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Foxp1/4 control epithelial cell fate during lung development and regeneration through regulation of anterior gradient 2

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SUMMARY

The molecular pathways regulating cell lineage determination and regeneration in epithelial tissues are poorly understood. The secretory epithelium of the lung is required for production of mucus to help protect the lung against environmental insults, including pathogens and pollution, that can lead to debilitating diseases such as asthma and chronic obstructive pulmonary disease. We show that the transcription factors Foxp1 and Foxp4 act cooperatively to regulate lung secretory epithelial cell fate and regeneration by directly restricting the goblet cell lineage program. Loss of Foxp1/4 in the developing lung and in postnatal secretory epithelium leads to ectopic activation of the goblet cell fate program, in part, through de-repression of the protein disulfide isomerase anterior gradient 2 (Agr2). Forced expression of Agr2 is sufficient to promote the goblet cell fate in the developing airway epithelium. Finally, in a model of lung secretory cell injury and regeneration, we show that loss of Foxp1/4 leads to catastrophic loss of airway epithelial regeneration due to default differentiation of secretory cells into the goblet cell lineage. These data demonstrate the importance of Foxp1/4 in restricting cell fate choices during development and regeneration, thereby providing the proper balance of functional epithelial lineages in the lung.

KEY WORDS: Foxp1, Foxp4, Endoderm, Goblet cell, Lung, Regeneration, Mouse

INTRODUCTION

The tracheal and bronchiolar airways of mammals are essential for providing defense against inhaled pathogens and external environmental insults, such as cigarette smoke and pollution. The pseudostratified epithelium lining the trachea and proximal bronchiolar airways is composed of multiple cell lineages, including secretory and ciliated epithelium as well as basal cells, which act as progenitors for these lineages (reviewed by Morrisey and Hogan, 2010). The molecular pathways controlling cell fate and the precise allocation of epithelial lineages during both lung development and lung regeneration are poorly understood. Such insight is crucial for developing better therapeutic options for lung diseases such as asthma and chronic obstructive pulmonary disease (COPD).

The proximal airways of the respiratory system are lined with several differentiated cell lineages, including secretory Clara cells, ciliated epithelial cells, neuroendocrine cells and, in large mammals such as humans, goblet cells. Goblet cells are large morphologically distinct cells containing large vacuoles needed for the secretion of mucus in response to airway irritation or injury. Goblet cells are thought to differentiate from other secretory lineages in the proximal airways such as Clara secretory cells (Park et al., 2007; Chen et al., 2009). In normal proximal airways of mammals, only a small number of goblet cells are found and in mice this lineage is absent unless an injury or disease model is provoked. However, in diseases such as asthma and COPD, an

increased numbers of goblet cells generate large amounts of mucus secretions in response to airway sensitization and to the chronic injury/repair cycle indicative of these diseases (reviewed by Fahy, 2001; Rogers, 2003; Tagaya and Tamaoki, 2007). The molecular pathways that regulate the cell fate of proximal airway epithelium, which generates the proper composition of the proximal airways, are poorly understood. However, previous studies have demonstrated important roles for the ETS-related transcription factor Spdef and the Notch signaling pathway (Park et al., 2007; Chen et al., 2009; Guseh et al., 2009; Tsao et al., 2009; Rock et al., 2011). Despite these findings, little is understood about how cell fate decisions are regulated in the developing and regenerating airway epithelium and whether there are factors that actively restrict cell lineage differentiation versus those that promote specific cell fates, such as Spdef (Park et al., 2007; Chen et al., 2009).

Foxp1, Foxp2 and Foxp4 transcription factors are conserved transcriptional repressors expressed in overlapping patterns in the epithelium and mesenchymal derivatives of the developing foregut (Shu et al., 2001; Lu et al., 2002; Shu et al., 2007). Foxp1/2/4 are each expressed at high levels during lung development as well as in the adult lung with Foxp1/4 being the predominant paralogs expressed in proximal bronchiolar and tracheal epithelium (Lu et al., 2002). Previous studies have shown that Foxp1/2 cooperatively regulate distal lung epithelial development as well as esophageal development (Shu et al., 2007). *Foxp2^{-/-}:Foxp1^{+/-}* mutants express lower levels of the key transcription factors N-myc (Mycn – Mouse Genome Informatics) and Hopx, leading to perinatal demise (Shu et al., 2007). However, the role of Foxp1/2/4 in development and homeostasis of the epithelium in the proximal airways of the respiratory system was unknown.

In this article, we show that conditional deletion of Foxp1/4 in developing lung epithelium leads to decreased secretory cell differentiation, in particular loss of Clara cells, but ectopic activation of the goblet cell program, thus indicating a key role for

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these factors in controlling secretory cell fate. Using ChIP-seq and microarray, we show that Foxp1/4 repress key factors in the goblet cell differentiation program, including the protein disulfide isomerase anterior gradient 2 (*Agr2*). Overexpression of *Agr2* leads to ectopic activation of the goblet cell gene expression program, indicating that this gene is sufficient to drive many aspects of goblet cell differentiation. Foxp1/4 are also required to restrict the goblet cell differentiation program during lung secretory cell homeostasis and regeneration after postnatal injury, as shown by the dramatic inhibition of airway epithelial regeneration in mutants lacking Foxp1/4 expression. Together, these data point to a crucial role for Foxp1/4 in restricting secretory cell fate determination during both lung development and epithelial regeneration, indicating a pivotal role for these transcription factors in defining epithelial cell fates within developing and regenerating foregut derivatives.

MATERIALS AND METHODS

Animals

Shh-cre and *Foxp1^{lox/lox}* mice were previously generated and genotyped as described (Harfe et al., 2004; Feng et al., 2010). *Foxp4^{lox/+}* mice were generated by introducing two loxP sites that flank exons 12 and 13 coding the forkhead DNA-binding domain (supplementary material Fig. S1) using standard recombination techniques. The targeting vector was linearized and electroporated into R1 embryonic stem (ES) cells. ES cell clones were isolated and DNA was extracted and Southern blot analysis was performed using both 5' and 3' probes to verify correct integration of both loxP sites. Two correctly targeted ES clones were injected into blastocysts to generate chimeric mice, which were further bred to C57BL/6 mice to generate germline transmission of the *Foxp4^{lox/+}* allele. The neomycin cassette was removed using Flpe mice. The *Scgbl1-cre* mouse was generated by homologous recombination in ES cells to insert the iCre cDNA with SV40 polyadenylation sequence at the *Scgbl1* start codon. Recombination in *Scgbl1-cre/R26R* animals shows activity in the majority of airway epithelial cells including the trachea, the bronchi and bronchioles. *Shh-cre* and *Scgbl1-cre* animals were used as controls in the relative experiments. Genotypes of all mutant lines were determined by PCR amplification using primers listed in supplementary material Table S1.

To generate *SFTPC-Agr2* mice, the full mouse *Agr2* coding region was cloned downstream of the human 3.7 kb *SFTPC* promoter and upstream of a SV40 polyadenylation sequence as previously described (Tian et al., 2011). The transgenic cassette was excised from the resulting plasmid, purified and injected into FVB/N fertilized oocytes. Transgenic positive embryos were collected at E18.5 and genotypes using PCR primers listed in supplementary material Table S1. Three *SFTPC-Agr2* genotype-positive animals were examined in these studies and the data were consistent amongst all three F0 founders. Genotype-negative littermates were used as controls for these experiments. All animal procedures were performed in accordance with the Institute for Animal Care and Use Committee at the University of Pennsylvania.

Naphthalene injury

Eight- to ten-week-old mice were injected interperitoneally with 200 mg/kg body weight of naphthalene dissolved in corn oil; control mice were injected with the same volume of corn oil. At days 3, 10 and 20 after injection, right lung lobe was collected for RNA extraction and left lobes were inflated fixed at 25 cm H₂O pressure with 10% formalin followed by an additional 24 hours of fixation in 10% formalin. Five animals were used for each genotype and time point tested.

Histology

Tissues were fixed in 10% formalin, embedded in paraffin wax and sectioned at a thickness of 5 μ m. Hematoxylin and Eosin (H&E) staining was performed using standard procedures. Immunohistochemistry was performed using the following antibodies: mouse anti-phospho-histone 3 (Cell Signaling Technology, 1:200), goat anti-Scgbl1a1 (Santa Cruz, 1:20), rabbit anti-SP-C (Chemicon, 1:500), rabbit anti-Nkx2.1 (Santa Cruz, 1:50),

rabbit anti-Sox9 (Santa Cruz, 1:100), rabbit anti-Sox2 (Seven Hills Bioreagents, 1:500), mouse anti- β -tubulin IV (BioGenex, 1:20), rabbit anti-e-cadherin (Cell Signaling, 1:100) and mouse anti-mucin5ac (Abcam, 1:100). The Foxp1 (1:200) and Foxp4 (1:200) polyclonal antibodies have been previously described (Lu et al., 2002). Slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). In situ hybridization was performed as previously described (Morrisey et al., 1996).

Microarray studies

RNA was isolated from E14.5 lungs from *Foxp1/4^{ShhcreDKO}* and *Shh-cre* control embryos and used to generate a biotinylated cRNA probe for Affymatrix Mouse Gene 1.0ST array. Data analysis and heatmap generation were performed as previously described (Zhang et al., 2008). Genes with 1.5-fold or greater changes over that of the experimental mean with a value of $P < 0.01$ (ANOVA) were considered to be statistically significant. Microarray data have been deposited in Gene Expression Omnibus under accession number GSE34584.

Quantitative PCR

Total RNA was isolated from lung tissue at the indicated time points using Trizol reagent, reverse transcribed using SuperScript First Strand Synthesis System (Invitrogen), and used in quantitative real-time PCR analysis with the primers listed in supplementary material Table S1.

ChIP and ChIP-seq analysis

For ChIP-seq analysis, each biological sample was generated from 20–30 embryonic day (E) 12.5 lungs. The ChIP and ChIP-seq analysis was performed as previously described (Zhang et al., 2010). Briefly, chromatin was prepared using a ChIP Assay Kit (Millipore), except protein-G agarose beads were used to pre-clear chromatin. The previously characterized Foxp1 polyclonal antibody as well as control rabbit IgG were used to immunoprecipitate protein–DNA complexes from this chromatin. The purified immunoprecipitated DNA was end-repaired, ligated to sequencing adapters, and enriched by PCR following the manufacturer's protocol (Illumina). The resulting DNA was sequenced on the Illumina Genome Analyzer II. Sequences were aligned to the mouse genome [University of California Santa Cruz Genome Browser on Mouse, July 2007 Assembly (mm9)] following pipeline processing. Peaks within a region covering 10 kb upstream and downstream from the transcriptional start site unique to the Foxp1 antibody and not found in the normal rabbit IgG were used as criteria for potential targets of Foxp1. ChIP-seq binding data for *Agr2* was confirmed by directed ChIP in chromatin samples from E12.5 and E18.5 lung using primers listed in supplementary material Table S1.

Cell culture transfection assays

The proximal 600 bp covering forkhead-binding sites within the Foxp1 ChIP-seq peak region (~8 kb upstream mouse *Agr2* transcriptional start site) was cloned into the pGL3 luciferase reporter plasmid to generate pGL3Agr2.luc. pGL3Agr2.luc was co-transfected into NIH 3T3 cells using Eugene 6 reagent with *Renilla* luciferase control expression plasmid and the indicated amount of Foxp1 expression vector. Forty-eight hours following transfection, cell extracts were assayed for luciferase expression using a commercially available kit (Promega). Relative reporter activities are expressed as luminescence units normalized for *Renilla* expression in the cell extracts. Data represent the average of three assays \pm s.e.m.

RESULTS

Loss of Foxp1/4 expression inhibits early secretory cell differentiation

Foxp1/4 are expressed in proximal airway epithelium in both the developing and adult lung (Lu et al., 2002). To assess the role of Foxp1/4 in lung epithelial differentiation, we generated a floxed allele of *Foxp4* (*Foxp4^{lox/lox}*) and, along with our previously described *Foxp1^{lox/lox}* allele (Feng et al., 2010), we deleted both genes using the *Shh-cre* line which is active throughout the developing lung epithelium from the earliest stages of lung development (Fig. 1A–E) (Goss et al., 2009). *Shh-cre*-mediated loss

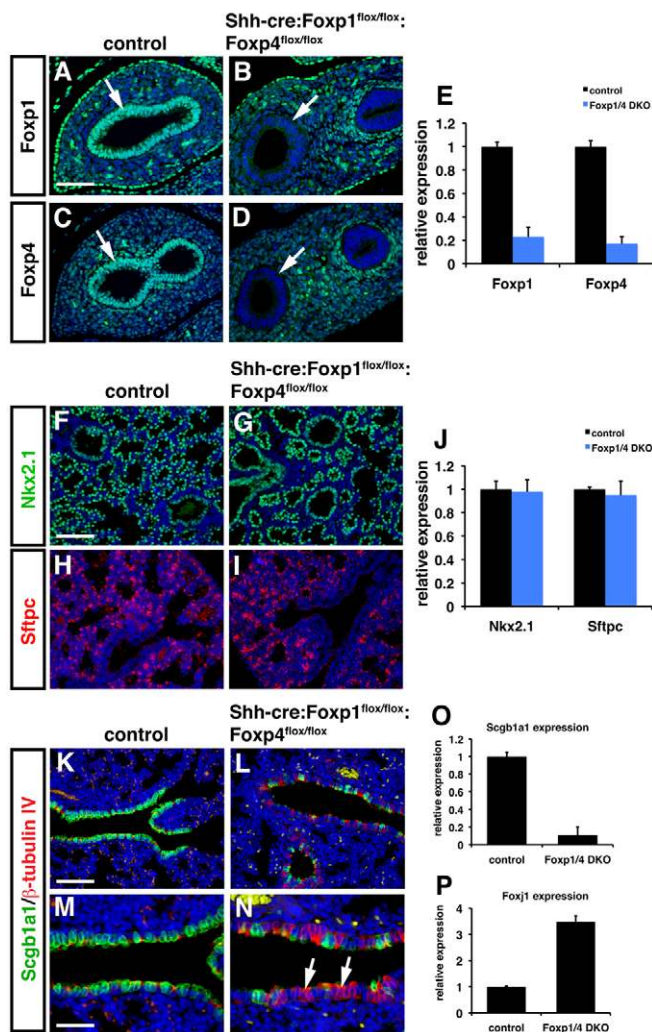


Fig. 1. Loss of Foxp1/4 leads to defective proximal airway epithelial development in mouse. (A-E) Foxp1 and Foxp4 were efficiently deleted using the *Shh-cre* line as noted by loss of expression in the developing airway epithelium using immunostaining (A-D, arrows) and Q-PCR (E) at E12.5. (F-J) Immunostaining for Nkx2.1 (F,G) and Sftpc (H,I) expression indicate normal lung endoderm specification and distal alveolar epithelial differentiation in *Foxp1/4*^{ShhcreDKO} mutants at E18.5. Q-PCR also indicates normal expression of both Nkx2.1 and Sftpc at E18.5 (J). (K-N) Expression of the proximal secretory cell differentiation marker Scgb1a1 was decreased in *Foxp1/4*^{ShhcreDKO} mutants as revealed by immunostaining whereas immunostaining for the ciliated epithelial marker β -tubulin IV was increased at E18.5 (K-N, arrows). Panels M and N represent higher magnification pictures of K and L to illustrate better the decrease in Scgb1a1-positive secretory cells (green) and an increase in β -tubulin IV-positive ciliated epithelial cells (red). (O,P) Q-PCR shows decreased expression of the secretory epithelial cell marker Scgb1a1 (O) and an increase in the ciliated epithelial marker Foxj1 (P) in *Foxp1/4*^{ShhcreDKO} mutants at E18.5. *Shh-cre* embryos were used as controls for all of these experiments. Error bars represent s.e.m. Scale bars: 100 μ m for A-D,M,N; 200 μ m for F-I,K,L.

of either Foxp1 or Foxp4 using *Shh-cre* did not result in any prenatal or postnatal lethality (data not shown). However, all *Shh-cre:Foxp1^{lox/lox}:Foxp4^{lox/lox}* mutants, from now on referred to as *Foxp1/4*^{ShhcreDKO}, die within minutes after birth of apparent respiratory distress. *Foxp1/4*^{ShhcreDKO} mutants expressed normal

levels of the important lung epithelial transcription factor Nkx2.1 and the alveolar epithelial type 2 cells marker surfactant protein C (Sftpc), suggesting that lung epithelial specification was not dramatically altered by Foxp1/4 deficiency (Fig. 1F-J). Although overall lung morphology of *Foxp1/4*^{ShhcreDKO} mutants appeared to be relatively normal, a reduction in Clara secretory cells was observed in the proximal airways of E18.5 *Foxp1/4*^{ShhcreDKO} mutants as determined by immunostaining and quantitative PCR (Q-PCR) for the Clara secretory cell marker gene *Scgb1a1* (Fig. 1K-O). This was associated with an increase in ciliated epithelial cell differentiation, as revealed by increased β -tubulin IV immunostaining and Q-PCR for the ciliated epithelial specific transcription factor *Foxj1* (Fig. 1K-N,P). These data indicate that secretory epithelial cell differentiation is defective in *Foxp1/4*^{ShhcreDKO} mutants with a decrease in Clara secretory cells and an increase in ciliated cells in the proximal airway epithelium.

Proximal airway epithelial differentiation defects in *Foxp1/4*^{ShhcreDKO} mutants

To understand better the genome-wide consequences of Foxp1/4 deficiency on secretory epithelial differentiation in the lung, we performed microarray analysis of *Shh-cre* control and *Foxp1/4*^{ShhcreDKO} mutant lungs at E14.5. Greater than 1.5-fold differences were observed for 49 upregulated genes and for 28 downregulated genes (Fig. 2A; supplementary material Table S2). Microarray, Q-PCR and in situ hybridization analyses indicate that the early secretory cell marker genes *Scgb3a2* and *Cbr2* were significantly downregulated in a fashion similar to *Scgb1a1*, further suggestive of early defects in the cell fate of the proximal airway epithelium of *Foxp1/4*^{ShhcreDKO} mutants (Fig. 2A-H). Interestingly, expression of *Agr2*, a protein disulfide isomerase expressed in goblet cells in the lung (Chen et al., 2009; Zhao et al., 2010), was upregulated in *Foxp1/4*^{ShhcreDKO} mutants using both microarray and Q-PCR assays (Fig. 2A,B).

In addition to loss of secretory cell marker genes, expression of the neuroendocrine (NE) cell marker *Ascl1* was shown to be decreased by microarray and Q-PCR analyses at E14.5 (Fig. 2B). NE cells are found in the large proximal airways of the lung and are thought to derive from a Sox2+ proximal airway precursor (Tompkins et al., 2009). In situ hybridization for *Ascl1* as well as immunostaining for Pgp9.5 (also known as Uchl1), an additional marker of the NE lineage, indicated that differentiation of the NE lineage is inhibited in *Foxp1/4*^{ShhcreDKO} mutants (Fig. 2I-K). Thus, loss of Foxp1/4 in the developing lung epithelium leads to a defect in cell fate choice in the proximal airway epithelium with decreased differentiation of both secretory and NE lineages but increased expression of the goblet cell gene expression program.

Lung endoderm progenitor allocation and Notch signaling are unaffected in *Foxp1/4*^{ShhcreDKO} mutants

Sox2+ endoderm cells are thought to act as precursors for most, if not all, of the proximal airway epithelial cell lineages, whereas expression of Sox9 is thought to mark an early distal endoderm progenitor cell lineage (Okubo et al., 2005; Lu et al., 2007). As defects in secretory and NE epithelial differentiation could arise from altered development of Sox2+ endoderm progenitors or from altered expression of Sox2 itself, we assessed expression of Sox2 as well as the distal epithelial progenitor marker Sox9 in *Foxp1/4*^{ShhcreDKO} mutants. Immunohistochemistry as well as Q-PCR showed that expression of Sox2 and Sox9 were unchanged in *Foxp1/4*^{ShhcreDKO} mutants (Fig. 3A-F). Moreover, the pattern of

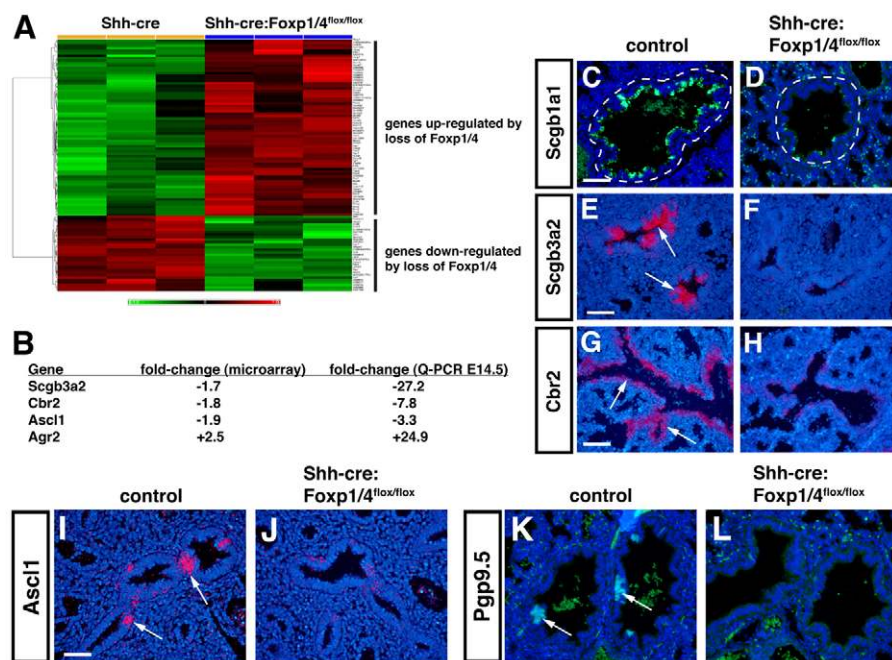


Fig. 2. Decreased secretory and neuroendocrine cell fates and an increase in goblet cell lineage markers in *Foxp1/4^{ShhcreDKO}* mutants. (A,B) Microarray and Q-PCR analyses show that several genes expressed in the Clara secretory cell (Scgb3a2, Cbr2) and neuroendocrine lineages (Ascl1) are decreased in *Foxp1/4^{ShhcreDKO}* mutants at E14.5. By contrast, the goblet cell marker gene *Agr2* is increased in both microarray and Q-PCR analyses. Red and green indicate up- and downregulated genes, respectively, versus *Shh-cre* controls. (C-H) Decreased expression of the secretory cell markers Scgb1a1 (C,D), Scgb3a2 (E,F) and Cbr2 (G,H) are observed in *Foxp1/4^{ShhcreDKO}* mutants at E18.5 using immunostaining (C,D) and in situ hybridization (E-H). Airways are encircled by a dashed line. (I-L) The neuroendocrine markers *Ascl1* (I,J) and *Pgp9.5* (K,L) are both decreased in *Foxp1/4^{ShhcreDKO}* mutants at E18.5 as revealed by in situ hybridization and immunostaining, respectively. Arrows indicate probe hybridization or immunostaining. *Shh-cre* embryos were used as controls for all of these experiments. Scale bars: 200 μ m for C-H,K,L; 150 μ m for I,J.

expression of these progenitor markers was not altered in *Foxp1/4^{ShhcreDKO}* mutants, with expression of Sox2 confined to the proximal lung endoderm and Sox9 expression confined to the distal lung endoderm (Fig. 3A-D). These data indicate that the defects in secretory epithelial cell fate and differentiation observed in *Foxp1/4^{ShhcreDKO}* mutants were not due to impaired allocation of Sox2+ or Sox9+ progenitors or from de-regulated expression of the *Sox2* or *Sox9* genes.

Previous studies have demonstrated that Notch signaling plays a key role in directing the cell fate and differentiation of proximal airway progenitors into the secretory cell lineage (Guseh et al., 2009; Tsao et al., 2009; Morimoto et al., 2010;

Rock et al., 2011). Loss of Notch signaling leads to a complete loss of the secretory cell lineage in the developing lung whereas increased Notch signaling promotes secretory cell differentiation (Guseh et al., 2009; Tsao et al., 2009; Morimoto et al., 2010; Rock et al., 2011). Because the phenotype in *Foxp1/4^{ShhcreDKO}* mutants was one of decreased secretory cell differentiation, we sought to determine whether Notch signaling was de-regulated in these mutants. Multiple Notch ligands, receptors, and transcriptional downstream effectors, such as *Hes1*, are expressed in the lung (Post et al., 2000; Tsao et al., 2009). Microarray and Q-PCR analyses detected no significant alterations in the expression of all Notch components tested in

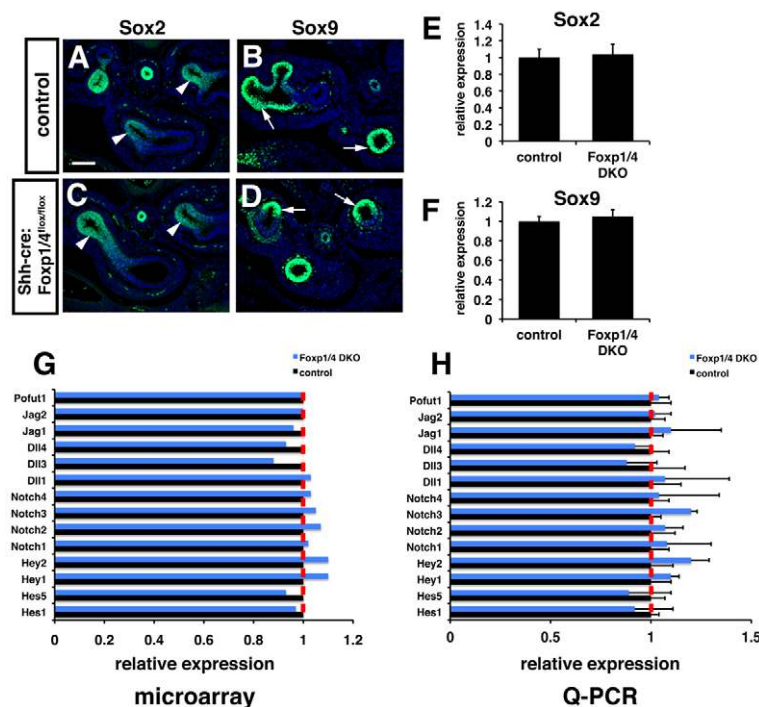


Fig. 3. Normal expression of early endoderm progenitor markers and Notch signaling components upon loss of *Foxp1/4* expression in lung endoderm. (A-D) The pattern of Sox2 (A,C; arrowheads) and Sox9 (B,D; arrows) expression appears normal by immunostaining in *Foxp1/4^{ShhcreDKO}* mutants at E12.5. (E,F) Q-PCR reveals normal expression of Sox2 and Sox9 in *Foxp1/4^{ShhcreDKO}* mutants at E12.5. (G,H) Microarray (G) and Q-PCR (H) analyses show normal expression of Notch signaling components, including the important Notch signaling activity readout genes *Hes1* and *Hes5*. *Shh-cre* embryos were used as controls for these experiments. Error bars represent s.e.m. Scale bar: 150 μ m.

Foxp1/4^{ShhcreDKO} mutants (Fig. 3G,H). These data suggest that *Foxp1/4* act either downstream or independently of Notch signaling to regulate secretory epithelial cell fate in the lung.

Ectopic activation of the goblet cell program in *Foxp1/4^{ShhcreDKO}* mutants

Increased expression of *Agr2* along with decreased expression of genes associated with the Clara secretory cell lineage suggested that *Foxp1/4* normally restricts the goblet cell lineage within the developing airway epithelium. Goblet cells do not form in mouse airways except during injury or in disease models such as those mimicking human asthma (Rogers, 2003; Sumi and Hamid, 2007). Moreover, goblet cells dramatically increase in number in the human lung after injury or in diseases such as asthma and COPD (Rogers, 2003; Sumi and Hamid, 2007). To assess the extent of activation of the goblet cell lineage program upon loss of *Foxp1/4* expression, we characterized E18.5 *Foxp1/4^{ShhcreDKO}* mutants for expression of additional goblet cell genes and proteins. Expression of *Muc5ac*, *Muc5b*, *Spdef* and *Foxa3* was upregulated although *Spdef* and *Foxa3* expression was increased less dramatically than the other genes (Fig. 4A). However, *Stat6*, which has been shown to regulate *Spdef* expression (Park et al., 2007; Chen et al., 2009), was not upregulated in *Foxp1/4^{ShhcreDKO}* mutants (Fig. 4A). In situ hybridization shows increased expression of *Agr2* in the proximal airways of *Foxp1/4^{ShhcreDKO}* mutants (Fig. 4B,C). These data suggested that *Foxp1/4* might act downstream of *Stat6* but upstream of *Agr2* to restrict the goblet cell lineage during lung development.

As noted above, previous models of goblet cell development have identified a hierarchical set of factors with Notch and *Stat6* residing at the top. These activate *Spdef*, which, in turn, activates additional goblet cell genes, including *Agr2* and the mucin-related proteins *Muc5ac* and *Muc5b* (Park et al., 2007; Chen et al., 2009). Of these, *Agr2* expression was increased the most and its expression was increased as early as E14.5 (Fig. 2). This is prior to full development of the secretory lineage in the proximal airways of the lung and before the increase of other goblet cell lineage genes, as noted by our microarray results (Fig. 2). *Agr2* is a protein disulfide isomerase that has been implicated in trafficking mucin proteins in goblet cells of the intestine (Zhao et al., 2010). Moreover, *Agr2* expression is essential for goblet cell development as *Agr2* null mice lack goblet cells in the intestine (Zhao et al., 2010). To determine whether *Agr2* is a direct transcriptional target of *Foxp1/4* in the lung, we performed ChIP-seq for *Foxp1* on chromatin from E12.5 mouse lungs (Zhang et al., 2010). The *Foxp1* ChIP-seq data reveal that *Foxp1* binds to an evolutionarily conserved region of the *Agr2* locus (located ~8 kb upstream of the predicted *Agr2* transcriptional start site) that contains two conserved forkhead DNA-binding sites (Fig. 4D). Directed ChIP experiments confirmed that both *Foxp1* and *Foxp4* bind to these evolutionarily conserved forkhead-binding sites in chromatin generated from both E12.5 and E18.5 lungs (Fig. 4E-G).

To assess whether *Foxp1* and *Foxp4* can directly repress *Agr2* gene expression, we overexpressed each transcription factor individually or in combination in the human lung epithelial cell line

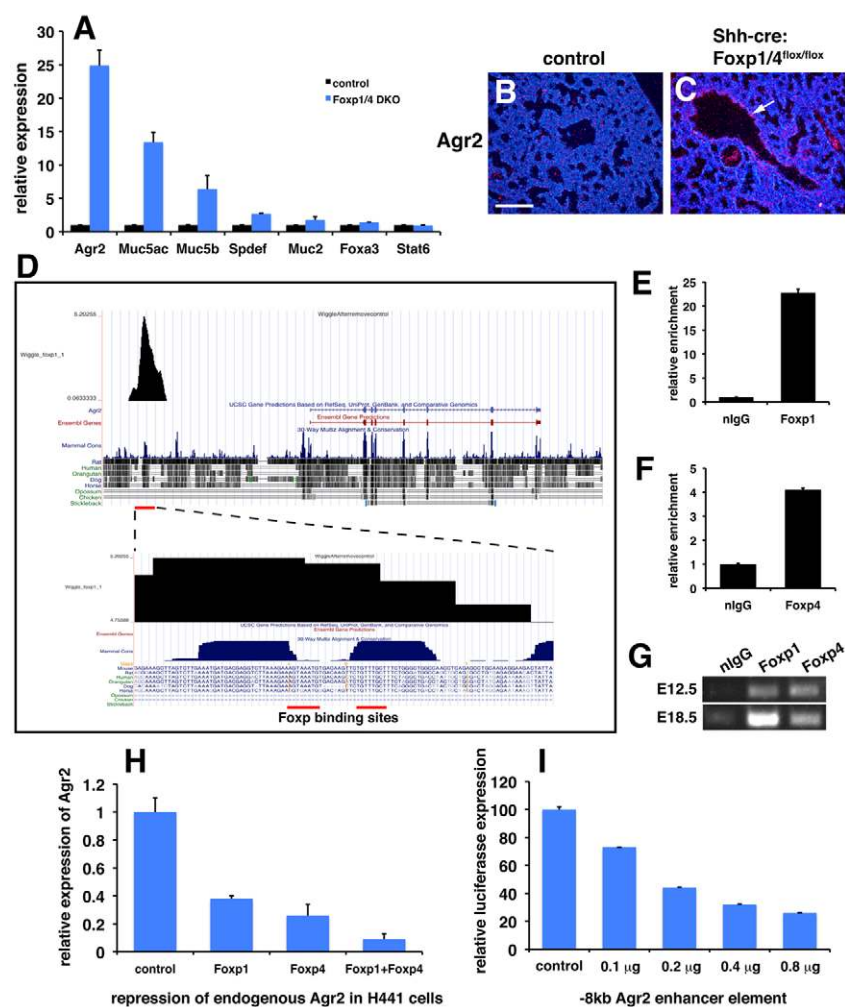


Fig. 4. Ectopic activation of the goblet cell lineage program in *Foxp1/4^{ShhcreDKO}* mutants and identification of *Agr2* as a direct target of *Foxp1/4*. (A) Q-PCR reveals increased expression of goblet cell related genes, including *Agr2*, in *Foxp1/4^{ShhcreDKO}* mutants at E18.5. (B,C) In situ hybridization showing increased *Agr2* expression in the proximal airways of *Foxp1/4^{ShhcreDKO}* mutants at E18.5 (arrow). (D) *Foxp1* ChIP-seq data reveals a single strong peak 8 kb upstream of the *Agr2* transcriptional start site that contains two evolutionarily conserved Foxp DNA-binding sites. (E-G) Directed ChIP for *Foxp1* and *Foxp4* on the *Agr2* -8 kb enhancer element using Q-PCR at E18.5 (E,F) and PCR agarose gel analysis at E12.5 and E18.5 (G). (H) *Foxp1* and *Foxp4* can cooperatively repress endogenous *Agr2* expression in H461 human lung epithelial cells. (I) The -8 kb *Agr2* enhancer element can be repressed by *Foxp1* in a dose-dependent manner when cloned into a luciferase reporter. Error bars represent s.e.m. Scale bar: 200 μ m.

H441, which has characteristics of Clara secretory epithelial cells (Sawaya et al., 1993). These studies show that Foxp1 and Foxp4 can cooperatively repress endogenous *AGR2* expression in lung secretory epithelium (Fig. 4H). Foxp1 can also repress this –8 kb *Agr2* enhancer element in a dose-dependent manner when cloned into a luciferase reporter construct (Fig. 4I). These data demonstrate that Foxp1 and Foxp4 can directly target and repress expression of *Agr2* in the secretory epithelium of the lung.

Overexpression of *Agr2* can elicit the goblet cell fate in the developing lung epithelium

Agr2 is required for intestinal goblet cell development and its expression is associated with metastasis and increased cellular stress responses (Zweitzig et al., 2007; Barraclough et al., 2009; Zhao et al., 2010). Because goblet cells numbers are increased upon epithelial stress in injury and disease in both mice and humans, the increased expression of *Agr2* might lead directly to increased expression of mucin genes along with other markers of the goblet cell program. Therefore, we generated transgenic mice using the human surfactant protein C (*SFTPC*) promoter to drive widespread ectopic *Agr2* expression in the early lung epithelium in order to determine whether increased *Agr2* expression could activate the goblet cell lineage gene expression program. Examination of transgenic F0 founders at E18.5 showed that three of the five total F0 genotype-positive founders expressed increased levels of *Agr2* in the proximal airway epithelium including the bronchi and smaller bronchioles (Fig. 5A–D). These *SFTPC-Agr2* transgenic animals expressed increased levels of *Muc5ac*, *Muc2* and *Clca3*, which are all expressed in the goblet cell lineage in the lung (Fig. 5E–H). Expression of the ciliated epithelial markers β -tubulin IV and Foxj1 was unchanged in *SFTPC-Agr2* transgenic lungs (Fig. 5I–K). The increase in *Clca3*, a calcium-activated chloride channel expressed in goblet cells (Zhou et al., 2002; Thai et al., 2005), suggests that ectopic expression of *Agr2* promotes a

broader goblet cell gene expression program than induction of mucin genes alone. Co-immunostaining for the secretory protein *Scgb1a1* and *Muc5ac* shows that both proteins are expressed in the secretory epithelium upon overexpression of *Agr2* (Fig. 5L,M). This is consistent with previous studies showing that goblet cells express both *Sgb1a1* and *Muc5ac* (Boers et al., 1999; Evans et al., 2004). These data demonstrate that increased *Agr2* is sufficient to promote many aspects of the goblet cell fate and that its repression in *Foxp1/4^{ShhcreDKO}* mutants is responsible, at least in part, for the ectopic activation of the goblet cell lineage program.

Foxp1/4 are required for regeneration of secretory cells in lung airways by restricting the goblet cell fate

Because Foxp1/4 are necessary for development of the early Clara secretory cell lineage while restricting the goblet cell lineage during lung development, we assessed whether Foxp1/4 were required to maintain the Clara cell secretory lineage and restrict the goblet lineage in the postnatal mouse. We employed a newly generated Clara secretory cell-specific cre line in which cre recombinase was inserted into the endogenous *Scgb1a1* locus (*Scgb1a1-cre*; Fig. 6A) to generate *Foxp1/4^{Scgb1a1creDKO}* mutants. The *Scgb1a1-cre* line is active by E18.5 and its activity is restricted to the proximal airway epithelium of the lung (Fig. 6B,C; data not shown). *Foxp1/4^{Scgb1a1creDKO}* mutants were viable and no significant lethality was observed for up to one year of age (data not shown). Histological analysis showed that although the bronchiolar epithelium of *Foxp1/4^{Scgb1a1creDKO}* mutants expressed *Scgb1a1*, many of these cells also expressed *Muc5ac* as noted by co-immunostaining (Fig. 6D,E; supplementary material Fig. S2). The large bulbous shape of these cells as well as their increased expression of mucins indicative of a goblet cell phenotype was noted by Alcian Blue staining (supplementary material Fig. S3). Other markers of the goblet cell lineage were significantly

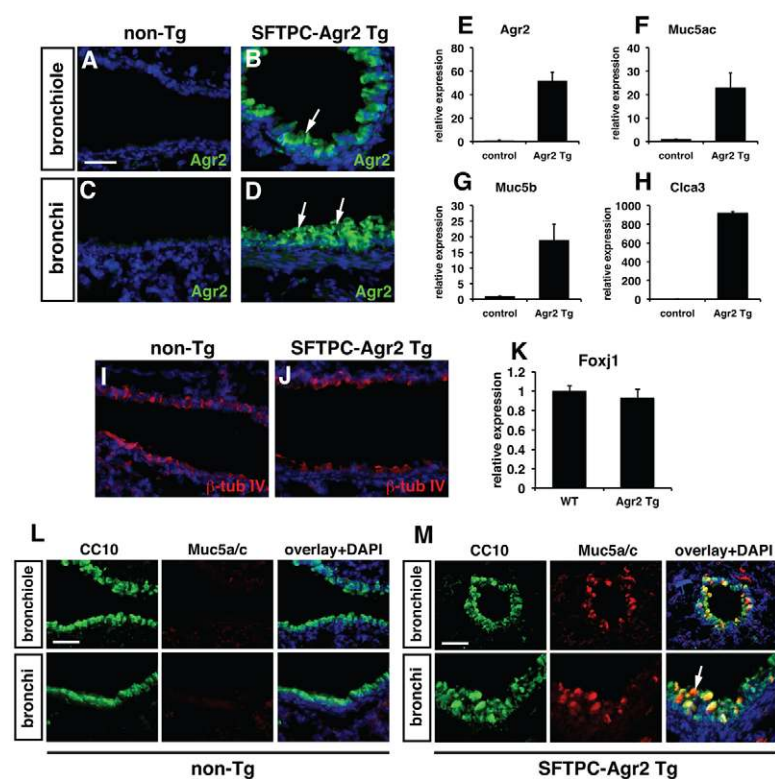


Fig. 5. Overexpression of *Agr2* can ectopically activate the goblet cell lineage gene expression program.

(A–D) *SFTPC-Agr2* transgenic lungs express elevated levels of *Agr2* protein in both small bronchiolar and larger bronchi at E18.5. (E–H) Q-PCR analysis of *Agr2* (E), *Muc5ac* (F), *Muc5b* (G) and *Clca3* (H) expression in *SFTPC-Agr2* transgenic lungs at E18.5. (I,J) Immunostaining for β -tubulin IV for non-transgenic (I) and *SFTPC-Agr2* transgenic (J) lungs. (K) Q-PCR for Foxj1 expression in non-transgenic and *SFTPC-Agr2* transgenic lungs. (L,M) Non-transgenic littermates do not express *Muc5ac* in proximal airway epithelium (L) whereas *Muc5ac* and *Scgb1a1* are co-expressed in epithelial cells of *SFTPC-Agr2* transgenic lungs at E18.5 (M, arrow). The Q-PCR data in E–H and K are the average \pm s.e.m. of the three different F0 founders at E18.5. Non-transgenic littermates were used as controls for these experiments. Scale bars: 150 μ m for A–D; 200 μ m for L,M.

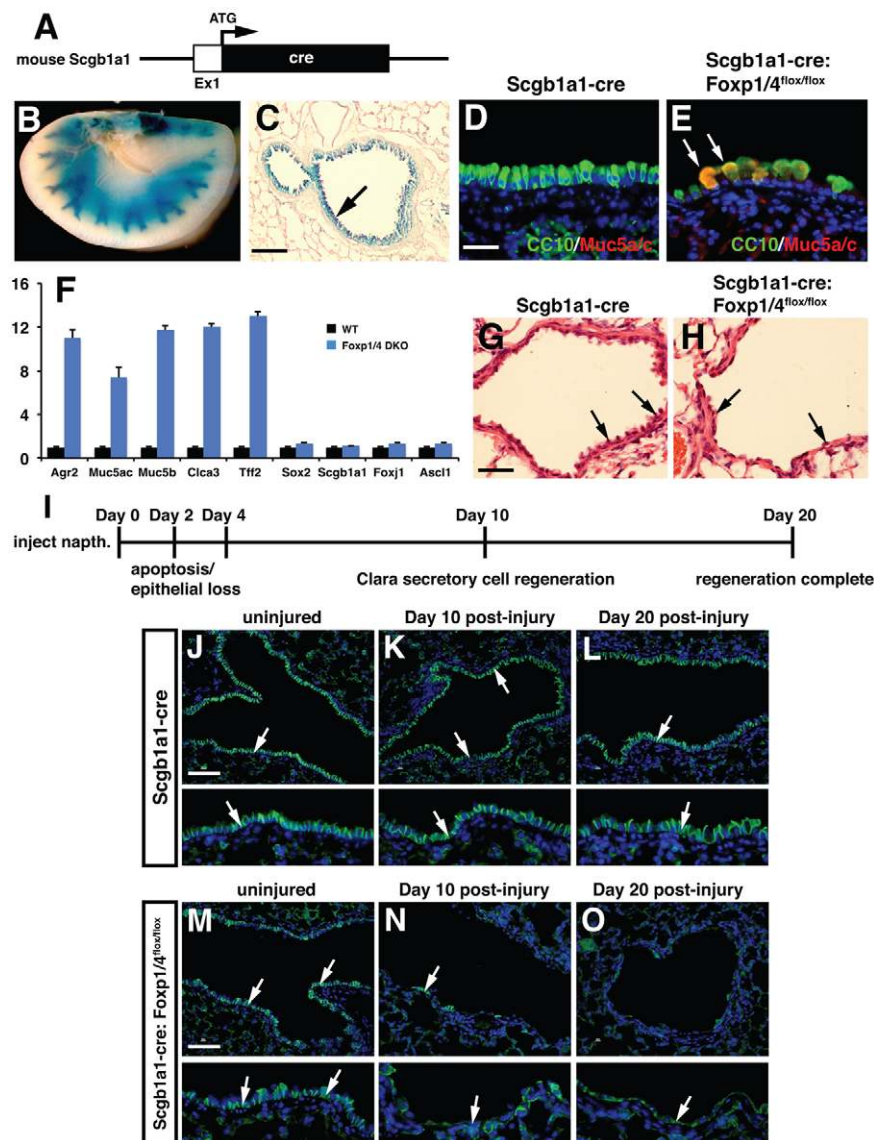


Fig. 6. Loss of Foxp1/4 expression in the mature secretory epithelial cell lineage leads to ectopic activation of the goblet cell lineage program and inhibits epithelial regeneration.

(A) Diagram of cre insertion into the *Scgb1a1* locus. (B,C) Activity of the *Scgb1a1*-cre: *R26R^{lacZ}* is confined to the proximal airway epithelium at two months as shown by whole-mount β -galactosidase histochemical staining (B) and histological sections from these lungs (C, arrow). (D,E) Loss of Foxp1/4 leads to ectopic activation of Muc5a/c expression in *Scgb1a1*-positive cells of the proximal airways of *Foxp1/4*^{*Scgb1a1*creDKO} adult mutants. (F) Q-PCR for markers of the goblet cell lineage program, including *Agr2* as well as the secretory, ciliated and neuroendocrine lineages in *Foxp1/4*^{*Scgb1a1*creDKO} adult lungs. Error bars represent s.e.m. (G,H) Naphthalene-induced secretory epithelial injury and regeneration leads to decreased numbers of airway epithelial cells 20 days after injury (arrows). (I) Schematic of the temporal nature of the naphthalene injury repair and regeneration model. (J-O) Immunostaining for E-cadherin shows normal regeneration of airway epithelium in *Scgb1a1*-cre control animals but reveals a dramatic loss of epithelial cells lining the injured airways of *Foxp1/4*^{*Scgb1a1*creDKO} mutants at both 10 and 20 days post-injury (arrows). Lower panels in J-O are higher magnification images to illustrate better the presence or absence of E-cadherin-positive epithelium in the airways. *Scgb1a1*-cre animals were used as controls for these experiments. Scale bars: 200 μ m for C,J-O; 100 μ m for D,E; 150 μ m for G,H.

upregulated in adult *Foxp1/4*^{*Scgb1a1*creDKO} mutants, including *Agr2*, whereas markers of Clara (*Scgb1a1*, *Sox2*), ciliated (*Foxj1*) and NE (*Ascl1*) cells were unchanged (Fig. 6F). These data suggested that Foxp1/4 are required to restrict the goblet cell lineage during secretory cell homeostasis in the lung.

We determined next whether Foxp1/4 were required for proper secretory cell repair and regeneration after naphthalene-induced injury. Naphthalene injury of the lung involves a severe and rapid loss of the majority of Clara secretory epithelium from the bronchiolar airways. A small population of Clara secretory cells that lack expression of the cytochrome Cyp2f2 are resistant to naphthalene injury and are thought to act as facultative progenitors during regeneration of the bronchiolar epithelium (Reynolds et al., 2000b; Giangreco et al., 2002). Loss of the secretory epithelium is complete after four days whereas regeneration and re-establishment of the airway epithelium is usually complete after two to three weeks. Naphthalene injury was induced in both *Scgb1a1*-cre control and *Foxp1/4*^{*Scgb1a1*creDKO} mutants at 8 weeks of age. Both control and *Foxp1/4*^{*Scgb1a1*creDKO} mutants survived the naphthalene treatment and displayed extensive airway apoptosis and loss of secretory cells three days after naphthalene treatment (Fig. 7A,B;

data not shown). In *Scgb1a1*-cre control animals, extensive regeneration of the airway epithelium was observed at both 10 and 20 days post-injury as judged by E-cadherin (cadherin 1) immunostaining (Fig. 6G,J-L). However, in *Foxp1/4*^{*Scgb1a1*creDKO} mutants, only a few airway epithelial cells were observed in the bronchiolar airways, indicating defective epithelial regeneration in the absence of Foxp1/4 expression (Fig. 6H,M-O).

To assess the lineage of the surviving and/or regenerated cells in the naphthalene-treated *Foxp1/4*^{*Scgb1a1*creDKO} mutants, *Scgb1a1* expression was measured by immunostaining and Q-PCR at 3 and 10 days after injury. At 3 days post-injury, very few *Scgb1a1* cells were present in either control or *Foxp1/4*^{*Scgb1a1*creDKO} mutants owing to naphthalene-induced depletion (Fig. 7A,B). By 20 days post-injury, *Scgb1a1* cells had readily regenerated in *Scgb1a1*-cre control animals whereas very few *Scgb1a1*-positive cells were observed in *Foxp1/4*^{*Scgb1a1*creDKO} mutants (Fig. 7C,D). This lack of regeneration of *Scgb1a1*-positive cells is supported by a dramatic decrease in *Scgb1a1* gene expression 10 days after injury in *Foxp1/4*^{*Scgb1a1*creDKO} mutants (Fig. 7E). There was also a decrease in the ciliated epithelial markers *Foxj1* and β -tubulin IV but no significant decrease in the neuroendocrine marker *Ascl1* (Fig.

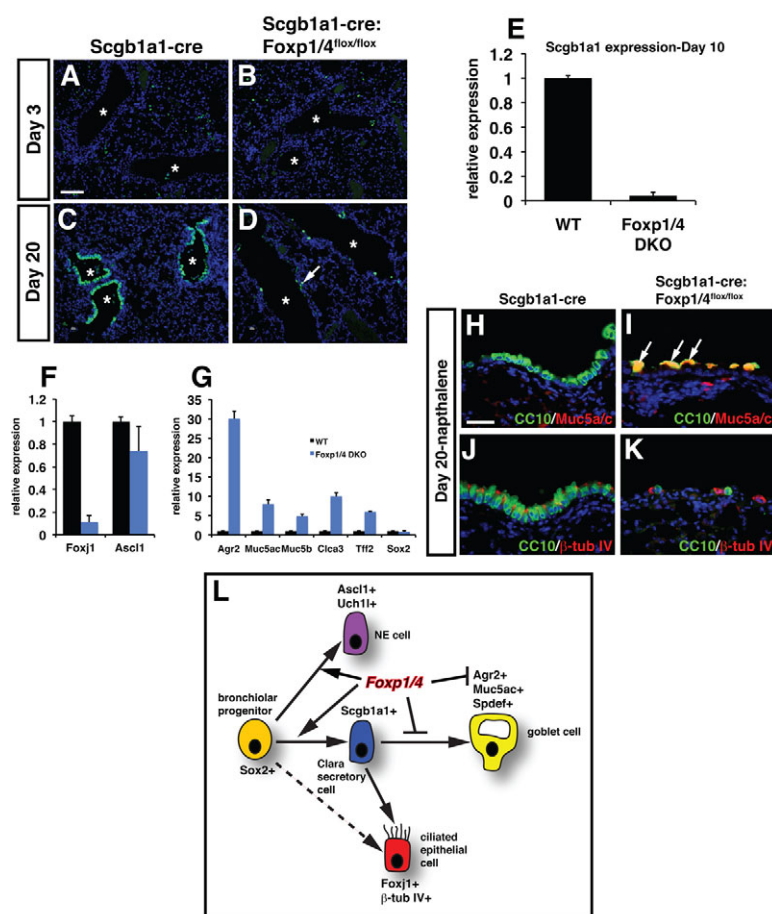


Fig. 7. Foxp1/4 are required for inhibition of the goblet cell lineage during secretory epithelial regeneration in the lung. (A–D) Scgb1a1-positive secretory cells are depleted at equal levels in Scgb1a1-cre and Foxp1/4^{Scgb1a1creDKO} mutants at 3 days post-injury (A,B). However, Foxp1/4^{Scgb1a1creDKO} mutants do not effectively regenerate Clara cell secretory epithelium after naphthalene injury as noted by Scgb1a1 immunostaining at 20 days post-injury (C,D). Asterisks denote airways. Arrow indicates the small number of Scgb1a1-positive cells in the Foxp1/4^{Scgb1a1creDKO} mutants. (E) Q-PCR for Scgb1a1 expression in control and Foxp1/4^{Scgb1a1creDKO} mutants at 10 days post-injury. (F) Q-PCR for Foxj1 and Ascl1 in control and Foxp1/4^{Scgb1a1creDKO} mutants at 10 days post-injury. (G) Goblet cell markers are expressed at high levels in Foxp1/4^{Scgb1a1creDKO} mutants 10 days post-naphthalene-induced injury, including high levels of Agr2. (H–K) The few epithelial cells in the proximal airways of Foxp1/4^{Scgb1a1creDKO} mutants are strongly positive for both Scgb1a1 and Muc5ac expression (H,I). There are only a few β -tubulin IV-positive ciliated cells in Foxp1/4^{Scgb1a1creDKO} mutants 10 days post-naphthalene-induced injury. Scgb1a1-cre animals were used as controls for these experiments. (L) Model of how Foxp1/4 act to restrict secretory cell lineage fate in the lung. Loss of Foxp1/4 leads initially to decreased secretory cell differentiation followed by ectopic activation of the goblet cell lineage program, which inhibits normal airway epithelial development and airway epithelial regeneration in the postnatal lung. Scale bars: 200 μ m for A–D; 100 μ m for H–K.

7F,J,K). Conversely, Agr2 expression was significantly increased relative to other goblet cell markers 10 days following naphthalene injury in Foxp1/4^{Scgb1a1creDKO} mutants (Fig. 7G). Immunostaining shows that the majority of the cells remaining after naphthalene airway injury in Foxp1/4^{Scgb1a1creDKO} mutants express Muc5ac, consistent with the acquisition of a goblet cell phenotype (Fig. 7G,H; supplementary material Fig. S2). This loss of secretory epithelial regeneration is not associated with changes in Cyp2f2 expression, absence of expression of which denotes a putative secretory epithelial progenitor capable of airway regeneration after naphthalene injury (supplementary material Fig. S3) (Reynolds et al., 2000a; Li et al., 2011). Thus, loss of Foxp1/4 in postnatal Clara secretory cells inhibits airway epithelial regeneration by ectopically activating the goblet cell fate. Together, these data indicate that Foxp1/4 are required for both development and maintenance of the secretory cell lineage but are also required to restrict the mature goblet cell lineage, in part, through direct repression of the protein

DISCUSSION

Lung diseases that involve dysfunctional development and regeneration of the pseudostratified proximal airway epithelium are a leading cause of morbidity and mortality. A hallmark of the response of the proximal airways to both injury and disease is an increase in the goblet cell lineage with a concomitant increase in mucin production and altered morphology of the pseudostratified epithelium. We show that Foxp1/4 play a central role in restricting secretory cell fate in the proximal airways of the lung by regulation of a novel pathway involving direct repression of the protein

disulfide isomerase Agr2. Ectopic expression of Agr2 alone can promote the goblet cell fate in the secretory epithelium of the lung. This result, along with previous findings that Agr2 is essential for intestinal goblet cell development (Zhao et al., 2010), suggests that Agr2 is a central player in defining goblet cell fate and function in the epithelium of multiple tissues including the lung and intestine. Given that increased goblet cell formation is associated with severe pulmonary diseases, such as asthma and COPD, our studies provide crucial insight into how secretory cell fate is established, maintained and regenerated in the lung, which may prove beneficial in developing future therapies for lung disease.

The mechanisms that control epithelial lineage fate, differentiation and regeneration are poorly understood. Within the proximal portion of the developing respiratory system, Sox2 is thought to act as an essential regulator of most, if not all, of the epithelial lineages. However, less is understood about the factors and pathways that direct specific epithelial lineage fate choices downstream of Sox2. Notch signaling has been shown to be essential for the fate choice between the secretory lineage and the ciliated lineage, both of which derive from Sox2+ progenitors (Que et al., 2007; Guseh et al., 2009; Tompkins et al., 2009; Tsao et al., 2009; Morimoto et al., 2010; Rock et al., 2011). The ETS transcription factor Spdef is sufficient and necessary for goblet cell differentiation in both gain- and loss-of-function experiments (Park et al., 2007; Chen et al., 2009). Whether other factors or pathways could regulate the balance of epithelial lineages by restricting certain cell fates was unclear. Our studies show that Foxp1/4 restrict the goblet cell fate through a novel pathway that appears to act either independently or does not appear to involve either

disruption in Notch signaling or a dramatic increase in Spdef. Foxp1/4 regulate the protein disulfide isomerase Agr2, which, when ectopically expressed, can promote the goblet cell fate.

Other forkhead factors have been implicated in regulating goblet cell formation. Loss of Foxa2 in the developing lung epithelia leads to goblet cell hyperplasia (Wan et al., 2004). Conversely, loss of Foxa1 and Foxa2 leads to decreased goblet cell formation in the developing intestine (Ye and Kaestner, 2009). The mechanism by which Foxa1/2 regulate goblet cell formation is likely to be very different to that of Foxp1/4, as Foxa1/2 are classic pioneer transcription factors responsible for opening compacted chromatin to allow for increased gene transcription (Cirillo et al., 2002). By contrast, Foxp1/4 are transcriptional repressors that interact with the NuRD chromatin-remodeling complexes and repress gene transcription through the action of histone deacetylases (Chokas et al., 2010). Thus, these two different subfamilies of forkhead transcription factors are not likely to act redundantly in the regulation of lung endoderm development or regeneration. In addition to defective airway epithelial development, *Foxp1/4^{ShhcreDKO}* mutants are likely to have additional defects in lung development, possibly in alveolar epithelial differentiation as they die prenatally owing to respiratory distress (data not shown). Therefore, further analysis of *Foxp1/4^{ShhcreDKO}* mutants is likely to uncover additional roles for Foxp1/4 in alveolar epithelial development.

We observed that de-repression of Agr2 expression closely correlated with loss of Foxp1/4 expression, both developmentally and in the postnatal Clara secretory cell lineage. Accordingly, ectopic expression of Agr2 can induce many characteristics of the goblet cell lineage, including increased expression of multiple mucin genes as well as Clca3, a chloride channel expressed in goblet cells (Zhou et al., 2002). However, the mechanisms by which increased levels of Agr2 lead to promotion of the goblet cell fate in the lung is unclear. Agr2 has been shown to bind mucin proteins directly and by managing their conformation as they traffick through the endoplasmic reticulum (ER), Agr2 might increase their stability and expression (Zhao et al., 2010; Higa et al., 2011; Niederreiter and Kaser, 2011). Expression of Agr2 is also induced by ER stress and is highly enhanced in multiple carcinomas, leading to stimulation of proliferation (Liu et al., 2005; Zweitzig et al., 2007; Ramachandran et al., 2008; Barraclough et al., 2009; Edgell et al., 2010; Maresh et al., 2010; Bu et al., 2011). More relevant in light of our results are previous findings showing that Agr2 can alter cell fates by inducing ectopic cement gland differentiation and anterior neural fate in *Xenopus laevis* (Aberger et al., 1998). Additional studies will be required to understand better how Agr2 controls cell fate decisions in lung epithelial cells.

Several human lung diseases exhibit increased levels of goblet cell differentiation. However, goblet cells are not apparent in the developing mouse lung and only develop in adult animals upon subjection to stressors, such as airway injury or asthma disease models induced with allergens (Fahy, 2001; Rogers, 2003; Izuhara et al., 2009). As previous studies have demonstrated that Foxp1/4 act as transcriptional repressors, our finding that loss of Foxp1/4 lead to ectopic activation of the goblet cell program and that this can be recapitulated by ectopic expression of Agr2 reveals that the developing secretory epithelium is under restrictive constraints to regulate cell fate decisions. This function of Foxp1/4 is independent of Notch signaling, which is known to regulate the secretory cell fate in the lung. Notch signaling also plays important roles in the developing and regenerating airway epithelium. In the developing lung, Notch signaling is essential for development of

the secretory cell lineage (Guseh et al., 2009; Tsao et al., 2009; Morimoto et al., 2010). In the postnatal lung, loss of Notch signaling leads to decreased Clara cell differentiation and increased goblet cell differentiation (Rock et al., 2011). Our findings show that Foxp1/4 promote initial development of the secretory epithelial cell fate and restrain the goblet cell fate in both the developing and postnatal secretory epithelium.

The secretory epithelium is the site of several debilitating human diseases including asthma and COPD. In these diseases, goblet cell formation is increased, which leads to excess mucus production and further degradation of airway function. The lack of airway epithelial regeneration in *Foxp1/4^{ScgblalcreDKO}* mutants demonstrates that the ectopic activation of the goblet cell fate leads to a failure of these cells to regenerate in response to airway injury. This would lead to decreased airway repair, exposure of the underlying basal lamina and promotion of an inflammatory response commonly observed in airway diseases such as asthma. Thus, Foxp1/4 are essential for generating the proper balance of epithelial lineages in the proximal airways, which is required for the response to injury and inhibition of diseases of the airway.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.079699/-DC1>

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