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Foxm1 Transcription Factor Is Critical for Proliferation and Differentiation of Clara Cells during Development of Conducting Airways

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SUMMARY

Respiratory epithelial cells are derived from cell progenitors in the foregut endoderm that subsequently differentiate into the distinct cell types lining the conducting and alveolar regions of the lung. To identify transcriptional mechanisms regulating differentiation and maintenance of respiratory epithelial cells, we conditionally deleted Foxm1 transcription factor from the conducting airways of the developing mouse lung. Conditional deletion of Foxm1 from Clara cells, controlled by the Scgb1a1 promoter, dramatically altered airway structure and caused peribronchial fibrosis, resulting in airway hyperreactivity in adult mice. Deletion of Foxm1 inhibited proliferation of Clara cells and disrupted the normal patterning of epithelial cell differentiation in the bronchioles, causing squamous and goblet cell metaplasia, and the loss of Clara and ciliated cells. Surprisingly, conducting airways of Foxm1-deficient mice contained highly differentiated cuboidal type II epithelial cells that are normally restricted to the alveoli. Lineage tracing studies showed that the ectopic alveolar type II cells in Foxm1-deficient airways were derived from Clara cells. Deletion of Foxm1 inhibited Sox2 and Scgb1a1, both of which are critical for differentiation and function of Clara cells. In co-transfection experiments, Foxm1 directly bound to and induced transcriptional activity of *Scgb1a1* and *Sox2* promoters. Foxm1 is required for differentiation and maintenance of epithelial cells lining conducting airways.

Keywords

Foxm1; Clara cells; airway epithelium; type II cells; Sox2; airway development

INTRODUCTION

The respiratory epithelium is derived from progenitor cells from the foregut endoderm. Respiratory tubules subsequently undergo branching morphogenesis, and epithelial cells

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differentiate into the multiple specific cell types that line the conducting airways and alveoli (Cardoso, 2001; Hogan and Yingling, 1998; Morrisey and Hogan, 2010; Warburton et al., 1999; Whitsett et al., 2004). Depending on species, composition of conducting airways as compared to alveolar regions of the lung are firmly established in the perinatal and early postnatal period of development. The respiratory epithelium remains proliferative until maturity as progenitor cells produce the complex pseudostratified epithelium lining cartilagenous airways and the simple columnar epithelium lining the bronchioles. While trachea and bronchi contain ciliated, basal, goblet, and secretory or Clara cells, pulmonary bronchioles are lined primarily by ciliated and Clara cells. Alveolar regions of the lung are lined by type II and type I epithelial cells. During lung morphogenesis and after lung injury, Clara cells and basal cells serve as airway progenitor cells that proliferate and re-differentiate to produce multiple airway epithelial cell types (Li et al., 2008; Morrisey and Hogan, 2010; Perl et al., 2005; Rawlins et al., 2009; Rock et al., 2011; Rock et al., 2009). Abnormalities in airway epithelial cell differentiation are associated with various lung disorders, including chronic obstructive pulmonary diseases, asthma, cystic fibrosis, interstitial lung disease and lung cancer (Warburton et al., 2010; Whitsett et al., 2004). Signaling pathways and transcriptional networks that control epithelial cell differentiation and the proximal/peripheral patterning of the developing lung have been increasingly studied. Signaling via FGF, Wnt, Bmp and Notch, and the activity of transcription factors of multiple families including SOX, FOX, NKX, ETS, RAR, and KLF families, all play important roles in the differentiation of the respiratory epithelium (Cardoso, 2001; Costa et al., 2001; Kalinichenko et al., 2001; Kim et al., 2005b; Morrisey and Hogan, 2010; Warburton et al., 1999; Whitsett et al., 2004). Lineage-tracing studies demonstrated that respiratory epithelial cell lineages specific to proximal (airway epithelial) as compared to distal (alveolar epithelial) cell fate are established relatively early in lung development, occurring well before the formation of definitive lung buds (E6.5–E8.5) (Perl et al., 2002). While factors controlling the ability of airway Clara cells to make and retain cell fate decisions later in development and during repair have not been fully elucidated, Clara cells retain the ability to be re-programmed to other airway epithelial cell fates even after differentiation. Recent studies demonstrated a critical role of SOX2 and SPDEF that are required for Clara/ ciliated and Clara/ goblet cell differentiation, respectively (Chen et al., 2009; Que et al., 2009; Tompkins et al., 2009). Whether airway Clara cells retain the ability to differentiate into alveolar type I and type II cells remains unknown.

The FOX family of proteins plays important roles in lung morphogenesis and respiratory epithelial cell differentiation (Costa et al., 2001; Kalin et al., 2011; Maeda et al., 2007; Morrisey and Hogan, 2010). Foxa1 and Foxa2 are expressed throughout the respiratory epithelium (Besnard et al., 2004) and have partially redundant functions during early lung formation being required for normal branching morphogenesis and epithelial cell differentiation (Maeda et al., 2007; Morrisey and Hogan, 2010; Wan et al., 2004). Later in development, expression of other Fox proteins become more restricted, Foxj1 playing a critical role in ciliagenesis (Brody et al., 2000) and Foxa3 marking a subset of goblet cells in conducting airways (Chen et al., 2009). Foxp2 is required for postnatal lung alveolarization (Shu et al., 2007). Mice haploinsufficient for Foxf1 exhibited lung hypoplasia, defects in peribronchial smooth muscle and alveolar capillaries, and increased mortality in the early neonatal period (Kalinichenko et al., 2004a; Kalinichenko et al., 2001; Kalinichenko et al., 2002). Genomic mutations in *FoxF1* gene locus were recently found in 30% of human patients with Alveolar Capillary Dysplasia (ACD), a congenital lethal lung disease (Stankiewicz et al., 2009).

Foxm1 transcription factor (previously known as HFH-11B, Trident, Win, or MPP2) is expressed in various tissues during embryogenesis, but is restricted to intestinal crypts, thymus, testes, regenerating tissues and malignancies in adult tissues (Kalin et al., 2011;

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Korver et al., 1997; Ye et al., 1997). *Foxm1*$^{-/-}$ mouse embryos exhibited embryonic lethal phenotype between E13.5 and E16.5 due to multiple abnormalities in various organ systems, including liver, lungs, blood vessels, and heart (Bolte et al., 2011; Kim et al., 2005b; Krupczak-Hollis et al., 2004; Ramakrishna et al., 2007). Abnormal accumulation of polyploid cardiomyocytes and hepatoblasts was found in *Foxm1*$^{-/-}$ embryos, implicating Foxm1 in the cell cycle regulation (Krupczak-Hollis et al., 2004; Ramakrishna et al., 2007). Majority of mice with smooth muscle-specific Foxm1 deletion (*smMHC-Cre Foxm1*$^{fl/fl}$) died immediately after birth due to severe pulmonary hemorrhage, structural defects in arterial wall, and esophageal abnormalities (Ustiyan et al., 2009). Deletion of Foxm1 from alveolar epithelium (*SPC-rtTA/ TetO-Cre Foxm1*$^{fl/fl}$) caused respiratory failure after birth due to impaired lung maturation, delayed differentiation of type I and type II alveolar epithelial cells and decreased expression of surfactant-associated proteins SP-B and SP-C (Kalin et al., 2008). Deletion of Foxm1 from alveolar type II cells impaired alveolar barrier repair after injury (Liu et al., 2011) and inhibited chemically-induced lung carcinogenesis (Wang et al., 2009). Although these studies demonstrated that Foxm1 is a critical transcriptional regulator of alveolar epithelial cells, the role of Foxm1 in the airway epithelium has not been identified.

MATERIALS AND METHODS

Mice

Animal studies were reviewed and approved by the Animal Care and Use Committee of Cincinnati Children's Hospital Research Foundation. Generation of *Foxm1*$^{flox/flox}$ (*Foxm1*$^{fl/fl}$) mouse line, which contains LoxP sequences flanking DNA binding and transcriptional activation domains of the *Foxm1* gene (exons 4–7), was previously described (Krupczak-Hollis et al., 2004). The *Foxm1*$^{fl/fl}$ mice were bred with *CCSP–rtTA*$^{tg/-}$/ *TetO-Cre*$^{tg/tg}$ mice (line 2, (Chen et al., 2009)) to generate *CCSP–rtTA*$^{tg/-}$/*TetO-Cre*$^{tg/-}$/*Foxm1*$^{fl/fl}$ mice. Doxycycline (625 mg/kg; Harlan Teklad, Madison, WI) was administered to the dams in the food starting at E16.5. Dox treatment continued until P5 or P30, causing conditional deletion of Foxm1 in Clara cells and their derivatives herein termed *CCSP-Foxm1*$^{-/-}$ mice. Less than 0.5% of distal epithelial cells expressed Cre protein at either P5 or P30. In separate experiments, Dox was given to adult (6–8 weeks old) mice for either 2 weeks or 5 months. *Foxm1*$^{fl/fl}$ littermates lacking either the *CCSP–rtTA*, the *TetO-Cre* or both transgenes were used as controls. Further controls included Dox-treated *CCSP–rtTA*$^{tg/-}$/ *TetO-Cre*$^{tg/-}$/*Foxm1*$^{wt/wt}$ mice as well as *CCSP–rtTA*$^{tg/-}$/*TetO-Cre*$^{tg/-}$/*Foxm1*$^{fl/fl}$ mice without Dox treatment. Airway structure and function in control mice were normal. For lineage-tracing experiments, the mice were bred with R26R reporter mice (Rosa26-LoxP-stop-LoxP-β-galactosidase; Jackson Lab).

To induce lung injury, 6–8 week old *CCSP–rtTA*$^{tg/-}$/*TetO-Cre*$^{tg/-}$/*Foxm1*$^{fl/fl}$ and *Foxm1*$^{fl/fl}$ male mice were treated with naphthalene (Sigma-Aldrich, 275 mg/kg as a single intraperitoneal injection) as described (Kida et al., 2008). Dox was given for 7 days prior to lung injury. Mice were sacrificed at days 0, 5 and 14 after naphthalene administration.

Immunohistochemical staining

Embryos were harvested, fixed in 10% buffered formalin, and embedded into paraffin blocks. Five-μm sections were either stained with hematoxylin and eosin (H&E) for morphological examination or used for immunohistochemistry as described (Kalin et al., 2008). The following antibodies were used for immunostaining: Foxm1 (1:1000, K-19, sc500, Santa Cruz Biotechnology); Cre recombinase (1:15000, #69050-3, Novagen); CCSP (1:5000, T-18, sc9772, Santa Cruz Biotechnology and 1:2000, WRAB-CCSP, Seven Hill Bioreagents); Foxj1 (1:1000, WMAB-319, Seven Hill Bioreagents); Ki-67 (1:500, clone

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Tec-3, Dako); PH3 (1:500, sc8656r, Santa Cruz Biotechnology); proSP-C (1:2000, (Kalin et al., 2008)); mature SP-C (1:1000, WRAB-MSPC, Seven Hill Bioreagents); mature SP-B (1:1500, generated in lab of J. A. Whitsett (Kalin et al., 2008)); ABCA3 (1:500, sc5361, Santa Cruz Biotechnology); T1α (1:500, DSHB 8.1.1., University of Iowa Hybridoma bank); TTF-1 (1:2000, WRAB-TTF1, Seven Hill Bioreagents); Foxa2 (1:4000, WRAB-FoxA2, Seven Hills Bioreagents); Foxa3 (1:200, sc5361, Santa Cruz Biotechnology); β-tubulin (1:100, MU178-UC, BioGenex); SPDEF (1:2000, generated in lab of J. A. Whitsett (Chen et al., 2009)); α-Smooth muscle actin (αSMA, 1:10000, clone A5228, Sigma); β-galactosidase (1:1000, ab9361, Abcam); Mucin5AC (1:100, 45M1, ab3649, Abcam); Sox2 (1:2500, generated in lab of J. A. Whitsett (Tompkins et al., 2009)); p63 (1:500, sc71827, Santa Cruz Biotechnology); Cytokeratin 5 (1:3000, generated in lab of J. A. Whitsett); pan-Cytokeratin (1:500, C1801, Sigma); Scgb3a1 (1:1000, sc49566, Santa Cruz Biotechnology); β-catenin (1:500, sc7199, Santa Cruz Biotechnology); E-cadherin (1:100, sc1499, Santa Cruz Biotechnology). Antibody-antigen complexes were detected using biotinylated secondary antibody followed by avidin-biotin-horseradish peroxidase complex (ABC), and DAB substrate (all from Vector Lab). Sections were counterstained with nuclear fast red (Vector Labs, Burlingame, CA).

For co-localization experiments, secondary antibodies conjugated with alexaFluor 488 or alexaFluor 594 (Invitrogen/ Molecular probes) were used as previously described (Ustiyan et al., 2009; Wang et al., 2010). Slides were counterstained with DAPI (Vector Lab). Fluorescent images were obtained using a Zeiss Axioplan2 microscope equipped with an AxioCam MRm digital camera and AxioVision 4.3 Software (Carl Zeiss Microimaging, Thornwood, NY).

Transmission Electron Microscopy

For ultrastructural analysis, P30 lungs from Dox-treated *CCSP-Foxm1*$^{-/-}$ mice and littermate controls were fixed in modified Karnovsky's fixative (2% glutaraldehyde, 2% paraformaldehyde and 0.1% calcium chloride in 0.1 M sodium cacodylate buffer; pH 7.3). The tissue was postfixed in 1% osmium tetroxide, stained with 4% aqueous uranyl acetate (UA), dehydrated, and embedded in epoxy resin (EMbed 812; Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were post-stained with 2% UA and saturated lead citrate and viewed in a Hitachi H-7600 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan). Digitized images were collected with an AMT Advantage Plus 2K × 2K digital camera (Advanced Microscopy Techniques, Danvers, MA).

Lung mechanics and measurements of saturated PC

Lung mechanics was assessed on tracheostomized 8-week old *CCSP-Foxm1*$^{-/-}$ and control mice (5 mice for each group) using a computerized Flexi Vent system (SCIREQ, Montreal, Canada) as described (Xu et al., 2009). Saturated phosphatidylcholine (Sat PC) was isolated from bronchoalveolar lavage fluid (BALF) or lung tissue homogenates using osmium tetroxide followed by measurement of phosphorus as described (Xu et al., 2009).

Quantitative real-time RT-PCR (qRT-PCR)

Lungs from *CCSP-Foxm1*$^{-/-}$ or control *Foxm1*$^{fl/fl}$ mice were used to prepare total RNA and analyze gene expression by qRT-PCR. StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) was used as described (Kalin et al., 2008). Samples were amplified with TaqMan Gene Expression Master Mix (Applied Biosystems) combined with inventoried TaqMan gene expression assays for the gene of interest (Suppl. Table 1). Reactions were analyzed in triplicates. Expression levels were normalized to β-actin mRNA from the same samples.

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Cotransfection studies

U2OS cells were transfected with either CMV-FoxM1b or control CMV-empty plasmids, as well as with luciferase (LUC) reporters driven by either −2.3 kb mouse *CCSP* promoter *(Scgb1a1)* or −5.7 kb mouse *Sox2* promoter. CMV-Renilla was used as an internal control to normalize transfection efficiency. Dual luciferase assay (Promega) was performed as described (Kalin et al., 2008).

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed using *in situ* cross-linked human bronchial epithelial BEAS-2B cells as described (Kalin et al., 2008; Malin et al., 2007; Wang et al., 2005). Antibodies used for ChIP: rabbit anti-Foxm1 Ab#1 (H-300, Santa Cruz), rabbit anti-Foxm1 Ab#2 (C-20, Santa Cruz) and control rabbit IgG (Vector Lab). The following sense (S) and antisense (AS) PCR primers were used to amplify promoter DNA fragments in ChIP assay: human *Scgb1a1* (S) 5'-AATTTTATTCTTTA-3' and (AS) 5'-ATGTTGAATAAAA-3'; human *Sox2* (S) 5'-AAAAAAATATGATTA-3' and (AS) 5'-TTAATTTTTATAA-3'.

Statistical analysis

Student's T-test was used to determine statistical significance. P values 0.05 were considered significant. Values for all measurements were expressed as the mean ± standard deviation (SD).

RESULTS

Conditional deletion of Foxm1 from Clara cells

Previous studies demonstrated that expression of Foxm1 transcription factor is transiently increased in the respiratory epithelium during the first week after birth, during which its expression is associated with increased proliferation of airway epithelial cells (Wang et al., 2010). To determine whether Foxm1 is critical for proliferation of airway epithelial cells, transgenic mice were produced containing *Scgb1a1(CCSP)–rtTA*$^{tg/-}$ and *TetO–Cre*$^{tg/-}$ transgenes, as well as the *Foxm1*$^{fl/fl}$ targeted allele (Krupczak-Hollis et al., 2004) in which loxP sites were inserted into exons 4–7 of the *Foxm1* gene (*Scgb1a1–rtTA*$^{tg/-}$/ *TetO–Cre*$^{tg/-}$/ *Foxm1*$^{fl/fl}$ or *CCSP-Foxm1*$^{fl/fl}$ mice). Scgb1a1 mouse (line 2) selectively expresses the reverse tetracycline transactivator (rtTA) in the majority of Clara cells without targeting other cell types in the lung (Chen et al., 2009). In the presence of doxycycline (Dox), rtTA binds to the TetO promoter and induces expression of Cre recombinase, deleting the DNA binding and transcriptional activation domains of the FOXM1 protein (Suppl. Fig. 1A) and causing selective deletion of *Foxm1* from airway Clara cells (*CCSP-Foxm1*$^{fl/fl}$ + Dox herein termed *CCSP-Foxm1*$^{-/-}$ mice).

Reduced proliferation of Clara cells in *CCSP-Foxm1*$^{-/-}$ mice

To address the efficiency of Foxm1 deletion and examine proliferation of Clara cells during the postnatal period, *CCSP-Foxm1*$^{-/-}$ and control mice (*Foxm1*$^{fl/fl}$ and TetO-Cre *Foxm1*$^{fl/fl}$) were given Dox-loaded food at E16.5 (after this Dox regimen, the first Cre-expressing cells are found between E18.5 and postnatal day 1 (P1)). Mice were harvested at P5 to assess the Foxm1 deletion. Foxm1 was readily detected in most airway epithelial cells of control mice (Fig. 1B). Consistent with previous studies (Chen et al., 2009), Cre was detected in the nuclei of airway epithelial cells of *CCSP-Foxm1*$^{-/-}$ mice after treatment with Dox, and was absent from the bronchiolar epithelium of control mice (Fig. 1A). Total numbers of Foxm1-positive epithelial cells were dramatically decreased in *CCSP-Foxm1*$^{-/-}$ airways (Fig. 1B and 1E), consistent with efficient deletion of Foxm1 by the Cre recombinase. Immunostaining for proliferation-specific Ki-67 antigen and phosphorylated histone H3

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(PH3) was reduced (Fig. 1C–D), and total numbers of epithelial cells undergoing the cell cycle were significantly decreased in *CCSP-Foxm1*$^{-/-}$ airways compared to the bronchiolar epithelium of control mice (Fig. 1F–G). Percentages of proliferative Clara cells were significantly decreased as demonstrated by co-localization experiments between Ki-67 and CCSP (Fig. 1I–J). The number of proliferative cells in distal lung saccules was unchanged (Fig. 1H). Thus, Foxm1 deletion reduced proliferation of airway Clara cells during the early postnatal period of lung development.

Deletion of Foxm1 decreased numbers of epithelial cells and caused squamous metaplasia

Previous studies demonstrated that Clara cells are important progenitor cells in conducting airways, being capable of cell renewal and differentiation into other airway epithelial cell types (Rawlins et al., 2009). Effects of Foxm1 deletion on differentiation of airway epithelial cells were assessed in *CCSP-Foxm1*$^{-/-}$ mice treated with Dox from E16.5 to P30. At this age, lung development is completed and airway structure firmly established. Body weight was similar in *CCSP-Foxm1*$^{-/-}$ and control mice (Suppl. Fig. 1C). Lung mechanics, including airway elastance, pulmonary compliance and tissue damping were not altered (Suppl. Fig. 1C). While deletion of *Foxm1* from Clara cells did not influence alveolar structure, the morphology of airway epithelium was dramatically altered (Fig. 2A). Extensive regions of the bronchioles lacked the normal cuboidal epithelium seen in control mice (Fig. 2A and Suppl. Fig. 1B). Bronchioles in *CCSP-Foxm1*$^{-/-}$ lungs were lined by regions of squamous and cuboidal cells and often contained cells with enlarged nuclei (Suppl. Fig. 1B).

Clara cells that persisted in the *CCSP-Foxm1*$^{-/-}$ mice expressed Cre, whereas Cre was not detected in control mice (Suppl. Fig. 2A–B). Bronchiolar epithelial cells of *CCSP-Foxm1*$^{-/-}$ mice expressed epithelial cytokeratins and epithelial-specific transcription factors TTF-1 and FOXA2 (Suppl. Fig. 2C–E). However, the number of epithelial cells lining the bronchioles was dramatically reduced (Suppl. Fig. 2C–E), a result consistent with reduced proliferation of Clara cells during the early postnatal period (Fig. 1). Expression of SOX2, a transcription factor critical for proliferation and maintenance of airway epithelium (Que et al., 2009; Tompkins et al., 2009), was reduced in *CCSP-Foxm1*$^{-/-}$ bronchioles (Suppl. Fig. 2F). Foxm1-deletion from Clara cells did not influence the morphology of the distal lung region. The number of alveolar type II and type I cells was similar in distal lung regions of *CCSP-Foxm1*$^{-/-}$ and control lungs (Suppl. Fig. 2G–I). Decreased staining for FOXJ1 and CCSP was consistent with decreased numbers of ciliated and Clara cells in *CCSP-Foxm1*$^{-/-}$ bronchioles (Fig. 2B–C and 2G–H). These results indicate that Foxm1 is required for proliferation of Clara cells and normal differentiation of the airway epithelium during the late embryonic/ postnatal period of lung development.

Increased numbers of goblet cells and airway remodeling in *CCSP-Foxm1*$^{-/-}$ lungs

While goblet cells were not detected in pulmonary bronchioles of control mice at P30, deletion of Foxm1 dramatically increased numbers of goblet cells as indicated by staining for Muc5AC (Fig. 2D) and Alcian blue (Suppl. Fig. 3A). Staining for SPDEF and FOXA3 transcription factors, associated with goblet cell differentiation (Chen et al., 2009), was increased in *CCSP-Foxm1*$^{-/-}$ mice (Suppl. Fig. 3B–C). Total numbers of goblet cells were increased in *CCSP-Foxm1*$^{-/-}$ airways (Fig. 2G–H). E-cadherin and β-catenin were present in epithelial junctions of control *Foxm1*$^{fl/fl}$ bronchioles at P30 (Suppl. Fig. 3E–F). Epithelial junctions were disrupted in Foxm1-deficient bronchiolar epithelium, causing abnormal localization of E-cadherin and β-catenin in basal membranes (Suppl. Fig. E–F). Increased thickness of stromal tissue and peribronchiolar fibrosis was observed in most of the *CCSP-Foxm1*$^{-/-}$ mice (Suppl. Fig. 1B). Consistent with this finding, airway resistance in *CCSP-*

Foxm1$^{-/-}$ mice was significantly induced (Suppl. Fig. 1C), and αSMA staining was increased (Suppl. Fig. 3D). Thus, loss of Foxm1 in Clara cells influences proper epithelial differentiation, causing squamous and goblet cell metaplasia, and airway remodeling.

Accumulation of alveolar type II cells in bronchioles after deletion of Foxm1

Bronchioles of *CCSP-Foxm1*$^{-/-}$ mice were lined by extensive regions of squamous cells expressing T1α (Fig. 2F) that are normally restricted to alveolar type I cells and a subset of basal cells in the proximal airway. A subset of bronchiolar epithelial cells in *CCSP-Foxm1*$^{-/-}$ mice expressed proSP-C, normally a cell-specific marker for alveolar type II cells (Fig. 2E). These ectopic type II cells in *CCSP-Foxm1*$^{-/-}$ bronchioles stained for mature SP-C, mature SP-B and ABCA3, consistent with differentiated features of normal alveolar type II cells (Fig. 3A–C). Neither T1α nor type II markers were present in bronchioles of control mice (Fig. 2E–F and 3A–C). Approximately 9% of epithelial cells lining the abnormal *CCSP-Foxm1*$^{-/-}$ bronchioles were type II cells, whereas squamous cells accounted for 12% of the bronchiolar epithelial cells (Fig. 2G–H). Numbers of cells lining bronchoalveolar duct junctions that stained for both proSP-C and CCSP were exceedingly rare and were not altered by deletion of Foxm1 (Suppl. Fig. 4).

In contrast to bronchiolar epithelium, no squamous or type II cells were found in the trachea of *CCSP-Foxm1*$^{-/-}$ mice after morphological examination or immunostaining with cell-specific markers (data not shown). Clara cell-specific markers, CCSP (Scgb1a1) and Scgb3a1, were reduced in *CCSP-Foxm1*$^{-/-}$ mice (Suppl. Fig. 5B and 5G), whereas the numbers and distribution of basal cells expressing p63 or CK5 were not changed (Suppl. Fig. 5C–E). Co-localization experiments were performed to identify cell types expressing Foxm1 in tracheal epithelium. In trachea of control *Foxm1*$^{fl/fl}$ mice, Foxm1 was detected in CCSP-positive Clara cells (Suppl. Fig. 5H) and p63-positive basal cells (Suppl. Fig. 5K), as well as in a rare subset (approximately 1%) of ciliated cells expressing β-tubulin (Suppl. Fig. 5J). Foxm1 was not found in tracheal goblet cells (Suppl. Fig. 5I). In *CCSP-Foxm1*$^{-/-}$ trachea, Foxm1 was present in basal and ciliated cells (Suppl. Fig. 5J–K), but was absent in a majority of Clara cells (Suppl. Fig. 5H), consistent with Clara cell-specific deletion of Foxm1 from tracheal epithelium. Thus, Foxm1 expression in Clara cells is required for development of bronchiolar (columnar) epithelium but dispensable for development of tracheal (pseudostratified) epithelium.

Ultrastructural abnormalities in the bronchioles of *CCSP-Foxm1*$^{-/-}$ mice

To examine the ultrastructure of *CCSP-Foxm1*$^{-/-}$ bronchioles, transmission electron microscopy (TEM) was performed at P30. Squamous epithelium was found in *CCSP-Foxm1*$^{-/-}$ but not in control bronchioles (Fig. 3D–E). Consistent with the presence of type II cell markers (Fig. 3A–C), ectopic type II cells were observed in *CCSP-Foxm1*$^{-/-}$ bronchioles (Fig. 3F–G). Ectopic type II cells contained lamellar bodies and apical microvilli, and were often located between ciliated and/ or Clara cells in the bronchioles of *CCSP-Foxm1*$^{-/-}$ mice (Fig. 3F–G). Type II cells were never found in the conducting airways of control mice (Fig. 3D). The ultrastructural features of ciliated cells were dramatically altered. Cells with cilia or Clara cells lacked normal columnar/cuboidal morphology and the number of cilia on the surface of ciliated cells was reduced (Suppl. Fig. 6). Abnormal Clara cells contained abundant characteristic mitochondria but lacked secretory granules (Suppl. Fig. 6D–F). The ultrastructure of basement membranes was unaltered, however, the thickness of stromal tissue in the bronchioles was markedly increased (Suppl. Fig. 6A–F), a finding consistent with light microscopic findings (Suppl. Fig. 1B), and the increased airway resistance (Suppl. Fig. 1C). Apoptotic cells were not detected in *CCSP-Foxm1*$^{-/-}$ epithelium by either TEM or immunostaining for activated caspase 3 (Suppl. Fig. 6 and data not shown).

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Foxm1 is critical for maintenance of bronchiolar epithelium and airway structure in the adult lung

Role of Foxm1 in adult airway epithelium was examined in *CCSP-Foxm1*$^{-/-}$ mice treated with Dox for 5 months. Foxm1 deletion caused extensive peribronchial fibrosis and enlargement of peripheral respiratory airspaces (Fig. 4A–C). CCSP expression and numbers of Clara cells were dramatically reduced by the Foxm1 deletion (Fig. 4E). SOX2 immunostaining was decreased (Fig. 4F), a finding consistent with diminished SOX2 expression in Foxm1-deficient lungs at P30 (Suppl. Fig. 2F). Ectopic squamous and type II cells were detected in the bronchioles of the *CCSP-Foxm1*$^{-/-}$ mice by immunostaining for T1α, proSP-C and mature SP-C (Fig. 4G–I). Thus, deletion of Foxm1 from the adult lung, induced peribronchial fibrosis, decreased the number of Clara cells and caused abnormal accumulation of squamous and type II cells in the bronchiolar epithelium. A decrease in Clara cells in Foxm1-deficient airways suggests a reduction in Clara cell proliferation. To directly test whether Foxm1 induces proliferation of Clara cells in the adult lung, 6–8 weeks old *CCSP-Foxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice were treated with Dox for 7 days followed by naphthalene administration. This short Dox treatment was insufficient to disrupt airway epithelium prior to naphthalene-induced injury (Fig. 5A–D and data not shown). After injury, proliferation of Clara cells was reduced in *CCSP-Foxm1*$^{-/-}$ mice compared to controls (Fig. 5F and 5J). An increase in the number of epithelial cells expressing proSP-C and mature SP-C were found in bronchioles of *CCSP-Foxm1*$^{-/-}$ mice at day 5 (Fig. 5G–H) and day 14 after the injury (Fig. 5K–L). These results are consistent with a critical role for Foxm1 in proliferation and differentiation of Clara cells in the adult lung.

Gene expression profile in *CCSP-Foxm1*$^{-/-}$ lungs

Expression of genes critical for respiratory epithelium was examined in total RNA from P30 lungs by quantitative real-time RT-PCR (qRT-PCR). Consistent with increased numbers of goblet cells, increased mRNA levels of *Foxa3* transcription factor were found in *CCSP-Foxm1*$^{-/-}$ lungs, while *Muc5AC* mRNA was not changed (Fig. 6A). While expression of Clara cell transcription factor Elf3 was increased in *CCSP-Foxm1*$^{-/-}$ lungs, no changes were found in *Ttf1, Foxa2, Spdef* and *Klf5* (Fig. 6A), all of which are important transcriptional regulators of gene expression in Clara cells (Chen et al., 2009; Morrisey and Hogan, 2010; Wan et al., 2008; Wan et al., 2004). *SP-C, SP-B* and *Abca3* mRNAs were increased in *CCSP-Foxm1*$^{-/-}$ lungs (Fig. 6A), consistent with the increased numbers of mature type II cells in the bronchioles of *CCSP-Foxm1*$^{-/-}$ mice (Fig. 3). In agreement with these data, increased concentration of saturated phosphatidylcholine, a main phospholipid of pulmonary surfactant, was found in bronchoalveolar lavage fluid (BALF) and lung tissue of *CCSP-Foxm1*$^{-/-}$ mice (Suppl. Fig 1C). There were no significant changes in total or differential counts of inflammatory cells in BALF (Suppl. Fig. 7A–B). Pulmonary inflammation was not observed in *CCSP-Foxm1*$^{-/-}$ lungs by morphological examination and TEM (data not shown).

Sox2 and CCSP (Scgb1a1) play important roles in differentiation and function of Clara cells (Que et al., 2009; Stripp et al., 2000; Tompkins et al., 2009). *CCSP* and *Sox2* mRNAs were significantly decreased in *CCSP-Foxm1*$^{-/-}$ lungs (Fig. 6A). Decreased staining for CCSP and SOX2 was also observed in *CCSP-Foxm1*$^{-/-}$ lungs at P5 (Fig. 6C), consistent with reduced expression of these genes at P30 (Fig. 2B and Suppl. Fig. 2F) and at 5 months of age (Fig. 4E–F). Since promoter regions of mouse *Scgb1a1* and *Sox2* genes contain potential Foxm1-binding sites (Fig. 6B), co-transfection experiments were performed to assess transcriptional regulation of these promoters by Foxm1. CMV-FoxM1b expression vector increased the activity of the −2.3 Kb *Scgb1a1* and the −5.7 Kb *Sox2* luciferase reporter plasmids (Fig. 6D). Chromatin Immunoprecipitation (ChIP) assay demonstrated that endogenous Foxm1 protein specifically binds to the promoter regions of *Scgb1a1* and *Sox2*

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genes in cultured bronchial epithelial BEAS-2B cells (Fig. 6E–F). Thus, Foxm1 can function as a transcriptional activator of *Scgb1a1* and *Sox2*.

Ectopic bronchiolar type II cells are derived from Clara cells

Lineage-tracing experiments were performed to determine whether ectopic type II cells in *CCSP-Foxm1*$^{-/-}$ airways originated from Clara cell lineage. To permanently label CCSP-expressing cells, *CCSP-rtTA/ TetO-Cre/ Foxm1*$^{fl/fl}$ triple-transgenic mice (*CCSP-Foxm1*$^{fl/fl}$) were bred with Rosa26-loxP-STOP-loxP-β-galactosidase reporter mice (*Rosa26R*) to generate *CCSP-Foxm1*$^{-/-}$/*Rosa26R* quadruple-transgenic mice. Mice were provided Dox from E16.5 until P30, simultaneously inducing Cre-mediated excisions of the Foxm1-floxed allele and the LoxP-STOP-LoxP cassette from the *Rosa26* locus. Thus, cells expressing Cre during this period were permanently labeled by β-galactosidase (β-gal). While β-gal was not detected in control lungs, cytoplasmic β-gal labeling was observed in CCSP-positive Clara cells of *CCSP-Foxm1*$^{-/-}$/*Rosa26R* bronchioles (Fig. 7A). The majority of ciliated and goblet cells lining *CCSP-Foxm1*$^{-/-}$/*Rosa26R* airways stained for β-gal (Fig. 7B–C), consistent with previous reports demonstrating that both ciliated and goblet cells differentiate from Clara cells (Chen et al., 2009; Rawlins et al., 2009). Neither squamous epithelial cells nor peribronchial smooth muscle cells in bronchioles of *CCSP-Foxm1*$^{-/-}$/*Rosa26R* mice stained for β-gal (7E–F). ProSP-C and β-gal were co-localized in ectopic type II cells (Fig. 7D). Likewise, β-gal staining was co-localized with ABCA3, mature SP-B and mature SP-C in bronchioles of *CCSP-Foxm1*$^{-/-}$/*Rosa26R* mice (Fig. 8A–C). All ectopic type II cells in *CCSP-Foxm1*$^{-/-}$/*Rosa26R* bronchioles expressed β-gal reporter (n=4 mice). Thus, ectopic type II cells were directly derived from CCSP-expressing cells after deletion of Foxm1, indicating that airway Clara cells are capable of differentiation into alveolar type II cells. Foxm1 is required for maintenance of normal bronchiolar cell differentiation and to restrict differentiation of the type II cells from conducting airway epithelial cells.

DISCUSSION

Foxm1 is critical for differentiation of Clara cells

The important contribution of present study is that Foxm1 regulates progenitor properties of Clara cells in the conducting airways. Previous studies demonstrated that secretory (Clara) cells in peripheral airways and basal cells in cartilaginous airways serve as epithelial progenitors that self-renew and give a rise to various types of airway epithelial cells, including ciliated, goblet and Clara cells (Rawlins et al., 2009; Rock et al., 2011; Rock et al., 2009). In the alveolar region, type II epithelial cells have been traditionally viewed as a progenitor cell from which type I and type II cells are derived following lung injury (Adamson and Bowden, 1974). While lineage specification of epithelial progenitors toward either conducting airway or alveolar epithelial cells occurs very early in lung development (Morrisey and Hogan, 2010; Perl et al., 2002), it is unknown whether airway Clara cells retain the ability to re-program and differentiate into alveolar type II cells in postnatal and adult lungs. In present study, proSP-C mRNA and protein were found in *CCSP-Foxm1*$^{-/-}$ bronchioles. Although proSP-C-positive cells were detected in airways of mice expressing activated β-catenin (Mucenski et al., 2005; Reynolds et al., 2008), highly differentiated type II cells were never found in conducting airways. The present ultrastructural and gene expression profiling data derived from the *CCSP-Foxm1*$^{-/-}$ bronchioles were consistent with a fully differentiated phenotype of the type II cells. These include the presence of cytoplasmic lamellar bodies and expression of ABCA3, secretion of mature (fully processed) SP-B and SP-C proteins, as well as increased SatPC in BALF. Lineage-tracing studies demonstrated that the ectopic type II cells identified in the present study were directly derived from cells in which the Scgb1a1 promoter is selectively active. Our findings are surprising and support the concept that Scgb1a1-expressing cells can acquire structural

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and functional characteristics of alveolar type II cells in the developing airways. Given the robust expression of Foxm1 during the late embryonic/postnatal period, Foxm1 expression in Clara cell progenitors may be required to prevent their differentiation into alveolar type II cells.

Previous studies demonstrated that Foxm1 deletion from type II cells (SPC promoter) causes decreased expression of SPC and SPB (Kalin et al., 2008). Foxm1 directly binds to promoter regions of *SPC* and *SPB* (Kalin et al., 2008), implicating Foxm1 in transcriptional activation of these genes in type II cells. In contrast to these studies, SPC and SPB mRNA and protein were increased in *CCSP-Foxm1*$^{-/-}$ lungs. One explanation for this discrepancy is that Foxm1 may function as a transcriptional activator of *SPC* and *SPB* in type II cells but repress these genes in Clara cells. Alternatively, it is possible that Foxm1 may induce expression of a Clara cell-specific transcriptional repressor(s), which directly inhibits SPC and SPB in Clara cells. In the absence of Foxm1, transcriptional repression of these genes is relieved, possibly contributing to increased SPB and SPC levels in a subset of airway Clara cells. Consistent with this hypothesis, Foxm1 was shown to differentially regulate many genes depending on tissue-specificity, developmental stage and biological context (reviewed in (Kalin et al., 2011)).

Foxm1 induces proliferation of Clara cells

In the present study, cell proliferation and decreased numbers of epithelial cells were observed in *CCSP-Foxm1*$^{-/-}$ airways during postnatal lung development and naphthalene-induced lung injury. In support of the role of Foxm1 in the regulation of cell proliferation, previous studies demonstrated that Foxm1 directly activates transcription of multiple cell cycle regulatory genes, such as *cyclin B1, Cdc25B, c-Myc, Plk-1 and Aurora B*, in cultured tumor cells and mouse embryonic fibroblasts (MEFs) (Costa et al., 2003; Costa et al., 2005; Laoukili et al., 2005; Wang et al., 2005). Expression of Foxm1 *in vivo* accelerated cellular proliferation during tissue regeneration/repair and tumor formation (Kalin et al., 2006; Kalinichenko et al., 2003; Kalinichenko et al., 2004b; Wang et al., 2008). However, no proliferative defects were found in the respiratory epithelium of either *Foxm1*$^{-/-}$ embryos (Kim et al., 2005b) or *SP-C-rtTA/TetO-Cre/Foxm1*$^{-/-}$ embryos (Kalin et al., 2008) at E15.5. Thus, Foxm1 is not required for proliferation of embryonic lung epithelium but is critical for proliferation of Clara cells after birth, findings consistent with increased proliferation of Clara cells and severe airway hyperplasia found in transgenic mice expressing activated FoxM1 mutant (Wang et al., 2010).

Foxm1 is critical for long-term maintenance of bronchiolar epithelium

In the present study, we found that long-term deletion of Foxm1 disrupted airway structure, caused peribronchial fibrosis and resulted in accumulation of ectopic type II cells in bronchioles of adult *CCSP-Foxm1*$^{-/-}$ mice. In contrast, the fibrosis and ectopic type II cells were not found two weeks after targeted deletion of Foxm1 in the airways (data not shown). Thus, Foxm1 is required for maintenance of airway epithelial differentiation and its loss did not cause rapid trans-differentiation of existing Clara cells into type II cells. These findings support the role of Foxm1 in self-renewal of airway epithelium. Increased airway resistance and peribronchial fibrosis seen in *CCSP-Foxm1*$^{-/-}$ mice are likely indirect consequences of airway epithelial defects caused by Foxm1 deletion. Since no significant changes in numbers of inflammatory cells were found in BALF of *CCSP-Foxm1*$^{-/-}$ mice, it is unlikely that pulmonary inflammation plays a role in the development of peribronchial fibrosis in *CCSP-Foxm1*$^{-/-}$ mice. Although our lineage-tracing studies ruled out EMT in Foxm1-deficient airway epithelial cells, Foxm1 deletion may influence epithelial homeostasis and/or cell-to-cell contacts in *CCSP-Foxm1*$^{-/-}$ airways, resulting in activation and proliferation of peribronchial fibroblasts and smooth muscle cells.

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Foxm1 deletion from Clara cells does not influence tracheal epithelium

Our lineage-tracing studies demonstrated that squamous cells expressing T1α in the bronchioles of Foxm1-deficient mice did not originate from Scgb1a1-expressing cells. Although the origin of squamous cells in *CCSP-Foxm1*$^{-/-}$ bronchioles remains unclear, previous studies demonstrated that a rare population of cells in bronchio-alveolar duct junctions (BASCs) can differentiate toward alveolar epithelial lineages under experimental conditions (Kim et al., 2005a; Nolen-Walston et al., 2008). Recent studies demonstrated that alveolar epithelial cells may also originate from α6β4-expressing cells that can serve as a multipotent epithelial progenitor during lung repair (Chapman et al., 2011). The finding that neither type II nor squamous cells were found in *CCSP-Foxm1*$^{-/-}$ trachea suggest that Foxm1 deletion did not result in re-programming of tracheal Clara cells. It is unlikely that this was due to inefficient Foxm1 deletion because robust Cre staining and reduced numbers of Clara cells were found in trachea of *CCSP-Foxm1*$^{-/-}$ mice. Since lineage-tracing studies demonstrated that the cell progenitors are distinct in trachea vs. epithelium from the peripheral lung (Perl et al., 2002), it is possible that Foxm1 requirements in tracheal and bronchiolar Clara cells are distinct. Alternatively, different genes can restrict differentiation of the Clara cell subtypes. Since the tracheal-bronchial epithelium is lined by a pseudostratified epithelium that includes basal cells, that are not targeted by Scgb1a1 promoter used in the present study, these non-targeted cells may serve as a source of the T1α^{+} cells that were seen in the airways of Foxm1-deficient mice. Basal cells are known to serve as progenitor cells from which both Clara and ciliated cells are derived in the proximal airways (Rock et al., 2011; Rock et al., 2009; Whitsett and Kalinichenko, 2011). Therefore, basal cells are likely to compensate for the loss of Scgb1a1-positive progenitor cells, contributing to maintenance of tracheal epithelium in *CCSP-Foxm1*$^{-/-}$ mice.

Foxm1 regulates expression of Sox2

Deletion of Foxm1 was associated with reduced *Sox2* expression in the bronchiolar epithelium. Deletion of Sox2 from Clara cells inhibited proliferation, decreased the numbers of bronchiolar epithelial cells, caused squamous metaplasia and prevented differentiation of Clara, ciliated and goblet cells (Que et al., 2009; Tompkins et al., 2009), findings similar to some of those in *CCSP-Foxm1*$^{-/-}$ mice. Thus, the loss of Sox2, a gene critical for differentiation of conducting airway epithelial cells, may contribute to the loss of normal epithelial cells after deletion of Foxm1. While decreased *Sox2* mRNA may be related in part to reduced numbers of epithelial cells in *CCSP-Foxm1*$^{-/-}$ airways, immunohistochemistry demonstrated reduced intensity of Sox2 staining in bronchioles. The finding that Foxm1 induced the *Sox2* promoter activity *in vitro* further supports a potential role of Foxm1 in the regulation of Sox2 gene. Previous studies did not observe alveolar cells in bronchiolar epithelium of Sox2-deficient mice (Tompkins et al., 2009), indicating that Sox2 and Foxm1 play distinct roles in airway epithelial cell differentiation.

Summary and Significance

Deletion of Foxm1 from bronchiolar Clara cells decreased proliferation and dramatically altered the cellular composition of the bronchiolar epithelium. Ectopic differentiation of type II alveolar cells in the bronchioles of *CCSP-Foxm1*$^{-/-}$ mice indicates that Foxm1 plays a critical role in the differentiation and maintenance of proximal vs. peripheral respiratory epithelial cell fate in the perinatal and adult lung. These studies demonstrate that Clara cells are capable of re-differentiation into alveolar type II cells in the absence of Foxm1 and that alveolar type II cells are not fully restricted from conducting airway epithelial fate by extrinsic cellular and intracellular cues. In summary, Foxm1 plays a critical role in the regulation of gene expression required for the restriction of bronchiolar cells to conducting vs. alveolar regions of the lung.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Foxm1 is required for differentiation and maintenance of airway epithelium.
- Foxm1 induces proliferation of Clara cells during lung development and lung injury.
- Foxm1 transcriptionally induces *Scgb1a1* and *Sox2*.
- Airway Clara cells retain the ability to re-program into mature type II cells.

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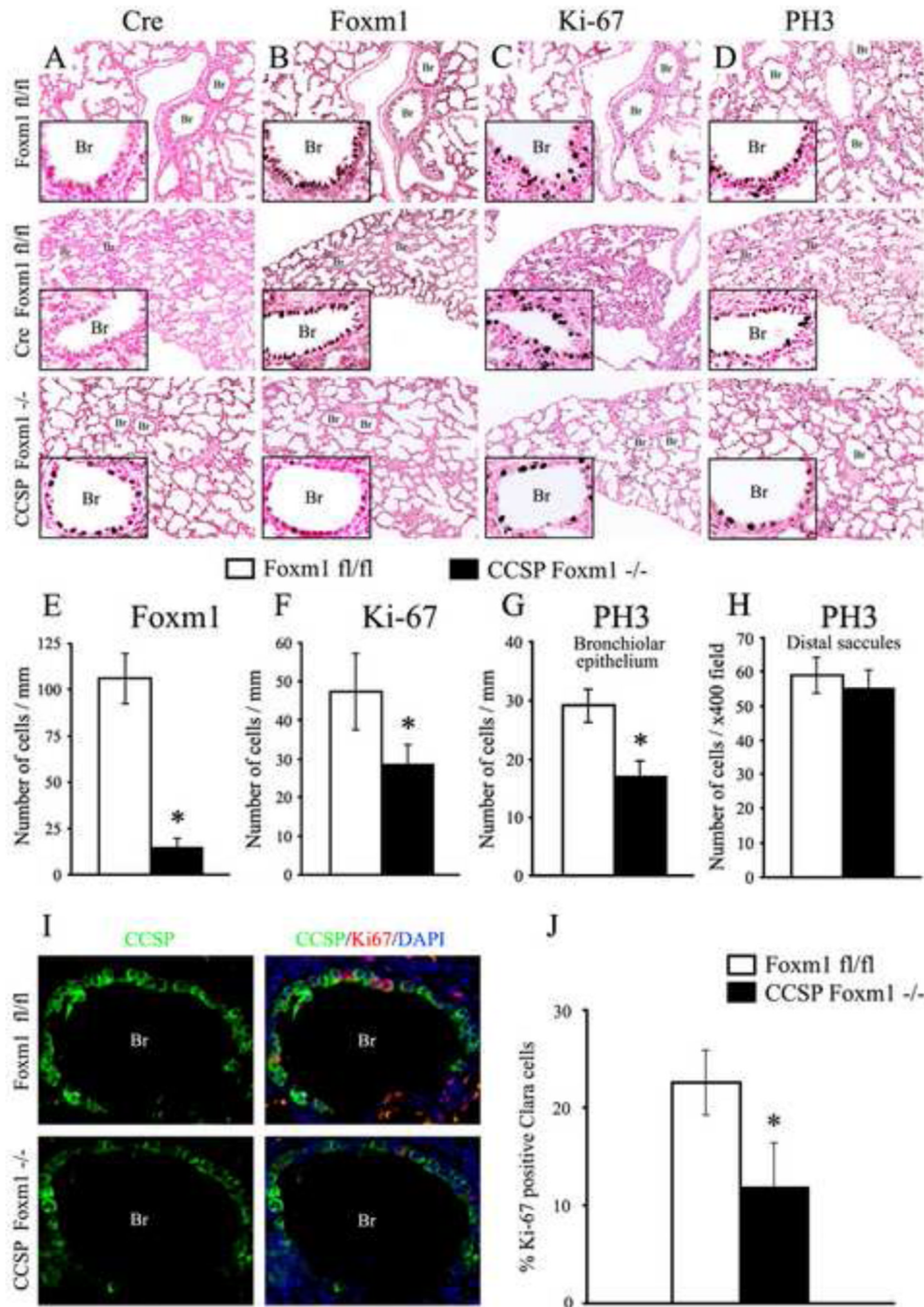

Figure 1. Efficient Foxm1 deletion and reduced proliferation of epithelial cells in *CCSP-Foxm1$^{-/-}$* airways

A–D, Foxm1 deletion from Clara cells reduces epithelial proliferation in P5 lungs. *CCSP-Foxm1$^{-/-}$* and control *Foxm1$^{fl/fl}$* and *TetO-Cre/ Foxm1$^{fl/fl}$* mice were treated with Dox at E16.5 and harvested at P5. Lung sections were stained with antibodies against Cre, Foxm1, Ki-67 and phosphorylated histone 3 (PH3). Sections were counterstained with nuclear fast red. *E–H,* Decreased numbers of epithelial cells expressing Foxm1 (E), Ki-67 (F) and PH3 (G) in *CCSP-Foxm1$^{-/-}$* bronchioles (Br). Numbers of Foxm1-positive, Ki-67-positive and PH3-positive bronchiolar epithelial cells (per 1 millimeter of airway length) were counted using ten random ×400 fields (5 mice in each group). The number of PH3-positive cells was

not altered in distal lung regions (H). *I–J,* Co-localization of CCSP (green) and Ki-67 (red) was performed in lung sections of P5 mice. DAPI was used to stain cell nuclei (blue). Ki-67-positive Clara cells were counted in 10 random microscope fields of 3 distinct mice (J). Percentages of cells are presented as mean±S.D. ($p<0.05$ is shown as *). Magnification: A–D, ×100 and ×400 (insets); I, ×630.

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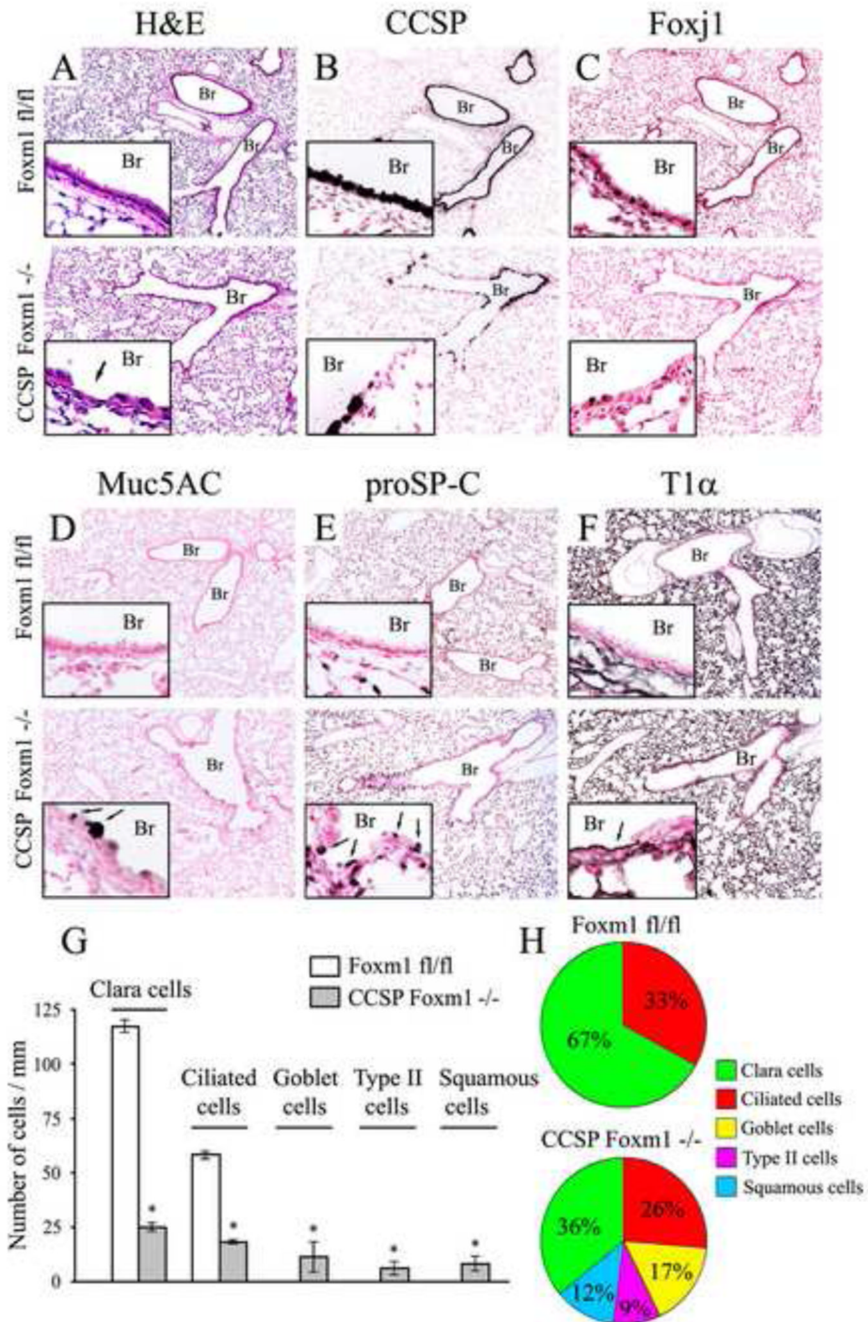

Figure 2. Decreased numbers of ciliated and Clara cells and the presence of squamous, goblet, and alveolar type II cells in airway epithelium of *CCSP-Foxm1*$^{-/-}$ mice

A–F, CCSP-Foxm1$^{-/-}$ and *Foxm1*$^{fl/fl}$ mice were treated with Dox from E16.5 to P30. Lung sections were stained with H&E (A) or used for immunohistochemistry (B–F). Slides were counterstained with nuclear fast red. Arrows show positive staining in *CCSP-Foxm1*$^{-/-}$ bronchioles (Br). *G–H,* Distribution of epithelial cells in *CCSP-Foxm1*$^{-/-}$ and *Foxm1*$^{fl/fl}$ bronchioles. Numbers of cells per 1 millimeter of bronchioles were counted using immunostained lung sections. Ten random 400× microscope fields were used. Mean ± S.D. was determined using 5 mice in each group. A p value < 0.001 is shown with asterisks (*). Magnification: A–F, ×50; all inserts, ×400.

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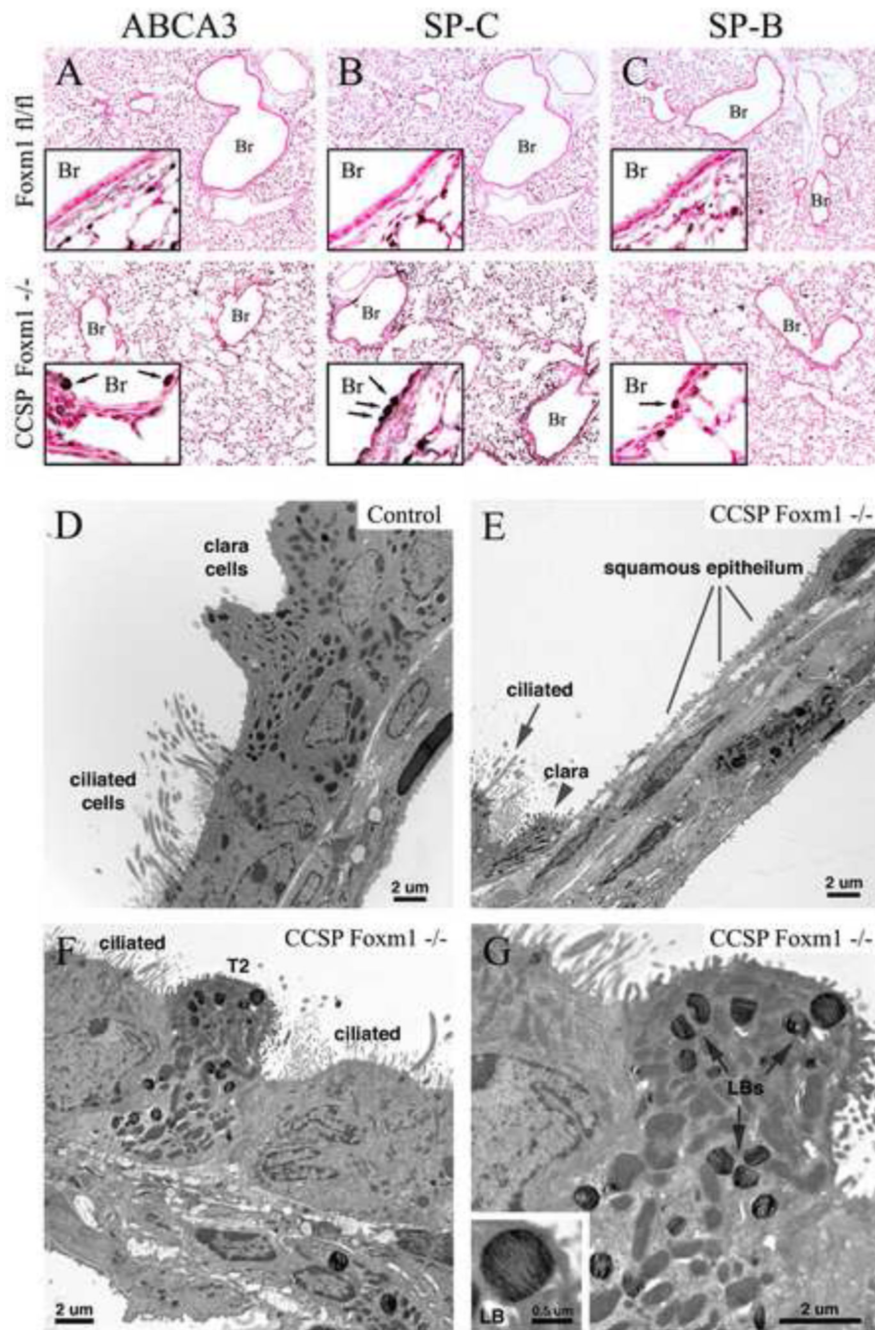

Figure 3. Presence of squamous and type II cells in bronchiolar epithelium of *CCSP-Foxm1*$^{-/-}$ mice

A–C, Immunohistochemistry was performed in P30 lung sections of *CCSP-Foxm1*$^{-/-}$ and *Foxm1*$^{fl/fl}$ mice treated with Dox from E16.5 to P30. Slides were counterstained with nuclear fast red. Arrows show positive staining in *CCSP-Foxm1*$^{-/-}$ bronchioles (Br). Magnification: ×50 and ×400 (insets). *D–G,* Transmission electron microscopy (TEM) was used to analyze P30 lung sections. Bronchiolar epithelium in control mice contained ciliated and Clara cells (D). In the *CCSP-Foxm1*$^{-/-}$ mice, extremely thin, elongated, squamous-like cells (extended lines) were found along the bronchiolar epithelium, interrupting the normal distribution of Clara (arrowhead) and ciliated cells (arrow) (E). Alveolar type II cells

containing mature lamellar bodies (LBs, arrows and insert in G) were found nestled between adjacent ciliated cells (F–G).

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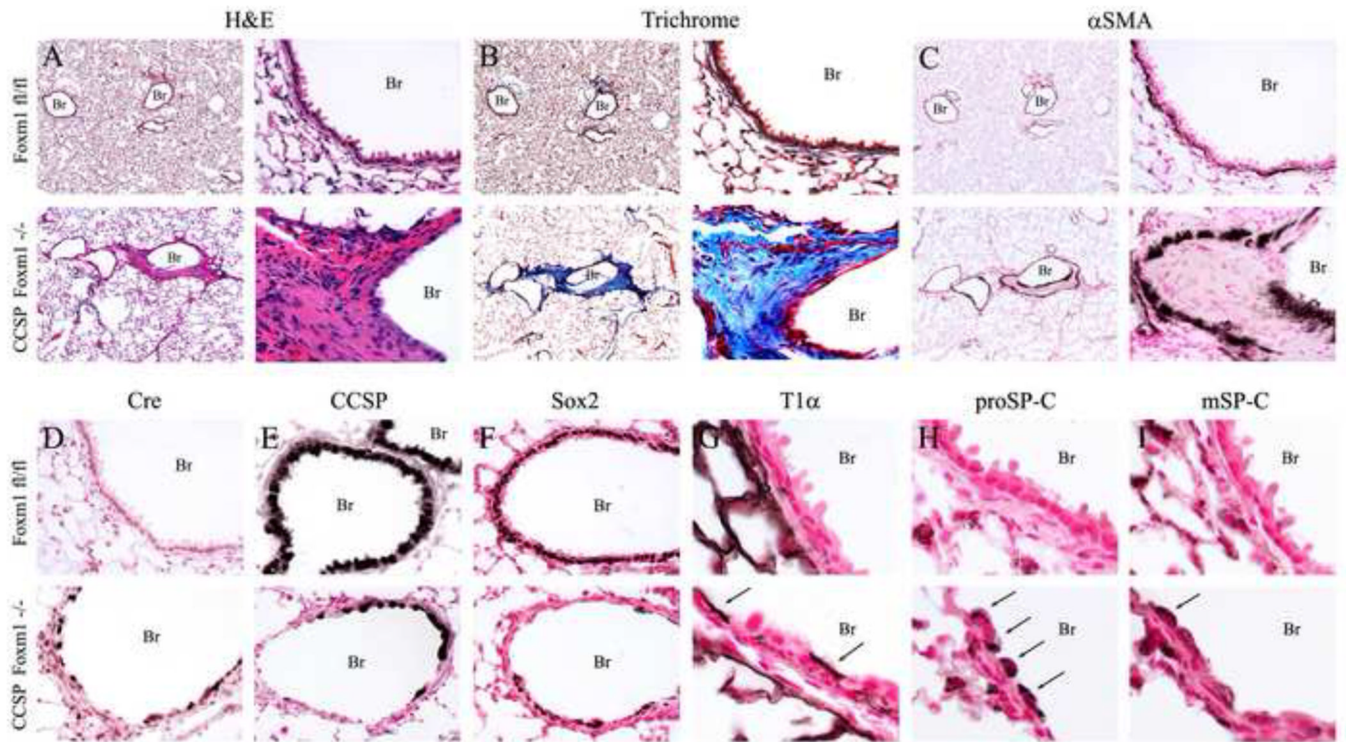

Figure 4. Extended deletion of Foxm1 in Clara cells causes peribronchial fibrosis and accumulation of ectopic type II cells

CCSP-Foxm1$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice were treated with Dox until 5 months of age. Lungs were fixed, paraffin-embedded, sectioned, and then stained with H&E (A) or trichrome (B). Slides were used for immunohistochemistry with antibodies specific to α-smooth muscle actin (C), Cre-recombinase (D), CCSP (E), Sox2 (F), T1α (G), proSP-C (H) and mature SP-C (I). Extended Dox treatment caused extensive peribronchial fibrosis (A–C) and resulted in accumulation of squamous and type II cells in Foxm1-deficient bronchioles (arrows in G–I). Decreased CCSP and Sox2 were observed in *CCSP-Foxm1*$^{-/-}$ lungs (E–F). Abbreviation: Br, bronchiole. Magnifications: left columns in A–C, ×50; D–F and right columns in A–C, ×400; G–I, ×1000.

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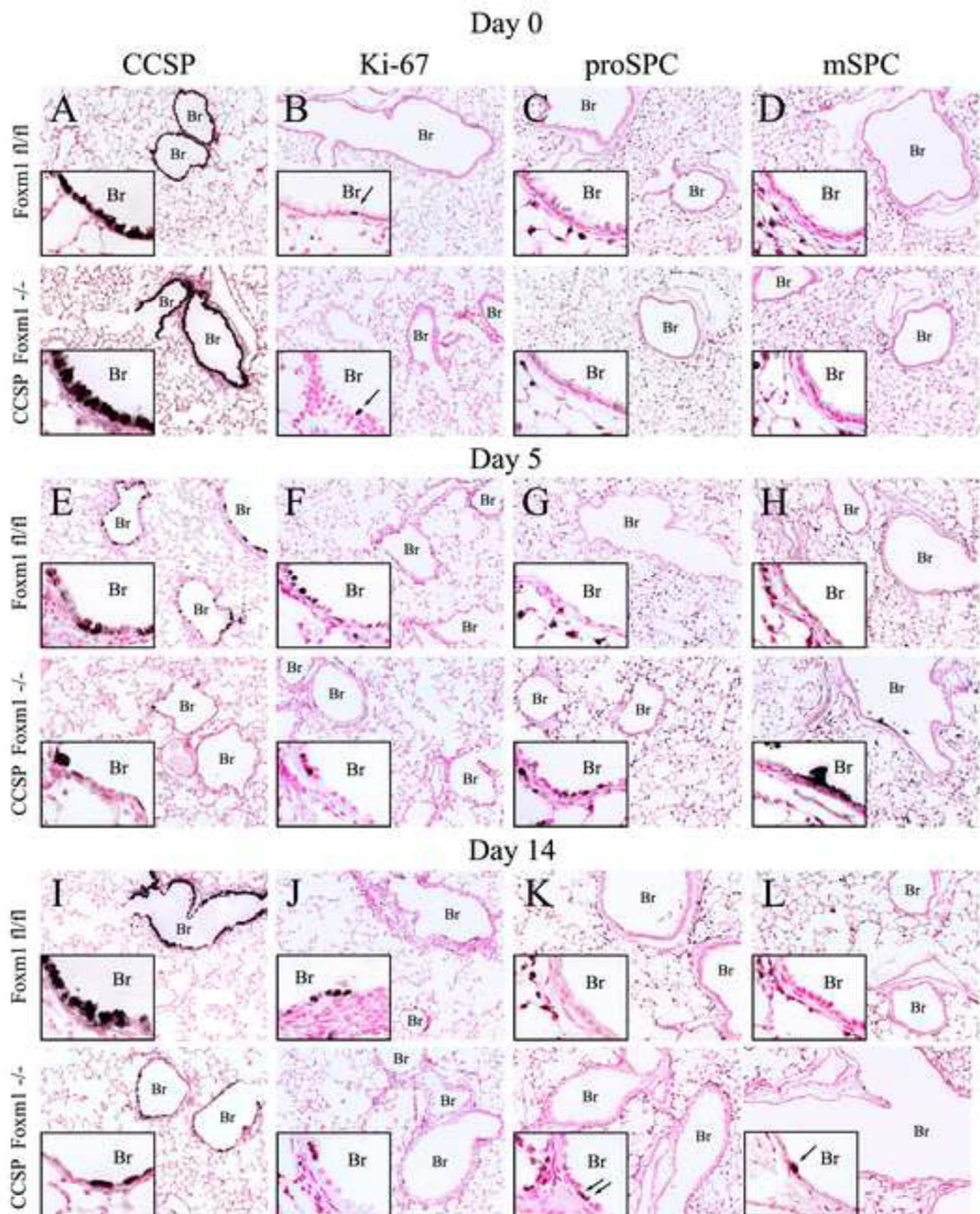

Figure 5. Deletion of Foxm1 reduces proliferation of Clara cells after naphthalene-induced lung injury

CCSP-Foxm1$^{-/-}$ and control *Foxm1*$^{fl/fl}$ male mice were treated with Dox for 7 days followed by naphthalene administration. Mice were sacrificed at days 0, 5 and 14 after injury. Lungs were fixed, paraffin-embedded, sectioned and used for immunohistochemistry. After injury, the number of Ki-67-positive Clara cells was reduced in *CCSP-Foxm1*$^{-/-}$ mice compared to controls (F and J). Airway epithelial cells expressing proSP-C and mature SP-C (mSPC) were found in bronchioles of *CCSP-Foxm1*$^{-/-}$ mice at day 5 (G–H) and day 14 after injury (arrows in K–L). Abbreviation: Br, bronchiole. Magnification is ×50 and ×400 (all inserts).

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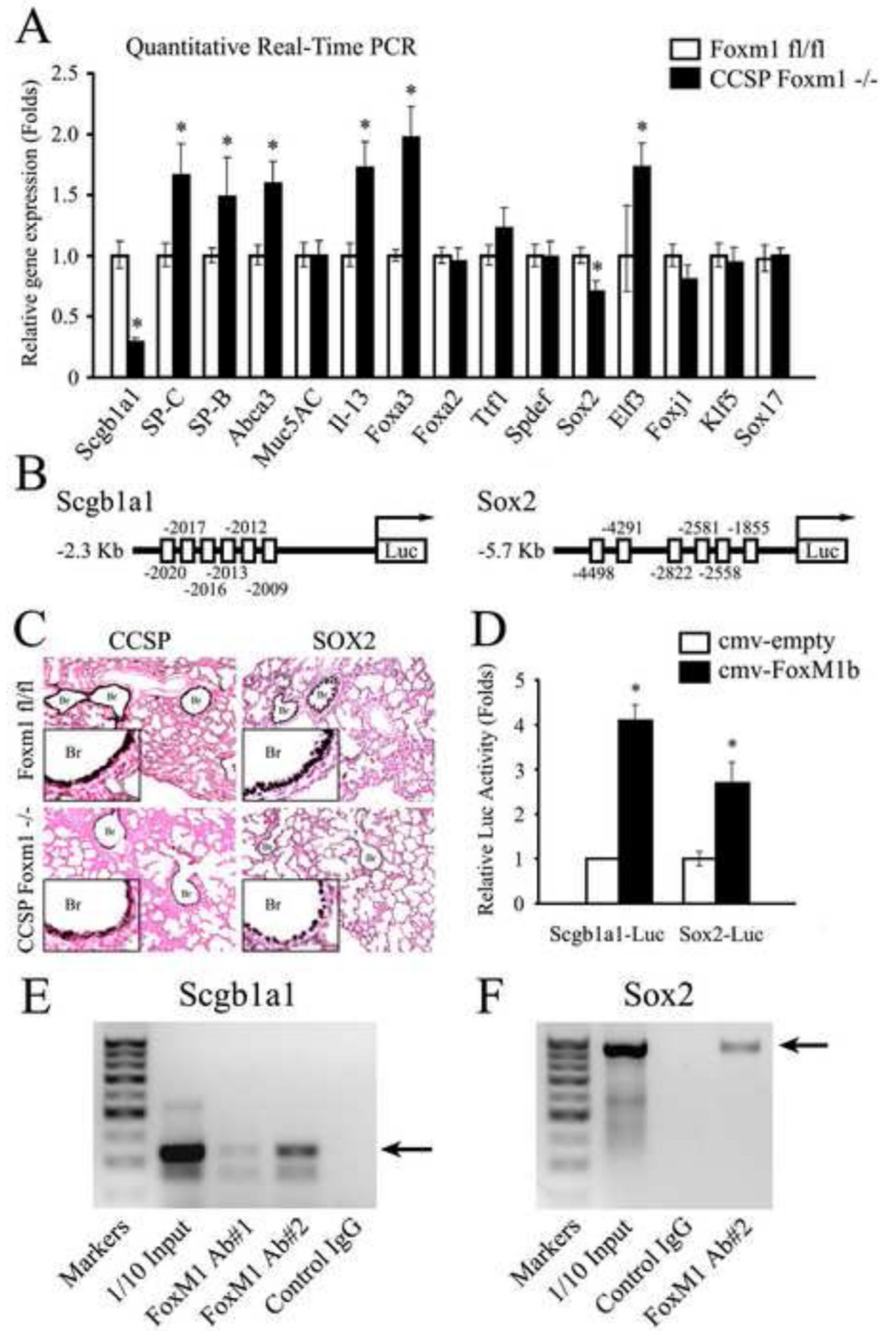

Figure 6. Foxm1 deletion from Clara cells decreases expression of Sox2 and CCSP in airway epithelium

A, Gene expression profile in *CCSP-Foxm1*$^{-/-}$ lungs. Total lung mRNA was prepared from *CCSP-Foxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice treated with Dox from E16.5 to P30 and then analyzed by qRT-PCR. *B,* Schematic drawings of −2.3 kb and −5.7 kb promoter regions of mouse *CCSP* (*Scgb1a1)* and *Sox2* genes. Locations of potential Foxm1 DNA binding sites are indicated (white boxes). *C,* Immunostaining of *CCSP-Foxm1*$^{-/-}$ lungs shows decreased protein expression of CCSP and Sox2 in bronchiolar epithelium at P5. Magnifications: ×50 and ×400 (inserts). *D,* Foxm1 induced the transcriptional activity of *Scgb1a1* and *Sox2* promoters. U2OS cells were transfected with CMV-FoxM1b expression vector and LUC

reporters driven by the *Scgb1a1* and *Sox2* promoter regions. Dual LUC assays were performed to determine LUC activity (* shows $p < 0.05$). *E–F,* Chromatin Immunoprecipitation (ChIP) assay was performed in cultured bronchial epithelial BEAS-2B cells. Endogenous Foxm1 protein specifically binds to the promoter regions of *Scgb1a1* (E) and *Sox2* genes (F).

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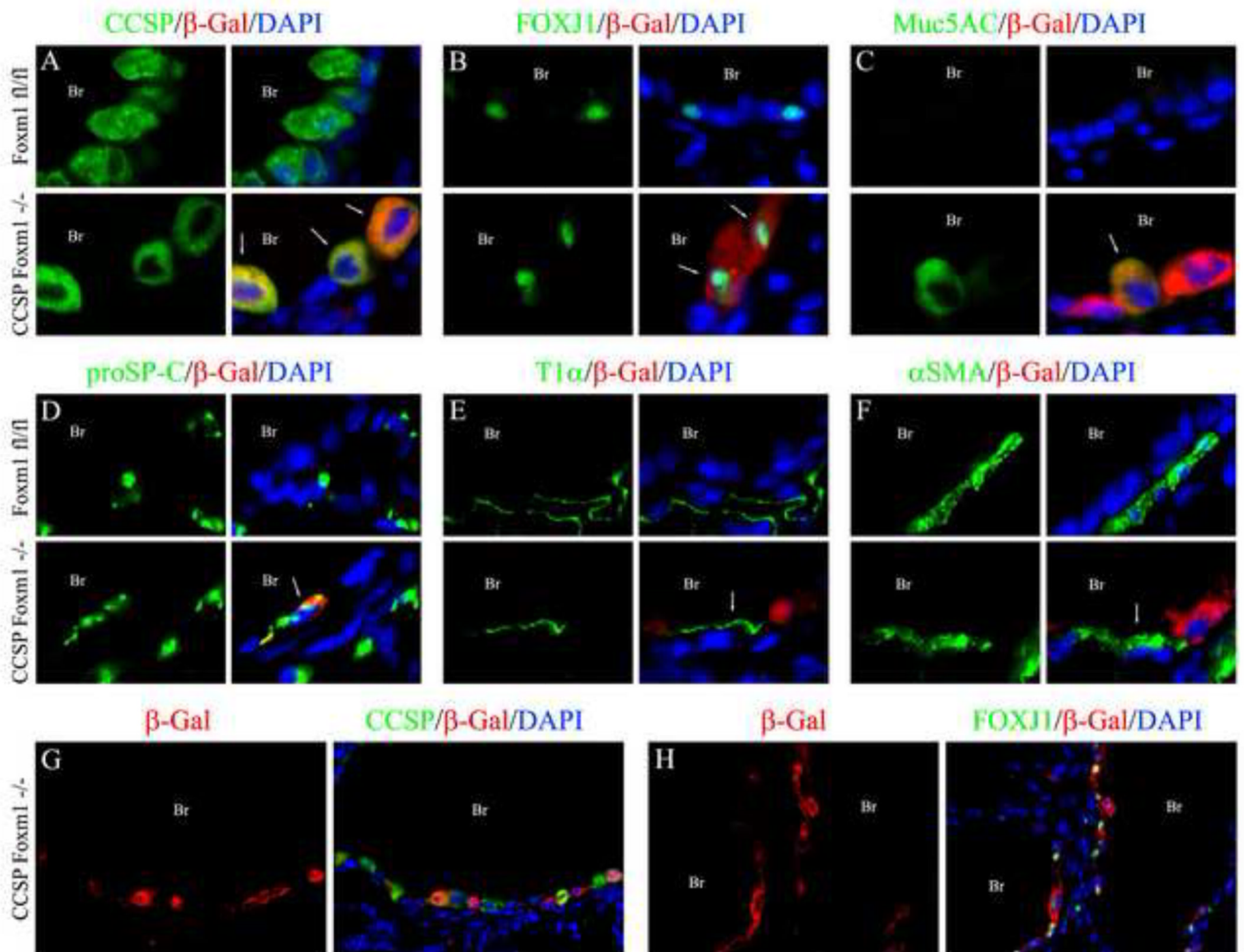

Figure 7. Epithelial cells expressing CCSP, FOXJ1, Muc5AC and proSP-C are derived from Clara cells in *CCSP-Foxm1*$^{-/-}$ airways

Rosa26R reporter mice were bred with *CCSP-Foxm1*$^{-/-}$ mice to generate *CCSP-Foxm1*$^{-/-}$/*Rosa26R* mice. Dox was given E16.5-P30 to label Cre-expressing cells with β-gal. Co-localization experiments were performed to identify cells expressing β-gal. Cytoplasmic β-gal labeling (arrows) was observed in Clara (A and G), ciliated (B and H), goblet (C) and type II cells (D) of *CCSP-Foxm1*$^{-/-}$/ *Rosa26R* bronchioles (Br) but was not found in control *Foxm1*$^{fl/fl}$ bronchioles. β-gal did not co-localize with T1α (E) or αSMA (F). Magnification: A–F, ×2000; G–H, ×400.

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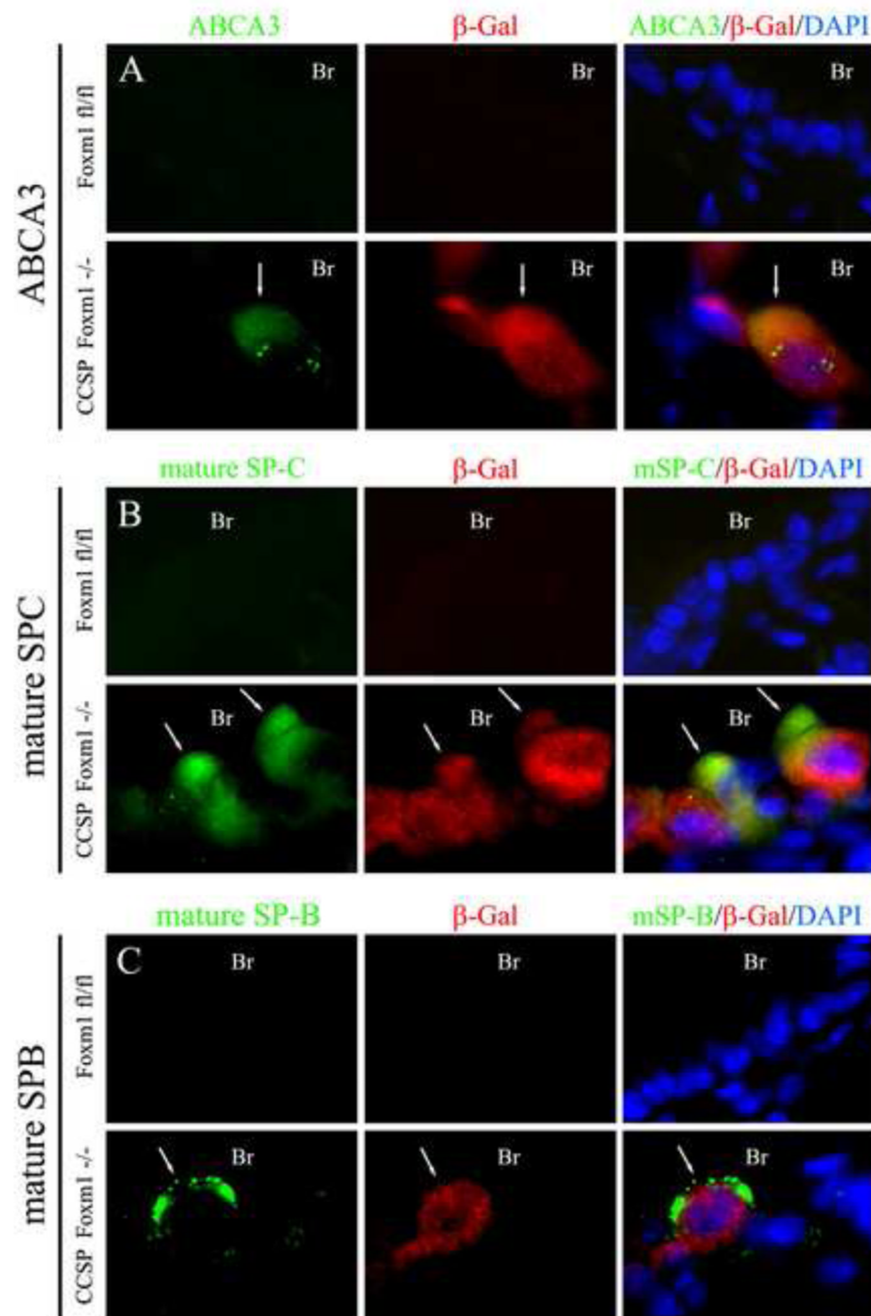

Figure 8. Type II alveolar epithelial cells expressing ABCA3, mature SP-C and mature SPB are derived from Clara cells in *CCSP-Foxm1*$^{-/-}$ airways

Rosa26-loxP-STOP-loxP-β-galactosidase reporter mice (Rosa26R) were bred with *CCSP-Foxm1*$^{-/-}$ mice to generate *CCSP-Foxm1*$^{-/-}$*/Rosa26R* quadruple-transgenic mice. Dox was given E16.5-P30 to label Cre-expressing cells with β-gal. Co-localization experiments were performed to identify cells expressing β-gal. In *CCSP-Foxm1*$^{-/-}$*/ Rosa26R* bronchioles (Br), cytoplasmic β-gal labeling (arrows) was observed in type II cells expressing ABCA3 (A), mature SP-C (B) and mature SP-B (C). Neither β-gal nor type II marker proteins were found in bronchioles of control *Foxm1*$^{fl/fl}$ *Rosa26R* mice. Magnification is ×2000.