). Data represent mean ± s.e.m. ***p<0.001; statistical analysis by unpaired two-tailed Student's *t*-test. Scale bar:100 µm. See also Figure S4.

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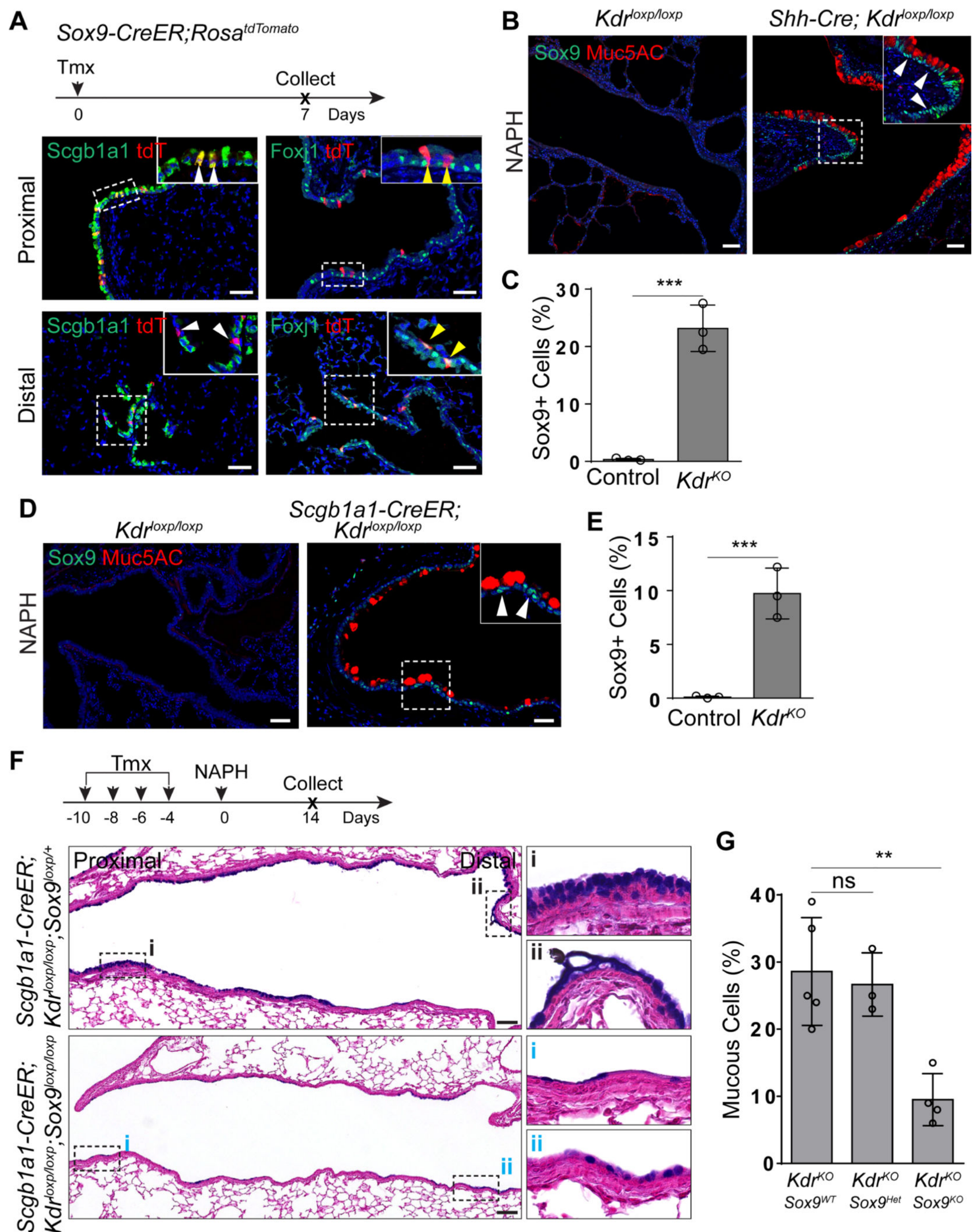

Figure 5: Sox9 mediates mucous metaplasia upon Kdr loss.

(**A**) Sox9 lineage-labeled cells include a limited number of club cells in the proximal airways and ciliated cells in the distal airways. (**B and C**) *Kdr* deletion leads to Sox9 expression in the metaplastic mucous cells of *Shh-Cre;Kdr*$^{loxp/loxp}$ mutants following NAPH treatment (n=3). (**D and E**) Sox9 is expressed in the airway mucous cells of *Scgb1a1-CreER;Kdr*$^{loxp/loxp}$ mutants following NAPH challenge (n=3). (**F and G**) *Sox9* deletion attenuates mucous metaplasia of the regenerated airway epithelium in *Scgb1a1-CreER;Kdr*$^{loxp/loxp}$*;Sox9*$^{loxp/loxp}$ mutants (n=4). Data represent mean ± s.e.m. ns: no

significant; $^{**}p<0.01$; $^{***}p<0.001$; statistical analysis by unpaired two-tailed Student's *t*-test. Scale bar: 50 μm. See also Figure S5.

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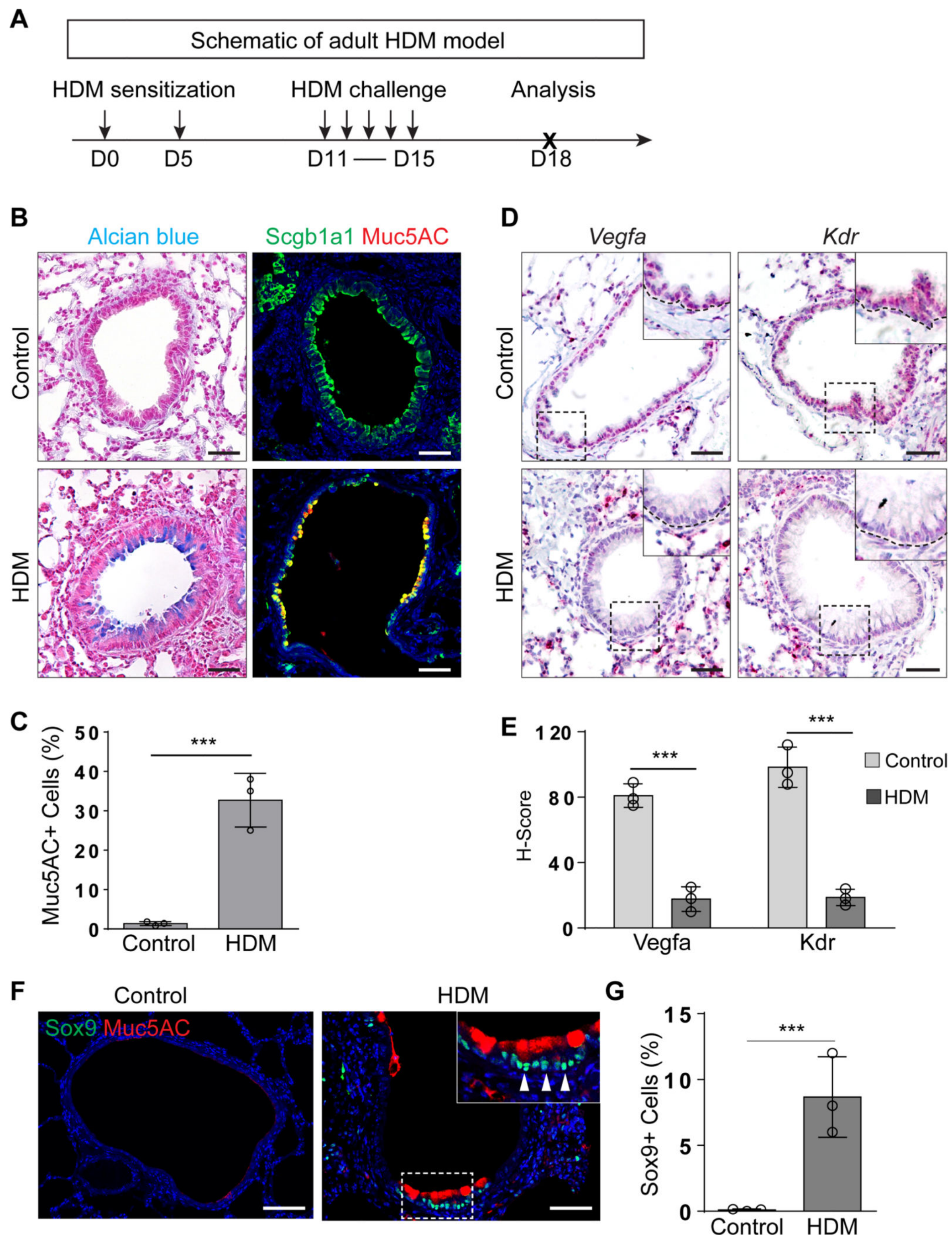

Figure 6: Asthmatic mucous metaplasia is associated with reduced *Vegfa* and *Kdr* transcripts.
(**A**) Schematic of the house dust mite (HDM)-induced adult mouse asthma model. (**B and C**) Mucous metaplasia occurs in the airway epithelium of asthmatic mice (n=3). (**D and E**) RNAscope analysis indicates the reduced transcripts of *Vegfa* and *Kdr* in the airway epithelium of asthmatic mice (n=3). (**F and G**) Sox9 is expressed in the airway mucous cells of HDM-induced asthmatic mice (n=3). Data represent mean ± s.e.m. ***p<0.001; statistical analysis by unpaired two-tailed Student's t-test.Scale bar:100 μm. See also Figure S6.

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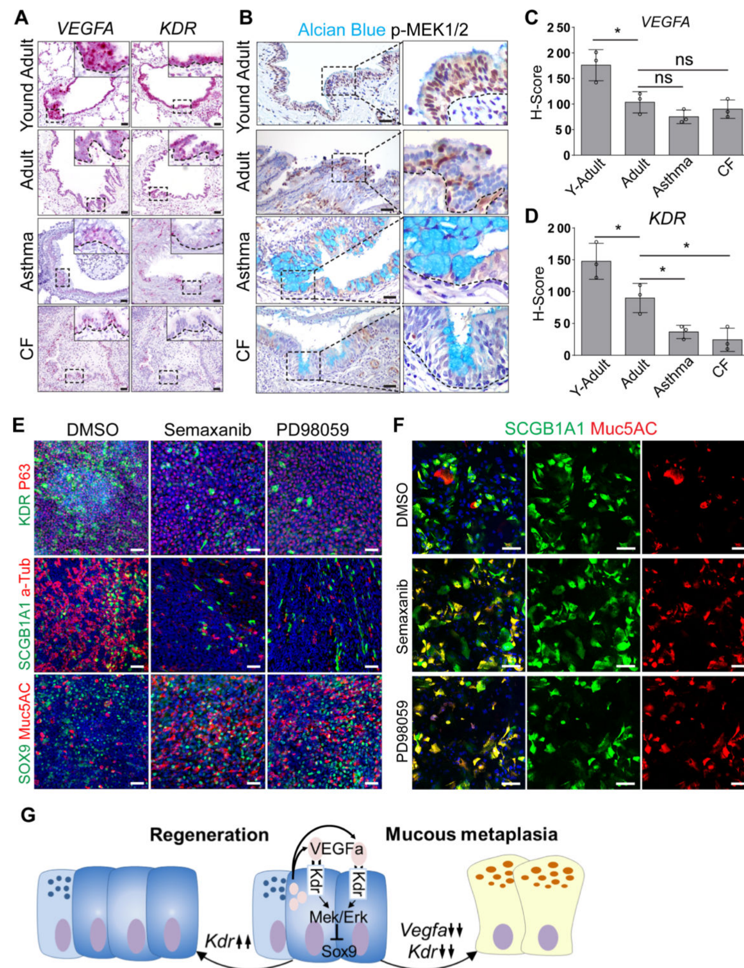

Figure 7: Suppressed VEGFA-KDR signaling promotes mucous metaplasia of human airway epithelial cells.

(**A**) *VEGFA* and *KDR* transcripts are expressed in the airway epithelium of young adults and reduced in adults (n=3). Note further reduced levels of transcripts in asthma (n=3) and cystic fibrosis (CF) patients (n=3). (**B**) Reduced p-MEK1/2 expression in the airway epithelium of asthma (n=3) and CF patients (n=3). (**C and D**) Reduced transcript levels of *VEGFA* (**C**) and *KDR* (**D**) in asthma and CF lung samples as measured by H-score. Data represent mean ± s.e.m. ns: no significant; $*p<0.05$; statistical analysis by unpaired two-tailed Student's *t*-test. (**E**) Inhibition of KDR (Semaxanib) and MEK (PD98059) promotes mucous differentiation of human small airway epithelial cells. Note the decreased numbers of club and ciliated cells following inhibitor treatments. (**F**) Inhibition of KDR or MEK promotes mucous metaplasia of club cells. (**G**) Transiently increased Vegfa-Kdr signaling is critical for airway epithelial regeneration. Reduced Vegfa-Kdr-Mek signaling promotes mucous cell differentiation of club cells through Sox9. Scale bar: 50 μm. See also Figure S7.

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KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-P63	Santa Cruz	Cat#sc-8343; RRID: AB_653763
Mouse monoclonal anti-P63	Biocare Medical	Cat# CM163; RRID: AB_10583039
Chicken polyclonal anti-GFP	Aves Labs	Cat#GFP-1020; RRID: AB_2307323
Rabbit polyclonal anti-Sox9	Millipore	Cat#AB5535; RRID: AB_2239761
Goat polyclonal anti-tdTomato	Biorbyt	Cat#orb182397; RRID: AB_2687917
Mouse monoclonal anti-Foxj1	eBioscience	Cat#14-9965-82; RRID: AB_1548835
Rabbit polyclonal anti-pMek1/2	Cell Signaling Technology	Cat#2338S; RRID: AB_490903
Rabbit polyclonal anti-pErk1/2	Cell Signaling Technology	Cat#4370S; RRID: AB_2315112
Rabbit polyclonal anti-pP38	Cell Signaling Technology	Cat#9215S; RRID: AB_331762
Rabbit polyclonal anti-pJNK	Cell Signaling Technology	Cat#9251S; RRID: AB_331659
Rabbit polyclonal anti-Kdr	Cell Signaling Technology	Cat#9698S; RRID: AB_11178792
Mouse monoclonal anti-Muc5AC	Lab Vision	Cat#MS-145-P0; RRID: AB_62735
Rabbit polyclonal anti-Clca3	Abcam	Cat#ab46512; RRID: AB_152837
Rabbit polyclonal anti-Scgb1a1	Millipore	Cat#07-623; RRID: AB_310759
Rat monoclonal anti-human SCGB1A1	R&D Systems	Cat#MAB4218; RRID: AB_394324
Mouse monoclonal anti-Acetylated tubulin	Sigma-Aldrich	Cat#T7451; RRID: AB_ 609894
Rat monoclonal APC anti-mouse Epcam	Biolegend	Cat#118213; RRID: AB_1134105
Mouse monoclonal APC anti-human p75	Biolegend	Cat#345107; RRID: AB_ 10639737
Chemicals, Peptides, and Recombinant Proteins		
PneumaCult-Ex Plus Medium	StemCell Technologies	Cat#05008
PneumaCult-ALI Medium	StemCell Technologies	Cat#05001
Collagen Type IV	Sigma-Aldrich	Cat#234154
Collagenase Type I	Gibco	Cat#17100-017
Elastase	Worthington Biochemical Corporation	Cat#LS002279
Dispase II	BD Biosciences	Cat#354235
Dnase I	Roche	Cat#10104159001
Naphthalene	Sigma-Aldrich	Cat#84679
HDM	Greer Laboratories	Cat#XPB70D3A25
Tamoxifen	Sigma-Aldrich	Cat#T5648
LIVE/DEAD Fixable Violet Dead Cell Stain Kit	Thermo Fisher Scientific	Cat#L34963
Semaxanib	Selleck	Cat#S2845
PD98059	Selleck	Cat#S1177
FR180204	Selleck	Cat#S7524
SB203580	Selleck	Cat#S1076
SP600125	Selleck	Cat#S1460

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
RNAscope 2.5 HD Assay-RED Kit	Advanced Cell Diagnostics	Cat#322360
mm-Kdr probe	Advanced Cell Diagnostics	Cat#414811
mm-Vegfa probe	Advanced Cell Diagnostics	Cat#405131
hs-KDR probe	Advanced Cell Diagnostics	Cat#312121
hs-Vegfa probe	Advanced Cell Diagnostics	Cat#423161
PPIB probe	Advanced Cell Diagnostics	Cat#313901
DapB probe	Advanced Cell Diagnostics	Cat#310043
Deposited data		
Single cell RNA sequencing data of Normal and NAPH treated lung epithelium	This paper	GEO: GSE#
Experimental Models: Cell Lines		
Primary Human Small Airway Epithelial Cells	ATCC	PCS-301-010
Experimental Models: Organisms/Strains		
Shh-Cre	(Harfe et al., 2004)	N/A
Scgb1a1-CreER	(Rawlins et al., 2009)	N/A
Sox9-CreER	(Soeda et al., 2010)	N/A
$Mek1^{loxp/loxp}$	(Bissonauth et al., 2006)	N/A
$Mek2^{-/-}$	(Belanger et al., 2003)	N/A
Kdr-GFP	Jackson Laboratory	017006
Kdr-Cre	Jackson Laboratory	018976
$Kdr^{loxp/loxp}$	Jackson Laboratory	018977
$Sox9^{loxp/loxp}$	Jackson Laboratory	013106
$R26^{tdTomato}$	Jackson Laboratory	007914
$R26^{lacZ}$	Jackson Laboratory	003474
Vegf-hyper	(Miquerol et al., 1999)	N/A
Vegf-hypo	(Damert et al., 2002)	N/A
$Vegf^{loxp/loxp}$	Kindly provided by Dr. Hoon-Ki Sung	N/A
Oligonucleotides		
Primer: Mouse Kdr Forward: CGAGACCATTGAAGTGACTTGCC	This paper	N/A
Primer: Mouse Kdr Reverse: TTCCTCACCCTGCGGATAGTCA	This paper	N/A
Primer: Mouse Scgb1a1 Forward: GGTTATGTGGCATCCCTGAAGC	This paper	N/A
Primer: Mouse Scgb1a1 Reverse: GCTTACACAGAGGACTTGTTAGG	This paper	N/A
Primer: Mouse Vegfa Forward: CTGCTGTAACGATGAAGCCCTG	This paper	N/A
Primer: Mouse Vegfa Reverse: GCTGTAGGAAGCTCATCTCTCC	This paper	N/A
Primer: Human Kdr Forward: GGAACCTCACTATCCGCAGAGT	This paper	N/A
Primer: Human Kdr Reverse: CCAAGTTCGTCTTTTCCTGGGC	This paper	N/A
Primer: Human p63 Forward: CAGGAAGACAGAGTGTGCTGGT	This paper	N/A

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REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Primer: Human p63 Reverse: AATTGGACGGCGGTTCATCCCT	This paper	N/A
Primer: Human Scgb1a1 Forward: TCATGGACACACCCTCCAGTTATGAG	This paper	N/A
Primer: Human Scgb1a1 Reverse: TGAGCTTAATGATGCTTTCTCTGGGC	This paper	N/A
Primer: Human Foxj1 Forward: GGCATAAGCGCAAACAGCCG	This paper	N/A
Primer: Human Foxj1 Reverse: TCGAAGATGGCCTCCCAGTCAAA	This paper	N/A
Primer: Human Sox9 Forward: AGGAAGCTCGCGGACCAGTAC	This paper	N/A
Primer: Human Sox9 Reverse: GGTGGTCCTTCTTGTGCTGCAC	This paper	N/A
Primer: Human Muc5AC Forward: GCACCAACGACAGGAAGGATGAG	This paper	N/A
Primer: Human Muc5AC Reverse: CACGTTCCAGAGGCCGGACAT	This paper	N/A
Software and Algorithms		
Prism 6	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
R	Bioconductor	https://www.bioconductor.org/

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