Deletion of *Pten* Expands Lung Epithelial Progenitor Pools and Confers Resistance to Airway Injury

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Rationale: *Pten* is a tumor-suppressor gene involved in stem cell homeostasis and tumorigenesis. In mouse, *Pten* expression is ubiquitous and begins as early as 7 days of gestation. *Pten*^{-/-} mouse embryos die early during gestation indicating a critical role for *Pten* in embryonic development.

Objectives: To test the role of *Pten* in lung development and injury.

Methods: We conditionally deleted *Pten* throughout the lung epithelium by crossing *Pten*^{flox/flox} with *Nkx2.1-cre* driver mice. The resulting *Pten*^{Nkx2.1-cre} mutants were analyzed for lung defects and response to injury.

Measurements and Main Results: *Pten*^{Nkx2.1-cre} embryonic lungs showed airway epithelial hyperplasia with no branching abnormalities. In adult mice, *Pten*^{Nkx2.1-cre} lungs exhibit increased progenitor cell pools composed of basal cells in the trachea, CGRP/CC10 double-positive neuroendocrine cells in the bronchi, and CC10/SPC double-positive cells at the bronchioalveolar duct junctions. *Pten* deletion affected differentiation of various lung epithelial cell lineages, with a decreased number of terminally differentiated cells. Over time, *Pten*^{Nkx2.1-cre} epithelial cells residing in the bronchioalveolar duct junctions underwent proliferation and formed uniform masses, supporting the concept that the cells residing in this distal niche may also be the source of procarcinogenic stem cells. Finally, increased progenitor cells in all the lung compartments conferred an overall selective advantage to naphthalene injury compared with wild-type control mice.

Conclusions: *Pten* has a pivotal role in lung stem cell homeostasis, cell differentiation, and consequently resistance to lung injury.

Keywords: *Pten*; lung progenitor cells; injury

Cell renewal is critical for maintenance of tissue homeostasis, aging, and repair after injury. It is currently believed that this ability is derived from resident progenitor cells that have long-term self-renewal capacity and the potential to regenerate highly specialized differentiated cell types (1, 2). Most of our understanding of the repair process has been obtained in mice. In particular, this model system has been used to study lung regeneration. The lung epithelium is a major target of insults and is organized into functional compartments along its proximal-distal axis. The proximal lung includes ciliated cells (β -tubulin^{pos}),

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

PTEN is a well-known tumor suppressor that plays a key role in stem cell homeostasis in multiple organs, including the lung. The role of PTEN in lung injury and repair has not been studied yet.

What This Study Adds to the Field

This study demonstrates that lung epithelial-specific deletion of *Pten* leads to the expansion of epithelial progenitor cells and allows increased protection as well as regeneration of the airways after injury.

Clara cells (CC10^{pos}), and a small number of innervated neuroendocrine (NE) cells (calcitonin gene related peptide, CGRP^{pos}). The cartilaginous airways (bronchi) include a relatively unspecialized basal cell type that expresses P63 and keratins 14 and 5. In the more distal bronchi and bronchioles, the epithelium consists mostly of Clara cells. Respiratory alveoli, the most distal compartments of the lung, are composed of alveolar type I (T1- α ^{pos}) and type II (SPC^{pos}) cells.

The lung contains both multipotent and lineage-restricted progenitor cells (3). Repair of tissue after injury or during normal aging entails different strategies and progenitor cells in each of the various lung compartments. In the proximal lung, the basal cells meet the criteria for "stemness" (4–6). A subpopulation of NE cells expressing both CGRP and CC10 may also have progenitor cell properties (7). In the airways, a variant type of Clara cells that lacks detectable cytochrome P450 2F2 isozyme (CYP2F2) proteins is known to restore the epithelium after naphthalene injury (8). In the distal lung, differentiated alveolar type II epithelial cells are likely facultative progenitor cells (9, 10). Recently, a rare population of progenitor cells referred to as the bronchioalveolar stem cells or BASC have been identified within the transition region between the terminal bronchioles and the alveoli, the bronchioalveolar duct junction (BADJ) (11). Rarity of progenitor cells represents a major technical block to the badly needed characterization of their functional properties.

Pten (phosphatase tensin homolog) was initially identified as a tumor-suppressor gene because of its link to Cowden's disease. *Pten* encodes a lipid phosphatase responsible for the degradation of phosphatidyl-inositol triphosphate. Through this action, PTEN counterbalances the activity of the phosphatidylinositol-3-kinase, a central pathway of growth factor signaling, providing a sensitive and critical counterbalance to growth factor stimulation. Through the inhibition of the phosphatidyl-

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inositol-3-kinase signaling pathway, PTEN controls cell growth, cell cycle and apoptosis, glucose oxidation, and cell migration (12). *Pten* is also expressed at high levels in embryonic stem cells and regulates their proliferation (13). *Pten* deletion in mouse causes early embryonic lethality (14, 15). Tissue-specific deletion of *Pten* in the brain causes a phenotype similar to macrocephaly in humans due to increased number of progenitor cells (16). Loss of *Pten* in the intestinal progenitor cells initiates polyposis, a condition characterized by precancerous neoplastic increase in the number of crypts, which contain intestinal progenitor cells (17). In the hematopoietic system, PTEN is required to maintain hematopoietic stem cells (HSCs) in a quiescent state and absence of PTEN drives the entry of HSC into cell cycle generating leukemic stem cells (18). PTEN, therefore, has an important role in self-renewal and stem cell homeostasis in several organs.

In the current study, we examined the consequences of epithelial-specific early deletion of *Pten* in lung development using a novel *Nkx2.1-cre* driver line. Deletion of *Pten* caused an expansion of all known progenitor/stem cell populations in the lung: in the proximal epithelial cells, *Pten* deletion increased the P63/K14 double-positive cells; in the progeny of *Pten*-deleted distal epithelial cells, both CC10/SPC double-positive cells in the BADJ and the CGRP/CC10 double-positive NE cells were more abundant.

Our data indicate that such increase of the progenitor cells in the lung leads to an arrest in cell differentiation in the proximal and distal compartments. Epithelial cells in the mutant lungs were more resistant to injury and recovered faster than the control lungs. Therefore, *Pten* deletion in the airway epithelium confers relative resistance to airway injury.

Some of the results of these studies have been previously reported in the form of abstracts (19, 20).

METHODS

Additional details on the methods are provided in the online data supplement.

Generation of *Nkx2.1-cre*

A novel transgenic mouse strain carrying the genomic integration of a modified bacterial artificial chromosome (BAC) in which the second exon of *Nkx2.1* is replaced by the *cre* recombinase was recently published (21). The *Nkx2.1-cre* transgenic mice are fertile and show no obvious abnormalities.

Generation of *Pten^{Nkx2.1-cre}* Mice

Pten^{flox/flox} females (BALBc background) were mated with *Nkx2.1-cre* male mice (C57BL6 background). We backcrossed the mice for five generations to obtain mice carrying *Nkx2.1-cre*; *Pten^{flox/flox}* (henceforth referred to as *Pten^{Nkx2.1-cre}*) in a pure BALBc background. *Pten^{flox/flox}* mice were used as control.

Genotyping of the *Nkx2.1-cre* mice (21) and of *Pten^{flox}*, *Pten^Δ*, and *Pten^{wt}* alleles was performed as previously described (22).

All animal experiments were approved by the University of Southern California Animal use and care committee.

Histological Analysis

Embryonic lungs from control and mutant embryos were collected at E15.5 and E18.5. Adult lungs were dissected, inflated at 20 cm water pressure with 4% paraformaldehyde, and fixed overnight. The lungs were then dehydrated through increasing ethanol gradient concentration and embedded in paraffin. Sections (5 μm) were mounted on slides for histological analysis.

After performing antigen retrieval and blocking, the lung tissues were incubated overnight with the primary antibodies at different concentration (see online supplement for more details). Signals were visualized with the Histostatin Rabbit or Mouse Primary Kit (Zymed-

Invitrogen, Carlsbad, CA) or with secondary antibodies from Jackson ImmunoResearch (West Grove, PA).

Naphthalene Treatments

Naphthalene treatments were performed as previously described (9).

Cell Proliferation Analysis

Cell proliferation was assessed using Ki67 staining on 2-month-old control and mutant lungs (n = 3).

Figure 1. *Nkx2.1-cre* expression during lung development. (A–K) Detection of *cre*-induced β-galactosidase activity at different embryonic stages. (A, B) E10.5 whole mount β-galactosidase staining of *Rosa26R^{Nkx2.1-cre}* detecting (A) activity at the level of the brain and the lung primordia (arrows). (B) Notice the strongest staining at the airway level (arrow). (C, D) β-galactosidase staining of WT and *Rosa26R^{Nkx2.1-cre}* embryos at E13.5. (C) The control does not present any staining, whereas (D) the *Rosa26R^{Nkx2.1-cre}* shows staining at the level of the brain (arrow), (E) thyroid (arrow), and (D) lung (arrow). (E–G) At E13.5, Lac-Z expression in the distal lung is heterogeneous, with areas more stained compared with others. The extrapulmonary and intrapulmonary airways were labeled completely, whereas the distal parenchyma presents some spots with a decreased degree of activity (G, higher magnification of F). (H, I) At E15.5, the majority of the cells were labeled in both of the compartments. (I) Vibrotome section through E15.5 lung. (J, K) At the adult stage, the majority of the cells in the distal compartment are stained. The airways present always a strongest β-galactosidase activity compared with the distal compartment (K, higher magnification of J). Br = brain; L = lung; T = thyroid.

Figure 2. Deletion of *Pten* does not affect branching morphogenesis during lung development. (A–D) Hematoxylin and eosin (H&E) staining of lung sections of control animals ($n = 4$, A and C) and mutant *Pten*^{Nkx2.1-cre} ($n = 4$, B and D) at E15.5, detecting no differences in branching between the two groups. Magnification A and B, $\times 10$; C and D, $\times 20$. (E–H) Lung sections were stained with PTEN antibodies and NKX2.1 antibodies (magnification $\times 40$); in the control, the cells (G) expressing NKX2.1 also (E) expressed PTEN. In the mutant, (H) these cells did not present PTEN staining, except (F) for very few cells (arrows). (I, J) H&E staining of trachea sections of wild type ($n = 4$, I) and mutant ($n = 4$, J) at E15.5, showing the epithelium hyperplasia present in the *Pten*^{Nkx2.1-cre} tra-

chea. (K, L) Trachea sections were stained with PTEN antibodies; in the control the cells were positive for PTEN, whereas (L) in the mutant there was an homogenous deletion of the gene in all the epithelial cells. (M) Tissue-specific deletion of *Pten* was also proved by polymerase chain reaction analysis. Primers for recombination analysis were designed as described previously (22). P1/P2 amplified the flanked-exon 5 ($\Delta 5$). e = epithelium, br = bronchi.

Protein Extraction and Western Blot

Total protein extracts were prepared from 3-week-old *Pten*^{fl/fl} and *Pten*^{Nkx2.1-cre} lungs with radio-immunoprecipitation assay buffer (Sigma, St. Louis, MO), separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then blotted to polyvinylidene difluoride membrane (Millipore, Billerica, MA). p-AKT was detected with antibodies purchased from Cell Signaling (Danvers, MA) (p-AKT) at the concentration suggested by the manufacturer.

RNA Extractions and Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated from lungs of transgenic mice and wild-type littermate control animals using a Qiagen (Carlsbad, CA) RNeasy kit and cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen). An ABI PRISM 7700 Sequence Detection System was used to detect the studied genes using pre-developed TaqMan assay reagents (Applied Biosystems, Foster City, CA). Data were normalized to β -actin (ACTB) mRNA levels as described previously (23).

Fluorescence-Activated Cell Sorting

Single lung cells were prepared from control and mutant lungs ($n = 3$) as described previously with some modifications (11). Sca-1/Lys6A, CD45, CD34, and CD31 antibodies were purchased from Pharmingen (San Jose, CA). Cell sorting was performed in a FACSaria Cytometer (BD Bioscience, San Jose, CA) and the data were analyzed by FACSDiva software version.

Data Presentation and Statistical Analysis

Data were presented as mean $\pm$ SEM unless otherwise stated. Statistical analyses were performed on the data with Student *t* test for comparison of two groups. *P* values 0.05 or less were considered as significant.

RESULTS

Nkx2.1-cre Recombinase Driver Mouse Line

Nkx2.1 encodes a key transcriptional regulator of lung morphogenesis whose onset of expression in the mouse occurs at

around embryonic Day E9.5 concomitant with the specification of the lung primordium (24). The murine *Nkx2.1* gene consists of three exons and a highly complex *cis*-active DNA region that controls its expression in the lung, brain, and thyroid (25). A novel transgenic cre mouse line was generated by inserting a modified BAC in which the second exon of *Nkx2.1* is replaced by the cre recombinase (21, 26). The pattern and efficiency of the *Nkx2.1-cre* line in mediating LoxP-dependent DNA excision in the lung epithelium was determined using *ROSA26R-LacZ* reporter mice. LacZ activity was virtually absent in the wild-type lungs (Figure 1C). In E10.5 *ROSA26R-LacZ*^{Nkx2.1-cre} embryos, LacZ activity was limited to the primordial lung and brain (Figures 1A and 1B, arrows). At E13.5, it was possible to detect LacZ activity in the lung epithelium, brain, and thyroid (Figures 1D and 1E, arrows) in the *ROSA26R-LacZ*^{Nkx2.1-cre} embryos. In E13.5 lungs, the pattern of LacZ activity was nearly homogeneous throughout the tracheal lung epithelium, with the exception of some random peripheral tips (Figures 1E–1G). In E15.5 and adult lungs (Figures 1H–1K), homogeneous epithelial staining was present in all epithelial cells, with the strongest expression proximally.

Thus, *Nkx2.1-cre* mice represent a highly useful tool for conditional deletion of epithelial genes very early in the course of lung development.

Epithelial-Specific Deletion of *Pten* by *Nkx2.1-cre*

To determine the potential role of *Pten* in lung morphogenesis, we used the *Nkx2.1-cre* mouse line to delete *Pten* in the lung epithelium. Homozygous deletion of *Pten* via *Nkx2.1-cre* was postnatally viable with a frequency consistent with expected mendelian ratios. Immunohistochemistry (IHC) analysis in *Pten*^{Nkx2.1-cre} lungs at E15.5 showed absence of PTEN protein in nearly 100% of epithelial cells with only rare positive staining in the mutant lungs (Figures 2E and 2F, arrows). PTEN-negative epithelial cells in the mutant lungs were positive for

Figure 3. Absence of *Pten* leads to bronchiolar hyperplasia secondary to an increase in proliferation rate and to a decrease in apoptosis. (A–D) Histological analysis through hematoxylin and eosin staining of lungs from wild type and mutants at E15.5 and E18.5 embryonic stage showing the presence of the epithelial hyperplasia. (E, F) E-cadherin staining for epithelial cells in the adult stage (PN60). (G–J) Ki67 staining in PN60 lungs detecting an increase of the Ki67-positive cells number in the *Pten^{Nkx2.1-cre}* mice compared with the control (G and H, magnification $\times 20$; I and J, magnification $\times 80$). (K, L) TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay in the mutant and control lungs. (M) Quantification revealed a statistically significant difference between the two groups ($n = 3$ mice per genotype), $*P \leq 0.01$ using the standard *t* test. (N) Statistical analysis of the apoptotic cells ($n = 3$ mice for genotype), $*P \leq 0.01$ using the standard *t* test. Lm = lumen.

Nkx2.1, indicating their lung epithelial cell identity (Figures 2G and 2H). At the trachea level the hyperproliferation of the epithelia was already present early during development (Figures 2I and 2J) in the *Pten^{Nkx2.1-cre}* mice. IHC for PTEN showed a homogenous deletion of the gene in approximately all the cells (Figure 2, compare L to K). We confirmed the deletion of *Pten* by polymerase chain reaction (PCR) using genomic DNA from lung tissue and two different sets of primers. Our results indicate the presence of the $\Delta 5$ allele that confirms *Pten* deletion (22) (Figure 2I). At E15.5 there were no detectable abnormalities in branching morphogenesis of the embryonic mutant versus control (*Pten^{lox/lox}*) lungs (Figure 2, compare A and C to B and D). However, quantification of the number of double-positive cells for E-cadherin (marker for epithelial cells) and phosphohistone H3 (marker of proliferation) in E15.5 lungs ($n = 3$ for each group) showed an increase in the proliferation rate of the epithelial cells in the mutant compared with the control ($1.51 \pm 0.14\%$ vs. $0.7 \pm 0.1\%$, $P \leq 0.01$, data not shown).

In the proximal lung epithelium, progressive epithelial hyperplasia extending from the trachea to the small bronchioles (Figures 3A–3F) was detected in the mutant embryos of all embryonic stages examined. In the adult stages, the epithelial cells positive for E-cadherin displayed a papillae-like structure with the apical side of the cells facing the airway lumen (Figures 3E and 3F). The hyperplastic epithelium showed evidence of increased cell proliferation, as documented by Ki67 immunostaining (Figures 3G–3M). The numbers of Ki67-positive cells in the mutant lungs exceeded by threefold the numbers found in the control lungs ($11 \pm 1.3\%$ vs. $4 \pm 0.4\%$, respectively; $n = 3$,

$P \leq 0.01$). In addition, analysis by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) revealed decreased apoptosis in the mutant lungs when compared with control lungs (Figures 3K and 3L). Further quantification of apoptosis (Figure 3N) confirmed the statistically significant decrease of the number of apoptotic cells in the mutants ($n = 3$) versus wild-type lungs ($n = 3$) ($0.3 \pm 0.06\%$ vs. $1 \pm 0.07\%$, $P \leq 0.01$). Therefore, early epithelial deletion of *Pten* causes airway hyperplasia that is detectable from early stages of lung development and in adult mice, due to increased cell proliferation and decrease of apoptosis.

Deletion of *Pten* Results in Expansion of Epithelial Cell Populations in Multiple Progenitor Cell Niches

When compared with control lungs, *Pten^{Nkx2.1-cre}* lungs showed expansion of cells within a number of previously defined progenitor cell niches. In the proximal lung the tracheal basal cells, defined by expression of P63 (Figures 4A and 4B) and keratin14 (Figures 4C and 4D), were significantly more abundant (Figures 4G and 4H, low magnification; Figures 4I and 4J, high magnification). Quantification confirmed the results obtained by immunofluorescence (IF) ($29 \pm 0.4\%$ vs. $51 \pm 1.1\%$, $n = 3$, $P \leq 0.01$; Figure 4K). More distally, in the bronchi, the neuroepithelial bodies (NEB), identifiable by CGRP/CC10 overlapping expression, were also increased in number in the *Pten^{Nkx2.1-cre}* versus control lungs (38 ± 2.04 vs. 12.5 ± 0.95 , $n = 3$, $P \leq 0.01$). Of note, although IF is not a quantitative technique, the NEB clusters were not only more numerous (Figure 5I) but also showed stronger immunoreactivity (Figures

Figure 4. Deletion of *Pten* increases number of basal cells in the *Pten^{Nkx2.1-cre}* trachea. (A–J) Lung sections from mutant *Pten^{Nkx2.1-cre}* and control littermates at 2 months of age were stained (A and B) for P63 and (C and D) for keratin14. Increased number of double-positive cells over the P63-positive cells was detected in the mutant lungs compared with control lungs (low magnification, G and H; high magnification, I and J). (K) Quantification analysis was performed using *t* test from three mice in each group, **P* ≤ 0.01.

5G and 5H). Real-time PCR data confirmed our IF data, showing a nearly 80-fold increase in CGRP expression in mutant compared with control lungs (Figure 5J). This observation suggests that either cells within the NEB clusters express higher levels of the two markers or that each cluster contains a larger number of cells.

When compared with control lungs, *Pten^{Nkx2.1-cre}* lungs also showed an expansion of progenitor cells occupying the BADJ region (Figure 6, compare A and C to B and D, respectively). Many of the *Pten^{Nkx2.1-cre}* cells were distinctly larger in size (Figures 6C and 6D, arrows). We used IF to determine whether any of the overexpanded cells in the BADJ were double positive for CC10 and SPC, a characteristic previously associated with putative progenitor cells in this region (11). Although in the control lungs these cells are extremely rare (Figures 6E and 6H), double staining for anti-CC10 and anti-SPC antibodies detected an increased number of CC10/SPC double-positive cells in the mutant lungs ($1.8 \pm 0.57\%$ vs. $0.3 \pm 0\%$, *n* = 3, *P* ≤

0.01; Figure 6, compare I and J to H; quantification analysis, Figure 6O). The CC10/SPC-positive cells were more convincingly revealed by confocal microscopy (Figures 6K–6N). Using fluorescence-activated cell sorter to further confirm this observation, we gated the BACs, defined as *Sca1⁺CD45⁻CD31⁻CD34⁺* cells, in the mutant and in the control lungs (*n* = 3 for each). The number of *Sca1⁺* cells in the *CD45⁻CD31⁻CD34⁺* cell population was more than threefold increased in *Pten^{Nkx2.1-cre}* compared with the control lungs (9.5 vs. 2.8%) (Figures 6P and 6Q). Thus, early epithelial deletion of *Pten* by *Nkx2.1-cre* expands several putative epithelial progenitor cell populations throughout the proximal-distal axis of the lung.

Pten^{Nkx2.1-cre} Cells Form Putative Progenitor Cell Masses in the BADJ

We examined the behavior of the *Pten^{Nkx2.1-cre}* cells residing within the BADJ in the mutant lungs over time. *Pten^{Nkx2.1-cre}* progenitor cells undergo proliferation as a function of time and within approximately 8 weeks of postnatal life, a mass consisting of *Pten^{Nkx2.1-cre}* epithelial cells around the BADJ area is detected in some, but not all, mutant lungs (Figures 7A and 7B). These masses are slow growing and do not interfere with viability or respiratory status of the animals (data not shown). Importantly, the cells within the mass express SPC (at higher level) and CC10 (at lower level) (Figures 7D and 7E). E-cadherin immunostaining showed that within the mass, the cells are organized into ductlike structures reminiscent of the pseudoglandular stage of early lung development and a distinct property of lung endodermal progenitor cells (Figure 7C). Interestingly, *N-myc*, a downstream target of activated β -catenin-dependent WNT signaling, was also highly expressed in the nuclei of the cells within the *Pten^{Nkx2.1-cre}* mass (Figure 7F).

Proliferation analysis inside the masses (Figure 7G) showed very few cells positive for Ki67: the quantification confirmed the slow proliferation rate inside the masses, not statistically significant compared with the parenchyma ($8.3 \pm 1.55\%$ vs. $12.3 \pm 1.85\%$, *P* > 0.05) (Figure 7H). These data indicate that the physiological role of *Pten* during normal lung development may be to constrain epithelial progenitor cell proliferation within the BADJ, an important lung progenitor cell niche.

Activation of AKT and β -Catenin in *Pten^{Nkx2.1-cre}* Cells

Deletion or inactivation of *Pten* is expected to cause increased activity (phosphorylation) of AKT. In addition, increased β -catenin activation has been associated with progenitor cell homeostasis and tumorigenesis in a number of tissues, including the intestine (17). Under normal physiological conditions, PTEN inhibits the stabilization of β -catenin by increasing the activity of GSK3. Recently, He and colleagues showed that the PTEN-AKT pathway phosphorylates β -catenin at the level of serine (Ser) 552, resulting in nuclear localization of this phosphorylated form of β -catenin in the intestinal stem cells. It is proposed that this nuclear form is acting as a transcription factor controlling stem cell homeostasis (17). Considering that the lung and the gastrointestinal tract share the same embryological origin, we therefore examined whether lung epithelial-specific deletion of *Pten* leads to increased phospho-AKT and nuclear β -catenin^{Ser 552} localization. Immunohistochemical staining of *Pten^{Nkx2.1-cre}* lungs showed increased level of phospho-AKT (Figure 8, compare C to D). Increased expression of P-AKT in mutant versus wild-type lungs was confirmed by Western blot analysis (Figure 8F). Double staining for E-cadherin and phosphorylated β -catenin^{Ser 552} was performed (Figures 8A and 8B). Quantification of nuclear phosphorylated β -catenin^{Ser 552}/E-cadherin double-positive cells over the total number of E-cadherin-

Figure 5. Inactivation of *Pten* leads to an increase of the neuroepithelial bodies in the bronchi. (A–H) Immunofluorescence for CC10 (A and B) and CGRP (C and D) in 8-week-old mutant and control lung sections. Increased size and brightness in the neuroepithelial bodies (NEB) (G and H, arrows) were observed in the *Pten^{Nkx2.1-cre}* lungs compared with the control animals. (I) Quantification analysis. The average and standard deviation from four mice were compared using the *t* test, **P* ≤ 0.01. (J) Relative expression of *CGRP* mRNA in the *Pten^{Nkx2.1-cre}* and control mice, confirming the statistically significant increase of the *CGRP* expression in the mutant compared with the control (data from three different animals, *P* ≤ 0.05). (K, L) Immunohistochemistry for CGRP in E18.5 control and mutant lungs, showing an increase of NEB numbers already during embryonic stages. Similar results are obtained in adult stages. Lm = lumen.

positive cells (*n* = 3 for each group) showed an increase of β -catenin^{Ser 552} nuclear localization in the epithelial cells in the *Pten^{Nkx2.1-cre}* compared with the *Pten^{lox/lox}* control lungs ($3.99 \pm 0.8\%$ vs. $1.13 \pm 0.34\%$, *P* ≤ 0.01, Figure 8E).

Finally, nuclear localized N-myc was increased in *Pten*-depleted epithelial cells compared with the surrounding parenchyma, which can be considered as an internal control (Figure 7F). Overall, these data indicate that β -catenin signaling has been functionally activated in *Pten^{Nkx2.1-cre}* lungs.

Impact of *Pten* Deletion on Epithelial Cell Lineage Determination

Expression of a number of cell markers was examined by IF and real-time PCR in *Pten^{Nkx2.1-cre}* versus *Pten^{lox/lox}* (control) lungs to determine the impact of epithelial *Pten* deletion on the emergence and differentiation of various lung epithelial cell lineages localized in the proximal and distal lung compartments. In the Clara cell lineage, IF for CC10 revealed a markedly increased number of Clara cells in the mutant lungs (Figures 9A and 9B). The increase in CC10-positive cells was associated with a decrease in the number of ciliated cells, believed to be their terminally differentiated progeny, as revealed by β -tubulin staining (Figures 9C and 9D, arrows). In the distal compartments, SPC, a Type II cell marker, was increased (Figures 9E and 9F), whereas T1- α and *Aqp 5*, two Type I cell markers, were decreased in *Pten^{Nkx2.1-cre}* lungs (Figures 9G and 9H), indicating

a block in transition from precursor to terminally differentiated cell types. The IHC results were validated by real-time PCR analysis of mRNA for the latter cell lineage markers. This analysis showed statistically significant increases of *CGRP*, *SPC*, and *CC10* and a decrease of β -tubulin IV (marker for ciliated cells) in the mutant versus control lungs. To better understand the mechanism underlying this phenomenon, we examined *hairless* and *enhancer of split 1* (*HES1*), known to be involved in cell determination in lung, particularly in the Clara cell lineage (27). Both IHC and real-time PCR showed increased *HES-1* in the mutant compared with the control lungs (Figures 9I and 9J). Therefore, *Pten* appears to play a necessary function in normal epithelial cell fate determination.

Impact of *Pten* Deletion on Airway Epithelial Cell injury

Conditional deletion of *Pten* causes airway epithelial hyperplasia in the trachea and in the bronchi. In the bronchi, these cells are CC10 positive. However, in the trachea, these cells are negative for CC10 as well as for all the known lung epithelial markers (data not shown). Based on this observation, we hypothesized that these cells are arrested during their differentiation process and, thus, may display a selective advantage in coping with airway injury. We therefore examined the response of the tracheal and bronchial airway epithelium of *Pten^{Nkx2.1-cre}* and control mice to naphthalene, a simple and well-defined model of lung injury. Corn oil was used as control.

Figure 6. Deletion of *Pten* increases the double-positive cells CC10/SPC in the BADJ. (A, D) Hematoxylin and eosin staining showed in the mutant an increase of cells at the BADJ level (compare A to B, lower magnification ×20; C to D, higher magnification, ×40) and these cells were also enlarged compared with the cells in the control (D, arrows). (E–J) Immunofluorescence for SPC and CC10 in control (n = 3) and mutant *Pten^{Nkx2.1-cre}* (n = 3) animals at PN60: the mutants presented an increased number of double-positive cells (E, F, and G, lower magnification, ×20; H, I, and J, higher magnification, ×80). (K–N) Double immunofluorescence was also detected in the mutant using a confocal microscope to confirm the staining in single double-positive cells (arrows). (O) Quantification analyses were performed in three mice from each group using the *t* test, **P* < 0.01. (P, Q) Fluorescence-activated cell sorter analysis of control (n = 3) and mutant (n = 3) lungs detecting CD45⁺CD31⁻CD34⁺Sca-1⁺ cells.

In *Pten^{fl/fl}* control mice, naphthalene injury was detected in the trachea (Figures 10A and 10D) and in the distal compartments (Figures 10G and 10J) when compared with the corn oil control animals. In the wild-type animals, intraperitoneally administered naphthalene denuded entirely the tracheal epithelium after 3 days (Figure 10B) with a partial reepithelization after 7 days (Figure 10C). In the distal airway, naphthalene caused epithelial cell death within 72 hours (Figure 10H) followed by reepithelization of the airways by presumably the P450^{neg} variant of Clara cells (28). After 7 days of injury, the epithelium was in part restored (Figure 10I). In contrast, after naphthalene administration, the proximal airway epithelium of *Pten^{Nkx2.1-cre}* lungs at 3 and 7 days post injury appeared to remain intact with no signs of injury (Figures 10E and 10F). At the bronchial level, injury was reduced (Figure 10, compare K to H) and repair enhanced (Figure 10, compare L to I) in the *Pten^{Nkx2.1-cre}* lungs, in comparison with the control group. Morphometric analysis of epithelial damage was carried out by measuring after CC10 IF staining, the ratio of nude surface (damaged surface) versus total epithelial surface in control compared with mutant lungs 1, 3, and 7 days after injury (Figure 10M). One day after injury, less epithelial damage is observed in mutant versus control lungs ($21 \pm 0.4\%$ vs. $79 \pm 0.4\%$, *P* ≤ 0.01). However, epithelial damage in mutant lungs increases at Day 3 after injury (from $21 \pm 0.4\%$ at Day 1 to $70 \pm 4.4\%$ at Day 3), whereas in the control, the extent of the epithelial damages remained similar (from $79 \pm 0.4\%$ at Day 1 to $88 \pm 3\%$

at Day 3). At 7 days after injury, epithelial damage was lower in mutant compared with control lungs ($37 \pm 4.7\%$ vs. $54 \pm 7\%$, *P* ≤ 0.05%). The ratio of epithelial damage (Day 7 vs. Day 3) can be considered as a measure of the efficiency of the repair process during this time period. Our results indicate indeed a better repair process in mutant versus wild-type lungs (0.53 vs. 0.61). The observed overall resistance to the injury observed at Day 1 correlates with the increased number of SPC/CC10 double-positive progenitor cells in the mutant compared with control lungs at this stage ($3.3 \pm 1\%$ vs. $0.2 \pm 0.06\%$, *P* ≤ 0.05) (Figure 10N). The improved repair process between Day 3 and Day 7 in mutant versus wild-type lungs correlates with the increased proliferation of CC10-positive cells ($2.9 \pm 0.8\%$ vs. $0.4 \pm 0.35\%$, *P* ≤ 0.05) (Figure 10O).

These results suggest that the *Pten^{Nkx2.1-cre}* airway epithelium has a significantly increased level of resistance to naphthalene and confers a better capacity to recover after injury.

DISCUSSION

Early *Pten* deletion in the lung does not affect branching morphogenesis but leads to conducting airway hyperplasia.

The results of our work confirm that *Pten* does not affect lung branching morphogenesis, but affects cell differentiation and blocks cells in a less differentiated status.

As this study was underway two reports outlined the results of epithelial-specific deletion of *Pten* on lung morphogenesis

Figure 7. The BADJ cells proliferate inside the parenchyma and give rise to masses. (A, B) Hematoxylin and eosin staining on PN180 *Pten^{Nkx2.1-cre}* lung showing that over time the cells in the BADJ grow in the parenchyma and give rise to a tumorlike mass. (C) E-cadherin staining showed that the tumor cells are epithelial cells acting as progenitors, forming structures (underscored) that resemble the branching happening during the pseudoglandular stage. (D, E) Immunofluorescence for SPC and CC10 detected double-positive cells (arrows) in the mass. (F) N-myc staining showed an increase of N-myc nuclear staining in tumor cells. Underscored line delimits the masse from the surrounding parenchyma. (G, H) Immunohistochemistry for Ki67 inside the masses showing very few cells positive for Ki67 (G). Statistical analysis did not show any statistically significant difference between the masses and the surrounding parenchyma (H). m = mass; p = parenchyma.

(29, 30). In both previous studies *Pten* deletion was achieved using the *SPC-rtTA;Tet(O)-cre* line.

Our findings are partially consistent with both reports: Yanagi and colleagues (2007), who induced *Pten* deletion in the distal lung epithelium from E10 to E16, found delayed lung branching along with impaired epithelial cell differentiation and neonatal lethality in 90% of mice, due likely to respiratory insufficiency (29). By contrast, Davé and coworkers (2007), used the same inducible *cre* model to effect epithelial *Pten* deletion

within a different time frame, from E0.5 to E14.5, and reported airway hyperplasia without impact on lung development or epithelial cell differentiation (30).

The differences in phenotype may simply be related to the different time points at which *cre* activation was effected or to the mixed genetic background of the mice; the latter has been well documented by observations that link onset and severity of tumorigenesis to the genetic background in *Pten* knockout mice (15). This dependence on the genetic background may well

Figure 8. Deletion of *Pten* increases p-AKT and β-catenin activation. (A, B) Immunofluorescence for β-catenin^{Ser522} and E-cadherin in E15.5 lung sections. Double-positive cells are increased in the mutant (B) compared with the control (A). (C, D) Immunohistochemistry for phospho-AKT in E15.5 lung sections: the *Pten^{Nkx2.1-cre}* lungs (D) showed up-regulation of phospho-AKT compared with the control (C). Pictures are representative of n = 3 separate mice per genotype, $P \leq 0.01$. (E) Statistical analysis of double-positive cells for β-catenin^{Ser522} and E-cadherin double-positive cells, showing the statistically significant increase of double-positive cells in the mutant compared with the control (n = 3 animals for each group). (G) Immunoblot for phospho-AKT in extract of whole lungs taken from control and mutants at 3 weeks of age, confirming the immunohistochemistry data. Data are representative of two animals for each group.

Figure 9. Absence of *Pten* impairs cell fate. (A–H) Immunofluorescence for (A, B) CC10, (C, D) β -tubulin, (E, F) SPC, and (G, H) T1- α from 2-month-old control and mutant lungs. (A, B) In the mutant, an increase of Clara cells (CC10 positive) is detected compared with the control (B). (C, D) β -tubulin staining showed a reduction in the ciliated cell number in the mutant (C, arrows) compared with the control (D). (E, F) Alveolar type II cells (SPC positive) were increased in the *Pten*^{Nkx2.1-cre} (E). (G, H) Decrease in alveolar type I cells (T1- α positive) was observed in the mutant. (I, J) Immunohistochemistry for HES-1 in 2-month-old control and mutant lung. (K–N) Related expression, as determined by real-time polymerase chain reaction of (I) CC10, (J) Tubulin IV, (K) SPC, and (L) Aqp-5 mRNAs in *Pten*^{Nkx2.1-cre} and control mice confirmed the lack of differentiation in the mutant lung (data from three different mice in each group). (O, P) Related expression of *Hes-1* mRNA in *Pten*^{Nkx2.1-cre} and control mice confirmed the increase of *HES-1* expression in the mutant. * $P \leq 0.05$.

apply to the role of *Pten* in organogenesis and could provide another potential explanation for the differences in lung phenotype observed in various studies.

In the current work, a homogeneous BALBc background was used to avoid the possible bias created by a mixed genetic background. *Nkx2.1-cre*, moreover, is not an inducible *cre* system and follows, with few exceptions, the pattern of endogenous *Nkx2.1* gene expression in the lung (Figure 1). In our hands, *Pten* deletion did not affect lung branching morphogenesis but caused epithelial airway hyperplasia. Moreover, none of the *Pten*^{Nkx2.1-cre} neonates experienced any respiratory distress, and any sporadic

death within the first 2 weeks of life was always associated with enlarged thyroid and obstruction of the trachea.

Thus, our results confirm a major role for *Pten* in proximal compared with distal lung morphogenesis. These data are supported by the fact that proliferation is affected only in the proximal airways of the mutant lungs, whereas there is no effect on the distal compartment.

Pten deletion through *Nkx2.1-cre*, therefore, represents a mixed phenotype between the two recent reports, without branching defects, but with airway epithelial hyperplasia and impaired cell fate.

Figure 10. Absence of *Pten* protects the airways from naphthalene injury. (A–F) Hematoxylin and eosin staining of a control and mutant trachea after corn oil or naphthalene injection. (A, D) Corn oil administration did not affect the tracheal structure in the control (A) or in the mutant (D). (B, E) Three days after naphthalene administration, tracheal epithelium in the control was completely denuded (B), whereas the *Pten^{Nkx2.1-cre}* tracheal epithelium did not show any sign of injury and the cells were able to survive (E). (C, F) At 7 days after injury, the *Pten^{fl/f}* trachea showed a reepithelization (C), whereas the mutant did not show any change in the morphology (F). (G–L) Immunofluorescence for CC10 in the bronchi. (G, J) Control (G) and mutant (J) bronchi did not present any damage after corn oil administration. (H, K) At 3 days after naphthalene injury, the injury in the control (H) was more extensive and severe compare with the mutant (K). (I, L) After 7 days, the *Pten^{Nkx2.1-cre}* (L) presented more cells compared to the control (I). Data from four different animals for each group. (M) Morphometric quantification of the epithelial damage at Day 1, 3, and 7 after naphthalene injury. Epithelial damage was measured as a ratio between nude surface and total surface in bronchi. Ten different fields from control and mutant lungs were considered (n = 4 animals in each group). **P* ≤ 0.05. (N) Quantification of the double-positive cells for SPC/CC10 versus total number of CC10-positive cells in control and mutant lungs at 1 day after injury. **P* ≤ 0.05. (O) Statistical analysis of the double-positive cells for PH3/CC10 versus total number of CC10-positive cells in the control and mutant lungs at 7 days after injury (n = 4). **P* ≤ 0.05. lm = lumen; p = parenchyma.

PTEN Controls Epithelial Progenitor Cell Pool Size in the Lung

Progenitor cells are localized along the proximal-distal axis of the lung, notably in specialized environments known as niches in the conducting airways and the BADJ. In *Pten*^{Nkx2.1-cre} lungs, a significant increase in K14/P63-positive cells localized in the trachea was observed (Figure 4). Other putative progenitor cells, including the CC10/CGRP double-positive NEB and SPC/CC10 double-positive cells in the BADJ were also increased (Figures 5 and 6). Yanagi and colleagues and Davé and colleagues also described an increased of NEB and BADJ cells, but an increase in progenitor cells in the *Pten*^{Nkx2.1-cre} trachea (area not affected by *SPC-rtTA;Tet(O)* cre driver line) reveals an additional role for *Pten* that had gone unnoticed by the previous studies (29, 30).

The increase in progenitor cells was also linked to impaired cell differentiation: in the proximal lung, the CC10-positive cells (called Clara cells) were present in higher numbers at the expense of ciliated cells, (recognized by β -tubulin staining). More distally we observed an increase in alveolar type II cells (SPC positive) at the expense of type I cells (T1 α positive). Both Clara cells and type II cells are considered to be progenitor cells, respectively, of the ciliated and the type I cells.

The increase in Clara cells is correlated with the increase of *Hes-1*, a transcriptional factor controlling the balance between endocrine and nonendocrine epithelial cell fate (27). Interestingly, in our study we did not observe a decrease in the neuroepithelial bodies, which is inconsistent with previous reports in which *Hes-1* inhibited neuroendocrine differentiation. Further studies are necessary to clarify the mechanisms underlying the impact of *Pten* in lung epithelial cell determination.

PTEN May Control Cell Fate and Progenitor Cell Homeostasis through β -Catenin

Absence of *Pten* in the cells leads to phosphatidyl-inositol triphosphate accumulation, which in turn leads to overactivation of several key signaling molecules, including AKT/PKB, mTOR, and S6 KINASE. AKT is the most characterized of these molecules. Numerous substrates for AKT have been identified that participate in control of cell metabolism, cell death, cell cycle progression, and cell differentiation (12). A primary target of AKT is GSK3, which destabilizes β -catenin and causes its degradation. Thus, deletion of *Pten* can activate β -catenin-dependent WNT signaling, a known regulator of progenitor cell behavior.

In addition, constitutive expression of a stable form of β -catenin in the lung epithelium leads to proximal airway hyperplasia similar to the one present in the *Pten*^{Nkx2.1-cre} lungs (C. Li, personal communication, 2008). Deletion of *Pten* increases β -catenin expression; thus it is possible to hypothesize that these cells may be arrested in a less differentiated state. Finally, deletion of *Pten* leads to an expansion of the progenitor cells and prevents the cells from undergoing terminal differentiation.

Absence of *Pten* at the BADJ Leads to Generation of Masses

Transformed cells, in which pathways related to self-renewal or stem cell homeostasis are activated, may be the source for tumor initiation, survival, and progression (31). Cancer may also arise from a selected number of progenitor cells that have in common the activation of selected pathways. This concept of "tumor stem cells" is already known in the hematopoietic system, where a rare group of stem cells (called leukemic stem cells), with an extensive capacity of self-renewal, can give rise to the majority of the leukemic cells (32).

Different candidate genes are suggested as regulator for the proliferative capacity of these cells. One of these is *Pten*, which

has a role in restricting the activation of hematopoietic stem cells as well as preventing leukemogenesis (33).

In our model it appears that, in the absence of PTEN, the CC10/SPC double-positive cells, considered as progenitor cells in the lung, in time give rise to slow-growing masses that do not interfere with respiratory mechanism. These cells proliferate inside the parenchyma, and at some point lose CC10 expression while retaining the more undifferentiated marker SPC. Over time, the cells form structures resembling the branching duct-like processes that are formed during early lung development, again indicating the less differentiated nature of these cells that may act as progenitors. A more detailed characterization of these cells is currently underway.

Pten^{Nkx2.1-cre} Airway Epithelium Exhibits Relative Resistance to Naphthalene Injury

Because mutant lungs showed an increase in the number of progenitor cells, we examined whether they may also demonstrate altered resistance to experimentally induced airway injury by naphthalene. In animal models of airway injury, exposure to naphthalene kills most Clara cells within the first 72 hours. Naphthalene is converted by the P450 (CytP4502F2) enzyme into its toxic derivatives, 1, 2-epoxide (34), a diepoxide (35), and quinines (36). A rare population of variant Clara cells (Clara^v cells) is believed to lack CytP4502F2 enzyme activity and hence is resistant to naphthalene killing. Clara^v cells are believed to act as progenitors, undergoing proliferation and subsequently repopulating the airway epithelium and reestablishing its cellular composition. In the absence of commercially available reagents for detecting CytP4502F2, an alternative, but functional assay for a putative progenitor cells in the airway may be their relative resistance to naphthalene killing. If expansion of the cells in the airway epithelium of *Pten*^{Nkx2.1-cre} lungs includes a larger number of such "progenitors," then their presence can be indirectly examined by assaying their relative resistance to naphthalene. Indeed, our results indirectly suggest the presence of an expanded population of progenitor Clara cells in the *Pten* mutant lungs as evidenced by their relative resistance to naphthalene injury and improved repair.

In summary, the current study, which was performed in a pure BALB genetic background, shows that epithelial deletion of *Pten* during early lung development does not affect lung branching morphogenesis. Deletion of *Pten* increased several progenitor pools in both proximal and distal lung compartments. In addition, absence of *Pten* inhibited cell differentiation of specialized epithelial cell types. The current study also shows that *Pten* has an important role in tracheal epithelial progenitor cell homeostasis. Finally, to our knowledge, our work has uncovered for the first time an impact of *Pten* deletion on lung epithelial airway injury.

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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