Conditional Deletion of *Pten* Causes Bronchiolar Hyperplasia

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Tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid phosphatase that regulates multiple cellular processes including cell polarity, migration, proliferation, and carcinogenesis. In this work, we demonstrate that conditional deletion of *Pten* (*Pten*^{Δ/Δ}) in the respiratory epithelial cells of the developing mouse lung caused epithelial cell proliferation and hyperplasia as early as 4 to 6 weeks of age. While bronchiolar cell differentiation was normal, as indicated by β -tubulin and FOXJ1 expression in ciliated cells and by CCSP expression in non-ciliated cells, cell proliferation (detected by expression of Ki-67, phospho-histone-H3, and cyclin D1) was increased and associated with activation of the AKT/mTOR survival pathway. Deletion of *Pten* caused papillary epithelial hyperplasia characterized by a hypercellular epithelium lining papillae with fibrovascular cores that protruded into the airway lumens. Cell polarity, as assessed by subcellular localization of cadherin, β -catenin, and zonula occludens-1, was unaltered. PTEN is required for regulation of epithelial cell proliferation in the lung and for the maintenance of the normal simple columnar epithelium characteristics of bronchi and bronchioles.

Keywords: PTEN; AKT; bronchiolar; hyperplasia; cell-cycle

Chronic lung inflammation associated with common respiratory diseases, including chronic obstructive pulmonary disease (COPD), asthma, and idiopathic pulmonary fibrosis (IPF), is associated with alterations in the differentiation and proliferation of epithelial cells lining the conducting airway. The cellular and genetic mechanisms controlling epithelial cell proliferation and differentiation during chronic lung injury of the airways are relatively poorly understood at present (1, 2). The conducting airways are lined by diverse epithelial cell types, including squamous, ciliated, nonciliated bronchiolar (Clara cells), basal, and neuroepithelial cells. The precise sites and numbers of the various epithelial cells are carefully regulated throughout the airways. These cell types contribute to the maintenance of the structure and function of the lung under normal conditions and after injury.

Proliferation and differentiation of the respiratory epithelium is precisely regulated during lung morphogenesis and during repair after epithelial cell injury in the post natal lung.

CLINICAL RELEVANCE

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) regulates epithelial cell proliferation and maintenance of epithelial cellularity in the conducting airways. PTEN may modulate dynamic changes in the conducting epithelial cells as observed in cystic fibrosis, chronic obstructive pulmonary disease, asthma, and idiopathic pulmonary fibrosis.

A number of signaling molecules, including Shh, Wnts, Notch, PI3K, FGFs, EGFs, BMPs, and TGF- β , play important roles during lung development and repair (3, 4). Lung injury and inflammation activate expression of various cytokines, growth factors, and signaling molecules that influence proliferation and differentiation of the airway epithelium. Sustained epithelial hyperplasia creates pre-cancerous lesions that predispose the lung to cancer (5, 6).

Deficiency of PTEN (phosphatase and tensin homolog deleted on chromosome 10) has been correlated in human cancers at a frequency comparable to that of p53 mutations (7). Loss of PTEN has been associated with hyperactivated PI3K-dependent signaling in non-small cell lung carcinoma (NSCLC). Multiple genetic events are involved in the pathogenesis of NSCLC, including amplification of *PIK3CA* and activating mutations in *PIK3CA*, *EGFR*, or *K-RAS* that serve to increase phosphatidylinositol-3,4,5-triphosphate (PIP3) levels, activating the AKT/mTOR pathway (8–10). Since PTEN is a critical regulator of PIP3, its deletion is likely to have similar molecular consequences that may predispose to pulmonary tumorigenesis.

PTEN is a lipid phosphatase that regulates the levels of PIP3 and negatively modulates the PI3K/AKT/mTOR pathway, exerting tumor suppressor activity (6). PI3K activates both AKT-mediated cell proliferation and survival pathways, and Rac1-mediated cell polarity. Thus PTEN deficiency causes dysregulation of the PI3K pathway, leading to uncontrolled cell proliferation, migration, and loss of cell polarity due to activation of Rac1 and Cdc42 GTPases in various cell types (11, 12). PTEN participates in diverse cellular processes that may be relevant to tumorigenesis, including regulation of cell proliferation, survival, migration, invasion, angiogenesis, genomic stability, loss of cell-cycle checkpoints, and enhancement of stem cell self-renewal (13).

Because *PTEN* deletion/mutations are found in only 10% of NSCLC (14, 15), *PTEN* was not considered as a gene of primary interest in lung cancer. However, recent studies demonstrated that PTEN expression was decreased in at least about 70% of NSCLC, likely mediated by epigenetic mechanisms, including *PTEN* promoter methylation and silencing (16, 17). In NSCLC, loss of PTEN, when associated with increased phospho-AKT and MKK4, conferred poor prognosis (18, 19). Restoration of PTEN was associated with increased sensitivity to gefitinib or erlotinib, suggesting that levels of PTEN modulated other pathways influencing tumorigenesis (20). Decreased expression

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of PTEN, with loss of expression of other tumor suppressor genes, was correlated with metastasis and shortened survival in NSCLC, supporting a role for PTEN in the progression of NSCLC (21). Taken together, these observations support the concept that lack of PTEN activity may cause hyperplastic lesions in the respiratory epithelium, increasing the susceptibility to lung cancer. A direct role for PTEN deficiency in the pathogenesis of airway epithelial hyperplasia, dysplasia, or lung tumorigenesis has not been defined.

In the present study, we produced transgenic mice in which *Pten* gene was conditionally deleted from the pulmonary epithelium in the fetal lung. Deletion of *Pten* caused marked hyperplasia characterized by a hypercellular epithelium lining papillae with fibrovascular cores that protruded into bronchial and bronchiolar lumens. Deletion of *Pten* activated the AKT/mTOR pathway via phosphorylation of AKT and increased Ki-67, phospho-histone-H3, and cyclin D1 expression in association with marked hyperproliferation. Thus, PTEN plays a critical role in the regulation of proliferation and homeostasis of the epithelial cells lining the conducting airways.

MATERIALS AND METHODS

Transgenic Mouse Lines Animal Husbandry and Doxycycline Administration

Because *Pten*-null mice are embryonic lethal, we developed triple transgenic mice in which *Pten* is conditionally inactivated in the fetal lung epithelium using the SPC-rtTA/TetO-Cre system (22). Mice bearing *loxP*-flanked exon V of *Pten* (Figure 1A) were produced and maintained as homozygotes in a mixed FVB/N/129S4/SvJae6 background. To achieve lung epithelial specific deletion of the *Pten* gene, these mice were first mated to *SP-C-rtTA*^{-tg} mice and *TetO-Cre*^{tg} mice expressing Cre recombinase. Further back-crossings gave triple-transgenic mice harboring *SP-C-rtTA*^{-tg}/*TetO-Cre*^{tg}/*Cnb1*^{flox/flox} alleles. Doxycycline in food (25 mg/g; Harlan Teklad, Madison, WI) was administered to dams from Embryonic Day (E)0.5 to E14.5, producing experimental animals: triple transgenic mice *SP-C-rtTA*^{-tg}/*TetO-Cre*^{tg}/*Pten*^{ΔΔ}, and control transgenic littermates: *SP-C-rtTA*^{-tg}/*Pten*^{flox/flox}, *Cre*^{tg}/*Pten*^{flox/flox}, *SP-C-rtTA*^{-tg} or *TetO-Cre*^{tg} that were killed at 6 weeks for biochemical and histochemical analyses. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Research Foundation. Mice were housed in humidity- and temperature-controlled rooms on a 12-hour light/12-hour dark cycle with food and water *ad libitum*. There was no serologic or histologic evidence of either pulmonary pathogens or infections in sentinel mouse colonies. Gestation was dated E0.5 by vaginal plug. Mice were killed by injection of anesthetic to obtain lung tissue between 4 and 6 weeks.

Histology, Immunohistochemistry and Immunoblotting

Lungs from experimental mice, *Pten*^{ΔΔ} (*n* = 10 total) and control littermates (*n* = 8 total) were inflation-fixed by gravity (25 cm of water pressure) with 4% paraformaldehyde in PBS, removed from the chest, and immersed in fixative overnight at 4°C. The tissue samples were rinsed in PBS, dehydrated, and then embedded in paraffin blocks. Sections were cut at 5-μm intervals and stained with hematoxylin and eosin or with a modified Masson's trichrome method and/or an Alcian blue staining kit (Poly Scientific R&D Corp., Bay Shore, NY) to assess histologic changes. Immunohistochemistry, using biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, or Southern Biotech, Birmingham, AL) and an avidin-biotin-horseradish peroxidase detection system (ABC reagent; Vector Laboratories), was performed on 5-μm-thick sections using rabbit polyclonal antibodies against: (1) TTF-1, FOXA2, proSP-C, CCSP (1:3,000, 1:6,000, 1:8,000, 1:1,000, respectively; all from Seven Hills Bioreagents, Cincinnati, OH), SP-B (1:1,000; Chemicon/Millipore, Billerica, MA), calcitonin gene-related peptide (CGRP) (1:4,000; Sigma-Aldrich, Inc., St. Louis, MO), cyclin D1 (1:250; Abcam Inc., Cambridge, MA), and phosphohistone H-3

Figure 1. Inactivation of *Pten* in the respiratory epithelium. (A) Deletion of *Pten* was achieved in mice bearing *loxP*-flanked exon V of *Pten* (*Pten*^{flox/flox}). *Pten*^{flox/flox} mice were mated to *SP-C-rtTA*^{-tg} mice and the *TetO-Cre*^{tg} mice. Administration of doxycycline to dams deleted exon V of *Pten* in the respiratory epithelium of the embryos, termed *Pten*^{ΔΔ} mice. (B) Genotype analysis using PCR on genomic DNA using primers flanking *loxP* sites in exon V identified *Pten*^{wt/wt}, *Pten*^{flox/flox}, and *Pten*^{flox/wt} genotypes. M, DNA molecular weight marker. (C) Hematoxylin/eosin staining of lung sections from *Pten*^{ΔΔ} mice demonstrated normal branching morphogenesis and postnatal lung formation at 6 weeks of age.

(1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); (2) rabbit monoclonal antibody against phospho-AKT (1:25, S473, clone 736E11; Cell Signaling Technology, Inc., Danvers, MA); (3) rat monoclonal antibodies against Ki-67 (1:500, clone TEC-3; Dako, Carpinteria, CA) and zonula occludens (ZO)-1 (1:150, clone R40.76; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); (4) goat antibodies against pancytokeratin and β-catenin (1:2,000, 1:2,000; both from Santa Cruz Biotechnology); and (5) mouse monoclonal antibodies against FOXJ1 (clone 319; Seven Hills Bioreagents), PTEN (clone 6H2.1; Cascade BioScience Inc., Winchester, MA), and β-tubulin IV (clone on1a6; Biogenex Laboratories Inc., San Ramon, CA). Antigen retrieval, using 0.1 M citrate buffer (pH 6.0) and heat, was used to detect TTF-1, FOXA2, FOXJ1, phospho-AKT cyclin D1, Ki-67, phosphohistone H-3, ZO-1, pancytokeratin, and β-catenin. A mouse-on-mouse blocking reagent (Vector Laboratories) was used with the mouse monoclonal antibodies. All secondary antibodies were used at a dilution of 1:200.

The enzymatic reaction product was enhanced using Ni-DAB and Tris cobalt to give a black precipitate, and the sections were counterstained with Nuclear Fast Red. On some sections, the primary antibody was omitted in order to check for nonspecific staining of the secondary antibody and/or the ABC reagent. For dual immunolabeling, primary antibodies from two different species were used as follows: (1) guinea pig anti-CCSP (1:10,000) and rabbit anti-CGRP (1:1,000); (2) rat anti-Ki-67 (1:100) and rabbit anti-TTF-1; (3) rat anti-Ki-67 (1:100) and guinea pig anti-CCSP (1:10,000); (4) rabbit anti-proSP-C (1:1,000) and guinea pig anti-CCSP (1:10,000); and (5) mouse anti-FOXJ1 (1:100) and guinea pig anti-CCSP (1:10,000). Secondary antibodies, conjugated with Alexa Fluor 594 (red) or Alexa Fluor 488 (green) fluorochromes (Molecular Probes, Invitrogen Corp., Carlsbad, CA), were used at a dilution of 1:200. Sections were mounted with anti-fade reagent containing DAPI (Vector Laboratories). On some sections, the primary antibodies were omitted to assess nonspecific fluorescence of the secondary antibody, while both the primary and secondary antibodies were omitted on other sections to assess autofluorescence from tissue. Micrographs in all figures are representative of $n \geq 4$ separate mice per genotype.

For phospho-AKT-S473 immunoblots, total protein from lung homogenates were normalized by Bradford Assay and loaded on a 4%–20% Tris/glycine SDS-PAGE (Invitrogen) and electroblotted to nitrocellulose membranes (0.45 μ m; Bio-Rad, Hercules, CA). Blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20) and incubated with rabbit monoclonal antibody against phospho-AKT (1:100; S473, clone 736E11; Cell Signaling Technology) or rabbit anti-actin antibody (A2066; Sigma-Aldrich) for normalization. Blots were washed in TBST and incubated with goat anti-rabbit heavy- and light-chain peroxidase-conjugated (Calbiochem, San Diego, CA; EMD Biosciences, Gibbstown, NJ) secondary antibodies and developed by chemiluminescence (Pierce Biotechnology, Rockford, IL) and quantitated using the volume integration function on a Phosphor-Imager software Imagequant 5.2 (Molecular Dynamics, Sunnyvale, CA).

Quantitation of Ki-67, Cyclin D1, and CGRP-Positive Cells

Five random fields from 5- μ m-thick sections of lungs containing bronchioles from *Pten* $^{\Delta/\Delta}$ and control mice ($n = 4$) immunostained for Ki-67 or cyclin D1 were digitally captured using a Nikon 90i microscope at $\times 20$ magnification and a DXM 1200C digital camera (both from Nikon, Tokyo, Japan). Number of total epithelial nuclei and Ki-67 or cyclin D1-positive epithelial nuclei in each airway were counted using Metamorph software (Universal Imaging Corp., Downingtown, PA). The Ki-67 mitotic index was calculated by dividing the number of Ki-67-positive nuclei by the total number of nuclei examined in the airway, and the value was expressed as percentage of Ki-67-positive cells. Likewise, the ratio of cyclin D1-positive cells to total epithelial cell was expressed as percentage. CGRP-positive cells were counted per NEB (6–12 NEBs for each genotype) from *Pten* $^{\Delta/\Delta}$ and control mice ($n = 4$ for each genotype). The averages and standard errors from four mice in each group were compared using the standard *t* test, $P < 0.01$ (Sigma-Stat, ver. 3.1; Systat Software, Inc., Point Richmond, CA).

RESULTS

Pten Is Not Required for Branching Morphogenesis and Postnatal Lung Formation

Mouse embryos homozygous for the *Pten*-inactivating mutation die between E6.5 and E9.5 (23). Triple-transgenic mice were produced containing three alleles: *loxP*-flanked exon V (*Pten*^{loxP/loxP}), which deletes the lipid-phosphatase domain of Pten in which many tumor-associated mutations were detected (24); *SP-C-rtTA*^{tg/+}; and *TetO-Cre*^{tg/+}. *Pten* was selectively deleted in the respiratory epithelial cells after administration of doxycycline (Dox) to the dam (Figure 1A). Since *Pten* is ubiquitously expressed during gastrulation, the human *SFTPC* gene promoter

Figure 2. Bronchial and bronchiolar hyperplasia. Selective deletion of *Pten* gene in the lung using the SPC-rtTA/TetO-Cre system caused epithelial hyperplasia within 6 weeks. As compared to control littermates, the bronchial epithelium in *Pten* $^{\Delta/\Delta}$ mice was hypercellular (A, B). Papillae consisting of fibrovascular cores lined by a hypercellular epithelium protruded into the bronchiolar lumens of *Pten* $^{\Delta/\Delta}$ mice. In contrast, bronchioles in control littermates lacked papillae and were lined by a pseudostratified or single layer epithelium (C, D). Papillae contained fibrovascular cores consisting of stroma with its associated collagen as highlighted by trichrome stain (E, F). $n \geq 4$ mice per genotype. Lung images shown are from 6-week-old mice, but are also representative of lungs from mice at 5 months of age (Figure E1).

was used to express Cre during embryonic lung development as previously described (22), deleting *Pten* specifically in precursors of bronchiolar and alveolar epithelial cells, generating "*Pten*^{ΔΔ} mice." The SFTPC promoter expresses reverse tetracycline trans-activator (rtTA), which in the presence of doxycycline activates the OTet-CMV promoter, activating expression of Cre recombinase (Figure 1A) (22). At birth, the transmission of all of the genes followed Mendelian inheritance (Figure 1B). Mice harboring *SPC-rtTA/Pten*^{fllox/fllox}, *Cre*^{tg}/*Pten*^{fllox/fllox}, *SPC-rtTA*, or *TetO-Cre*^{tg} were used as controls. Mice expressing *rtTA*, or bearing *TetO-Cre*^{tg} without the *Pten*^{fllox/fllox} allele, were normal. Deletion of *Pten* in the developing fetal lung did not perturb branching morphogenesis, structural lung maturation, or postnatal lung formation. Pups were viable and reached maturity with fully developed lungs after deletion of *Pten* as assessed by light microscopy at 6 weeks of age (Figure 1C).

Deletion of *Pten* in the Respiratory Epithelium Caused Bronchiolar Hyperplasia

Loss of *Pten* in the respiratory epithelial cells caused bronchial and bronchiolar epithelial hyperplasia, producing papillae composed of fibrovascular cores lined by a hypercellular epithelium

protruding into the airway lumens consisting of enlarged cells (Figures 2A–2F). Hyperplastic epithelial cells lacked significant dysplasia and maintained morphologic features of differentiation. Epithelial papillary hyperplasia was observed as early as 4 to 6 weeks of age, and was noted throughout the conducting airways consistent with the site of deletion mediated by the *SPC-rtTA/TetO-Cre* system (22). Lungs from *Pten*^{ΔΔ} mice at 5 months of age showed papillary epithelial hyperplasia morphologically similar to that seen at 4 to 6 weeks of age. Overt pulmonary tumors were not observed at 6 weeks of age. Pro-SPC and TTF-1 staining were relatively unperturbed in epithelial cells in the alveolar region (data not shown).

Activation of the AKT/mTOR Survival Pathway in the Respiratory Epithelium after Deletion of *Pten*

Loss of PTEN is known to increase PIP₃ levels, phosphorylating AKT, thereby activating the AKT/PKB survival pathway (25). Increased phosphorylation of AKT protects cells from apoptosis and enhances proliferation, a finding associated with tumorigenesis (6). Deletion of *Pten* in the respiratory epithelium was associated with increased phosphorylation of AKT at Ser473 as

Figure 3. PTEN Regulates PI3K/AKT signaling and cell cycle in the conducting airway epithelium. Absence of *Pten* increased PI3 kinase activity as assessed by increased phosphorylation of its downstream effector AKT (A, enlarged insets). Immunoblotting of lung homogenates from *Pten*^{ΔΔ} mice demonstrated increase of phosphorylation of AKT at Ser473 at least approximately 3.5-fold. Mean $\pm$ SD, $n = 4$ mice of each genotype, * $P < 0.02$, unpaired two-tailed Student's t test, Excel, ver. 2003 (B, C). Increase in cell cycle associated proteins (Ki-67, phospho-histone H3, and cyclin D1) was detected in respiratory epithelial cells of *Pten*^{ΔΔ} mice (D). Mitotic index as assessed by Ki-67-positive nuclear staining, mean $\pm$ SE ($n = 4$ mice per genotype), * $P < 0.01$ using the standard t test (E). Cyclin D1-positive nuclei were increased approximately 2-fold, mean $\pm$ SE ($n = 4$ mice per genotype), * $P = 0.017$ using the standard t test (Sigma-Stat, ver. 3.1) (F). Increased staining for phospho-AKT at Thr308 was not observed (data not shown).

Figure 4. Co-expression of Ki-67 with TTF-1 and CCSP. Nuclear staining for Ki-67 (red, shown by white arrows) was co-localized with cytoplasmic staining for CCSP (green) as detected by confocal microscopy, demonstrating that proliferation occurred in nonciliated bronchial and bronchiolar Clara cells (A). Nuclear co-localization of TTF-1 (green) and Ki-67 (red) immunostaining was observed in airway epithelial cells (white arrows), as demonstrated by yellow fluorescence in the merged images by confocal microscopy, indicating that proliferation occurred in the epithelial cells lining conducting airways after deletion of *Pten* (B). Micrographs are representative of $n \geq 4$ separate mice per genotype.

assessed by immunohistochemical staining (Figure 3A). Phosphorylation of AKT-Ser473, as quantified by immunoblot analysis, increased approximately 3.5-fold (Figures 3B and 3C), indicating that activation of the PI3K/AKT/mTOR pathway may participate in epithelial cell hyperplasia in this model.

Deletion of *Pten* Influenced Cell Cycle and Increased Cell Proliferation

Bronchiolar epithelial cell hyperplasia and enlarged cell size was observed in *Pten* Δ/Δ mice. Since PTEN is known to influence cell proliferation in many organs (26), phospho-histone H3, Ki-67, and cyclin D1 staining was performed on lungs from *Pten* Δ/Δ mice. Phosphorylation of histone H3 at Ser10 is mitosis

specific and coincident with chromosome condensation in the G2 phase (27). Expression of Ki-67 a cell proliferation-associated antigen, occurs preferentially during late G1, S, G2, and M phases but not in G0 phase of the cell cycle (28). Increased phospho-histone H3, Ki-67, and cyclin D1 staining was detected in the bronchiolar epithelium of *Pten* Δ/Δ mice (Figure 3D). While increased Ki-67-positive cells were detected in the lung periphery, alveolar regions of the lung were not markedly hyperplastic. Proliferative (mitotic) index, as assessed by Ki-67-positive nuclei and expression of cyclin D1 in the conducting airways of the *Pten* Δ/Δ mice, increased approximately 2-fold after deletion of *Pten* (Figures 3E and 3F), demonstrating that PTEN influences cell division in the bronchial and bronchiolar

Figure 5. Deletion of *Pten* leads to expansion of PNECs. Lung sections from control littermates and *Pten* Δ/Δ mice at 6 weeks of age were stained with CCSP (green) and CGRP (red) antibodies and examined using confocal microscopy. Increase in the size of NEBs (red) expressing CGRP was associated with an approximately at least 2-fold increase in number of PNEC-positive cells observed after deletion of *Pten* (A and B). No double-positive (CCSP and CGRP) respiratory epithelial cells within the NEBS were detected in controls or *Pten* Δ/Δ mice. The averages and standard errors from four mice in each group were compared using the standard *t* test, * $P < 0.01$ (Sigma-Stat, ver. 3.1).

epithelium. Confocal microscopy was used to demonstrate that Ki-67 was co-localized with cytoplasmic staining for CCSP, indicating that proliferation occurred primarily in nonciliated bronchial and bronchiolar Clara cells (Figure 4A). Ki-67 was co-expressed with TTF-1 in the nucleus, indicating that proliferation occurred in the epithelial cells lining conducting airways after deletion of *Pten* (Figure 4B).

Deletion of *Pten* Caused Pulmonary Neuroepithelial Cell Hyperplasia

Pulmonary neuroendocrine cells (PNEC) reside as solitary cells and as clusters at discrete locations such as airway junctions in the bronchioles, forming neuroepithelial bodies (NEBs) in the conducting airways (29). While the cellular and molecular mechanisms underlying the origin and differentiation of neuroepithelial cells are unknown, PNEC shares gene expression patterns with those of small cell lung cancer (SCLC) and may be a progenitor giving rise to neuroendocrine lesions, including bronchial carcinoid tumors and SCLC (30). Deletion of *Pten* in respiratory epithelium caused an increase in the size of NEBs that was associated with an approximately at least 2-fold increase in number of PNECs as assessed by CGRP-positive cells (Figures 5A and 5B). Putative airway epithelial stem cells that are double positive for CCSP and CGRP that were previously identified in injury models (31) were not detected in the *Pten*^{Δ/Δ} mice.

Pten Is Not Required in Normal Respiratory Epithelial Cell Differentiation

Since PTEN is known to influence cell differentiation in many tissues (32–34), we assessed whether respiratory epithelial cell differentiation was perturbed in *Pten*^{Δ/Δ} mice at 6 weeks of age. While hyperplasia and enlarged cell size was observed, Alcian blue and periodic acid Schiff's staining for mucins (Figure 6 and data not shown) were not overtly altered. Bronchiolar epithelial cell differentiation, as assessed by expression of FOXJ1, CCSP, FOXA2, and TTF1 (Figure 6), and β -tubulin (Figure 7), were not altered in the *Pten*^{Δ/Δ} mice. Expression of TTF-1, FOXA2, and pro-SPC in the alveolar region of the lung were relatively unperturbed in the *Pten*^{Δ/Δ} mice (data not shown). Thus, *Pten* was not required for normal respiratory epithelial cell differentiation.

Deletion of *Pten* Did Not Lead to Loss of Respiratory Epithelial Cell Polarity

Very little is known about regulation and maintenance of cell polarity. PTEN modulates PIP3 levels. Apical-basal axis and attachment of epithelial cells with the extracellular matrix (ECM) are controlled by intracellular PIP3 concentration gradients (12, 35). Intracellular localization, activity, and levels of *Pten* may control cell polarity, a finding observed in various cell types, including kidney epithelial cells (12). PTEN associates with Par3/aPKC complex involved in biogenesis of adherens and tight junction (36, 37) participating in epithelial cell polarity. PTEN contains a PDZ (PSD95, Dlg, and ZO-1)-binding motif that associates with protein complexes involved in cell polarity. Therefore, localization of apical protein ZO-1, and of epithelial junctional proteins cadherins and β -catenin, was assessed by immunostaining. Expression and localization of cadherin and β -catenin was not disrupted, indicating that the polarization of the cytoskeleton was maintained (Figures 7A–7D). Formation of epithelial tight junctions was not perturbed, as evidenced by the normal localization of ZO-1 in *Pten*^{Δ/Δ} mice (Figures 7E and 7F). Staining of β -tubulin, an apical marker of ciliated epithelial cells was unaltered, indicating that while loss of *Pten* in respiratory epithelial cells affected cell size and

Figure 6. Respiratory epithelial cell differentiation in *Pten*^{Δ/Δ} mice. Immunostaining for FOXJ1 (ciliated cells) and CCSP (nonciliated cells) lining the conducting airway was unaltered in *Pten*^{Δ/Δ} mice (A–D). Staining for FOXA2 and TTF-1 expression was unaltered in *Pten*^{Δ/Δ} mice (E–H). Alcian blue staining in epithelial cells lining the conducting airways of *Pten*^{Δ/Δ} mice and control littermates was unaltered, indicating that while loss of *Pten* induced airway hyperplasia, it did not cause goblet cell differentiation. Micrographs are representative of *n* = 4 separate mice per genotype.

increased proliferation; it did not influence cell polarity in ciliated cells.

DISCUSSION

Chronic lung inflammation and injury cause dynamic changes in the numbers and characteristics of respiratory epithelial cells as observed in common respiratory diseases, including cystic fibrosis, COPD, asthma, and idiopathic pulmonary fibrosis. Depending on the site of injury, subsets of stem/progenitor cells in the conducting airways proliferate, serving to repopulate the respiratory epithelium after injury (38–40). Dysregulation in

Figure 7. Pten does not influence respiratory epithelial cell polarity. Immunostaining for cadherin and β -catenin proteins involved in maintaining cell-cell adhesion, and cell polarity was unperturbed in $Pten^{\Delta/\Delta}$ mice when compared with lungs from littermate controls (A–D). Apical staining of ZO-1 was unperturbed in $Pten^{\Delta/\Delta}$ mice when compared with control littermates. There was no ZO-1 staining detected in the cytoplasm, indicating that the hyperplastic cells had not lost their polarity (E, F). Staining for β -tubulin demonstrated normal distribution pattern in the apical region of ciliated cells that was unaltered in $Pten^{\Delta/\Delta}$ mice compared with their littermate controls (G, H). Micrographs represent $n = 4$ separate mice per genotype.

this process results in hyperplasia and dysplasia that predisposes to lung cancer (41). Selective deletion of *Pten* gene activated PI3K/AKT pathway, causing papillary epithelial cell hyperplasia and cell enlargement in the bronchial and bronchiolar lumen, providing evidence that PTEN is required for the normal regulation of epithelial cell proliferation in the lung.

Absence of *Pten* activated the AKT/mTOR pathway via phosphorylation of AKT, consistent with the role of Pten as a negative modulator of PI3K/AKT pathway (6). AKT is activated by phosphorylation at Thr308 and Ser473 by PDK1 and PDK2, respectively. In the $Pten^{\Delta/\Delta}$ mice, AKT was phosphorylated at Ser473 but not at Thr308, as assessed by Western blotting (Figure 3), consistent with previously observed inverse correlation between PTEN expression and phosphorylation of AKT on Ser473 (42), suggesting that absence of PTEN activates a distinct pathway via PDK2, mediating AKT phosphorylation on Ser473. With identification of mTOR-Rictor (mTORC2) as the PDK2 kinase that phosphorylates Ser473, it is plausible that bronchial hyperplasia observed after PTEN deletion is, in part, caused by PDK2 (mTORC2)-mediated phosphorylation of AKT on Ser473 (43). Indeed, increased phospho-AKT-Ser473

expression was observed in human bronchial dysplasia and NSCLC (44), suggesting that mTORC2 activation plays a critical role in lung tumorigenesis. Little is known regarding the regulation of mTORC2, except that its activation is independent of the PDK1 pathway (45).

While the adult lung is not mitotically active, deletion of PTEN increased cell proliferation as detected by increased expression of Ki-67, phospho-histone-H3, and cyclin D1, indicating that airway epithelial cells became mitotically active. PTEN is known to directly coordinate G_1 arrest by down-regulating cyclin D1 and up-regulating p27 in models of breast cancer (46). Adenovirus-mediated delivery of *PTEN* inhibited S-phase transition via recruitment of p27 into cyclin E complexes (47). While loss of PTEN in the respiratory epithelium caused hyperplasia in the airway lumen, it is not known that PTEN activity modulates the rapid proliferation and repopulation of the airway epithelium after injury. In the $Pten^{\Delta/\Delta}$ mice, the PI3K/AKT pathway was activated, both enhancing the S-phase transition and conferring resistance to apoptotic signals (48). Finally, AKT phosphorylates FoxO transcription factors (FoxO1a, FoxO3a, and FoxO4), inhibiting their activity on the

cell cycle (49). Thus, deletion of PTEN in respiratory epithelium likely enhances cell cycle entry and progression, causing uncontrolled epithelial proliferation and hyperplasia.

Deletion of PTEN caused pulmonary neuroepithelial cell hyperplasia, significantly increasing the size of the NEBs. Previous studies with this transgenic system demonstrated that SPTPC promoter was not active in the pulmonary epithelial cells of the neuroendocrine lineage (22); therefore, Cre-mediated *Pten* deletion is not likely to occur in PNE cells. The observed neuroepithelial cell hyperplasia in *Pten*^{ΔΔ} mice is not caused by a cell autonomous mechanism, but is likely due to paracrine signaling from adjacent cells that are deficient in PTEN, consistent with previous studies in which epithelial cells that are adjacent to NEBs contribute to PNE cell hyperplasia (31). Deletion of PTEN in fibroblast and mammary tumor cells *in vivo* enhanced secretion of growth factors, including pleiotrophin, that may influence cell adhesion, migration, survival, growth, and differentiation via a paracrine mechanism (50).

While PTEN is required for differentiation of the endodermal, ectodermal, and mesodermal tissues in early embryogenesis and is essential for germ cell, neuronal, and osteoclast differentiation (23, 33, 34), deletion of *Pten* in the present study did not alter cell differentiation as assessed by several respiratory epithelial cell type specific markers. This result is consistent with findings in *Drosophila* (51) and in the mammalian brain (52). Thus, PTEN-deficient respiratory epithelial cells remain responsive to exogenous and endogenous differentiation cues.

Cell polarity was generally maintained in the respiratory epithelial cells of the conducting airways despite deletion of *Pten*. Spatial distribution of cadherin and β-catenin remained unchanged. Apical localization of ZO-1 was observed in respiratory epithelial cells of the conducting airway in *Pten*^{ΔΔ} mice, suggesting that its membrane distribution was unperturbed. Likewise, β-tubulin, an apical marker for ciliated epithelial cell in the conducting airway was unaltered. Thus, increased proliferation and loss of tissue polarity as a consequences of increased PI3K signaling may be influenced by distinct cellular processes in respiratory epithelial cells of *Pten*^{ΔΔ} mice as previously demonstrated (53).

In summary, deletion of *Pten* in the developing lung did not disrupt branching morphogenesis, epithelial cell differentiation, or postnatal lung formation. Loss of PTEN activated the AKT/mTOR pathway and induced both initiation and progression of cell cycle, causing respiratory epithelial cell hyperplasia, producing a hypercellular papillary bronchial and bronchiolar epithelium. These findings support the concept that PTEN participates in the regulation of normal bronchial and bronchiolar epithelial cell proliferation and may play critical roles in normal regeneration and maintenance of the conducting airways after injury.

Since deletion of *Pten* was not sufficient for tumor formation at 6 weeks, *Pten*^{ΔΔ} mice were kept for 5 months to determine if deletion of *Pten* would produce any additional changes in the respiratory epithelium, including dysplasia, preneoplastic lesions, or tumors. While papillary epithelial hyperplasias were readily observed in lungs of *Pten*^{ΔΔ} mice at 5 months of age, they were morphologically similar to those seen in lungs from mice at 4 to 6 weeks of age. Airways in littermate control mice were lined by single-layered epithelium, whereas epithelial hyperplasia associated with papillary projections protruding into the airway lumens was observed in the bronchioles of *Pten*^{ΔΔ} mice at 5 months of age (see Figures E1A–E1D in the online supplement) that were similar to those of 6-week-old mice. No tumors were detected grossly or microscopically, suggesting that loss of PTEN alone is not sufficient for tumor formation, even in older mice. Thus,

loss of PTEN activity may act in concert with secondary genetic lesions, including mutations in *RAS*, *MYC*, and *P53* and/or various epigenetic inactivations as observed in *TIMP-3*, *p16^{INK4A}*, *p14^{ARF}* genes in human lung cancers (54).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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