PTEN is a tumor suppressor gene mutated in many human cancers. We generated a bronchioalveolar epithelium–specific null mutation of Pten in mice [SP-CrtTA/((tetO)−Cre/Ptenfl/fl (SOPtenfl/fl) mice] that was under the control of doxycycline. Ninety percent of SOPtenfl/fl mice that received doxycycline in utero [SOPtenfl/fl(E10–16) mice] died of hypoxia soon after birth. Surviving SOPtenfl/fl(E10–16) mice and mice that received doxycycline postnatally [SOPtenfl/fl(P21–27) mice] developed spontaneous lung adenocarcinomas. Urethane treatment accelerated number and size of lung tumors developing in SOPtenfl/fl mice of both ages. Histological and biochemical examinations of the lungs of SOPtenfl/fl (E10–16) mice revealed hyperplasia of bronchioalveolar epithelial cells and myofibroblast precursors, enlarged alveolar epithelial cells, and impaired production of surfactant proteins. Numbers of bronchioalveolar stem cells (BASCs), putative initiators of lung adenocarcinomas, were increased. Lungs of SOPtenfl/fl (E10–16) mice showed increased expression of Spry2, which inhibits the maturation of alveolar epithelial cells. Levels ofAkt, c-Myc, Bcl-2, and Shh were also elevated in SOPtenfl/fl(E10–16) and SOPtenfl/fl(P21–27) lungs. Furthermore, K-ras was frequently mutated in adenocarcinomas observed in SOPtenfl/fl(P21–27) lungs. These results indicate that Pten is essential for both normal lung morphogenesis and the prevention of lung carcinogenesis, possibly because this tumor suppressor is required for BASC homeostasis.

Introduction
Lung cancer is the leading cause of death due to cancer all over the world, and the 5-year survival rate remains relatively poor despite aggressive medical therapy (1). Adenocarcinoma is the most common type of lung cancer in the United States, and its frequency is increasing rapidly (2, 3). As with most cancers, lung cancer appears to arise by a transformation of organ-specific stem cells or progenitor cells that results in the selective expression of genes enhancing self-renewal potential (2, 4). In this light, stem cells can be considered proto-tumorigenic, in that they must first undergo genetic mutation before they can initiate cancers (2).

Three types of organ-specific stem cells are thought to play key roles in maintaining the epithelial layers of lung tissue. The first type is the basal cell, which generates Clara cells and ciliated cells and is located at the submucosal gland duct junctions or intra-cartilaginous boundaries of the proximal airway. The second stem cell type is the variant Clara cell, which also generates Clara cells as well as bronchial and bronchiolar neuroendocrine cells and is located adjacent to neuroepithelial bodies in the bronchi/bronchioles. The third stem cell type is the bronchioalveolar stem cell (BASC), which generates Clara cells and alveolar type I (AE-I) and AE-II epithelial cells and is located at the bronchioalveolar duct junction (2). Among these putative stem cell types, it is the BASCs that have recently been proven to exhibit self renewal and multipotency, properties characteristic of stem cells. Importantly, BASCs have been shown to give rise to lung adenocarcinomas (5). Overexpression of K-ras increases the proliferation of BASCs both in vitro and in vivo, whereas this increased proliferation is not seen in cultured differentiated alveolar cells that overexpress K-ras (5). It is not known what molecules in addition to K-ras have positive or negative regulatory effects on BASCs.

Pten is a multifunctional phosphatase whose major substrate is phosphatidylinositol-3,4,5-trisphosphate (PIP3) (6), a lipid second messenger molecule. PIP3 is generated by the action of PI3K, which has been activated in response to various growth factors (7). PIP3 in turn activates numerous downstream targets, including the serine/threonine kinase Akt/protein kinase B involved in apoptosis, proliferation, and oncogenesis (8). By using its lipid phosphatase activity to dephosphorylate PIP3 at the cell membrane, Pten negatively regulates the PI3K/Akt pathway and exerts tumor suppression. In addition, recent studies have shown that
Pten is required for the maintenance of organ-specific stem/progenitor cells in adult brain (9), prostate (10), and blood cells (11), as well as germ cells in the embryo (12).

While mutation of the PTEN gene itself is an infrequent event in lung adenocarcinomas (13, 14), loss of PTEN protein expression is seen in 39%–77% of these tumors (14, 15). Indeed, loss of PTEN expression leading to Akt activation correlates positively with an increased area of lung adenocarcinoma cells treated with siRNA to inactivate Akt fail to proliferate in response to fibronectin (16). These observations support a prosurvival role for Akt activation in lung adenocarcinomas.

In addition to its function as a tumor suppressor, Pten plays a critical role in the development of various murine tissues, including T cells (17), B cells (18), endothelial cells (19), and germ cells (20). Whether Pten also influences lung development is currently unknown. The development of lung tissue is an intricate process that involves branching morphogenesis and complex pathways of epithelial and mesenchymal cell differentiation. Based on histology, embryonic lung development can be divided into the pseudoglandular stage (E9.5–E16.5), the canalicular stage (E16.6–E17.4), and the terminal sac stage (E17.5–P0) (20). To date, the only information available on Pten function in the lung is that high levels of Pten expression occur in the respiratory epithelium at the terminal sac stage (21). To thoroughly investigate the roles of Pten in normal lung development and the prevention of tumorigenesis, we generated mice deficient for Pten specifically in bronchioalveolar epithelial cells. Our results provide the first comprehensive evidence that Pten is essential for normal lung morphogenesis, the homeostasis of BASCs, and the prevention of lung adenocarcinomas.

### Results

**Generation of bronchioalveolar epithelium-specific Pten-deficient mice.** Bronchioalveolar epithelium–specific Pten-deficient mice [SP-C-rTetO/-(tetO)-Cre/Pten<sup>flox/flox</sup> mice, referred to here as SOPten<sup>flox/flox</sup> mice] were generated by mating (tetO)-Cre transgenic mice (22) with SP-C-rTetO (23) and Pten<sup>flox/flox</sup> mice, in which Pten exon 5 encoding the phosphatase domain is flanked by loxP sequences (24). Administration of doxycycline either in utero (E10–E16) or postnatally (P21–P27 or P84–P90) activates expression of Cre in combination with reverse tetracycline transactivator (rtTA) (23), which triggers recombination and deletion of the floxed Pten gene. Southern blotting of DNA from whole lung tissue of 8-week-old SOPten<sup>flox/flox</sup> (E10–E16) mice showed that Cre-mediated recombination of the loxP sites had deleted most of the 6.0-kb Pten<sup>flox</sup> allele in a majority of cells, leaving the 2.3-kb Pten<sup>Δ</sup> allele (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI31854DS1). Western blotting confirmed a dramatic reduction of Pten protein in lungs of 8-week-old SOPten<sup>flox/flox</sup> (E10–E16) mice (Supplemental Figure 1B).

**Impaired lung morphogenesis in SOPten<sup>flox/flox</sup> (E10–E16) mice.** Interbreeding of (tetO)-Cre/Pten<sup>flox/flox</sup> and SOPten<sup>flox/flox</sup> mice generated SOPten<sup>flox/flox</sup> pups that were born at the expected Mendelian ratio. However, about 90% of SOPten<sup>flox/flox</sup> (E10–E16) mice died within 2 hours of birth (Table 1). In contrast to WT (E10–E16) neonates, which breathed regularly, were well oxygenated, and actively moved their limbs, SOPten<sup>flox/flox</sup> (E10–E16) neonates breathed irregularly without gasping and had a cyanotic skin color (Figure 1A). Arterial blood gas analyses confirmed the presence of markedly hypoxic and respiratory acidosis in SOPten<sup>flox/flox</sup> (E10–E16) neonates (Table 2). SOPten<sup>flox/flox</sup> (P21–P27) and SOPten<sup>flox/flox</sup> (P84–P90) mice did not show any neonatal lethality.

### Table 1

<table>
<thead>
<tr>
<th>SOPten&lt;sup&gt;flox/flox&lt;/sup&gt; (E10–E16)</th>
<th>Alive (n = 28)</th>
<th>Dead (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (E10–E16)</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>SOPten&lt;sup&gt;flox/flox&lt;/sup&gt; (E10–E16)</td>
<td>1</td>
<td>21</td>
</tr>
</tbody>
</table>

Genotyping of P0 mice derived from (tetO)-Cre/Pten<sup>flox/flox</sup> female and SP-C-rTetO/(tetO)-Cre/Pten<sup>flox/flox</sup> male intercrosses.

Histologically, lungs of WT(E10–E16) mice showed normal sacular expansion and septal thinning at birth, whereas lungs of SOPten<sup>flox/flox</sup> (E10–E16) mice showed notable alveolar septal and bronchiolar hyperplasia with reduced airspaces (Figure 1B). Cell counting confirmed that total cell numbers in SOPten<sup>flox/flox</sup> (E10–E16) neonatal lungs were increased 1.5-fold over WT(E10–E16) levels at birth (Figure 1C). Further histological analyses of lungs from WT(E10–E16) and SOPten<sup>flox/flox</sup> (E10–E16) embryos at various stages of gestation revealed that loss of Pten delayed lung development at the terminal sac stage (E17.5 to about P0; Figure 1D) but did not affect the pseudoglandular (E14.5; Figure 1D) or canalicular (E16.6; Figure 1D) stages. Specifically, terminal sac–stage (E17.5 to about P0) lungs from WT(E10–E16) animals showed normal progression with the formation of the sac-like structures that serve as precursors of the alveoli (Figure 1D, upper panels). However, terminal sac–stage lungs from SOPten<sup>flox/flox</sup> (E10–E16) mice showed a significant delay in the dilatation of the distal tubules (Figure 1D, lower panels).

Ultrastructure analysis by transmission electron microscopy showed marked increases in numbers of both alveolar epithelial cells and mesenchymal cells in lungs of E19.5 SOPten<sup>flox/flox</sup> (E10–E16) embryos (Figure 2A). In WT(E10–E16) lungs the septa contained 2 layers of capillaries juxtaposed to the alveolar lumen (Figure 2A, upper left panel). The flat AE-I cells and capillary endothelial cells constituting the blood-air barrier that facilitates efficient gas exchange were clearly visible (Figure 2A, arrowheads). In contrast, the septa of SOPten<sup>flox/flox</sup> (E10–E16) lungs contained an increased number of cuboidal undifferentiated alveolar epithelial cells and mesenchymal cells. The size of each alveolar epithelial cell was also increased. Consequently, the blood-air barrier was much thicker in SOPten<sup>flox/flox</sup> (E10–E16) lung tissue (Figure 2A, right panels) than in WT(E10–E16) lungs. The increased numbers of alveolar epithelial cells and mesenchymal cells in the mutant lung were caused by enhanced cell proliferation rather than reduced apoptosis at E19.5, as determined by BrdU incorporation (Supplemental Figure 2A) and TUNEL assays (Supplemental Figure 2B). To determine the origin of the expanded mesenchymal cell population, immunohistochemical (IHC) analysis was performed using anti-CD31, anti-α-SMA, anti-desmin, and anti–glial fibrillary acidic protein (anti-GFAP) antibodies. As shown in Supplemental Figure 3, the septa of SOPten<sup>flox/flox</sup> (E10–E16) lungs exhibited a marked increase in cells that were α-SMA positive but desmin negative. Cells with this surface marker expression pattern are reportedly precursors of myofibroblasts (25).

Taken together, these results indicate that the abnormal thickness of the blood-air barrier in SOPten<sup>flox/flox</sup> (E10–E16) lungs is the result of the in utero and postnatal expression of the floxed Pten gene, indicating that Pten is essential for normal lung morphogenesis and the homeostasis of BASCs, and the prevention of lung adenocarcinomas.
result of increases in the number and size of alveolar epithelial cells as well as elevated numbers of myofibroblast precursors in the septa. These defects in the blood-air barrier are likely responsible for the respiratory failure of SOPten<sup>flox/flox</sup>(E10–E16) mice.

**Impaired alveolar epithelial cell differentiation in SOPten<sup>flox/flox</sup>(E10–E16) embryos.** Bronchioalveolar epithelial cells differentiate during the terminal sac stage, resulting in distinct cell lineages generated along a proximodistal axis (20). To determine the level of maturity of alveolar epithelial cells in SOPten<sup>flox/flox</sup>(E10–E16) lungs, we examined the expression of lung epithelial cell–specific marker proteins by Western blotting and IHC analysis. The expression levels of the AE-II cell marker surfactant proteins A (SP-A), B, C, and D as well as that of the AE-I cell marker aquaporin-5 (AQP5) were severely attenuated in SOPten<sup>flox/flox</sup>(E10–E16) lungs compared with WT(E10–E16) lungs at E19.5 (Figure 2, B and C). The defect in alveolar epithelial cell differentiation in SOPten<sup>flox/flox</sup>(E10–E16) lungs was confirmed by transmission electron microscopy. In WT(E10–E16) lungs at E19.5, AE-I cells are distinctive in appearance and characterized by their squamous morphology. WT AE-II cells are recognized by their characteristic apical microvilli and lamellar bodies, which are involved in the storage and exocytosis of SPs (Figure 2A, lower left panel). In addition, surfactant materials are observed within the sacculair spaces of WT(E10–E16) lungs (Figure 2A, arrow). In contrast, no differences were detected between the WT and mutant embryos at E14.5 or E16.6, but dramatic differences were visible from E17.5 onward. WT(E10–E16) lungs showed dilatation of distal tubules and mesenchyme thinning at E17.5, with progression of septation from E17.5 to P0. SOPten<sup>flox/flox</sup>(E10–E16) lungs showed fewer sacculair structures during this period. Scale bars: 200 μm.
Defective expression of molecular markers in SOPten\textsuperscript{flx/flx}(E10–E16) embryos. To examine the expression of key molecules involved in lung morphogenesis and distal epithelial cell differentiation, we performed RT-PCR analyses of whole lung tissue from E19.5 WT(E10–E16) and SOPten\textsuperscript{flx/flx}(E10–E16) embryos. The expression of Spry2, a Sprouty family protein that negatively regulates lung morphogenesis and SP-A, -B, and -C expression in murine embryonic lungs (27, 28), and Shh, a soluble factor that positively regulates epithelial and mesenchymal cell proliferation during lung development (29, 30), were dramatically upregulated in SOPten\textsuperscript{flx/flx}(E10–E16) lungs (Figure 3A). There were no differences between the genotypes in the expression of signaling molecules such as FGFs, bone morphogenetic protein, and Wnt7b, or in various transcription factors important for alveolar epithelial cell differentiation. Immunostaining of SOPten\textsuperscript{flx/flx}(E10–E16) lungs with anti-Spry2 and anti-Shh antibodies revealed markedly elevated expression of these molecules compared with their levels in WT(E10–E16) lungs, especially in mutant alveolar epithelial cells (Figure 3B). These data suggest that abnormally increased expression of Spry2 and Shh in alveolar epithelial cells may underlie the impaired alveolar epithelial cell differentiation and epithelial and mesenchymal cell proliferation seen in SOPten\textsuperscript{flx/flx}(E10–E16) mice.

Bronchioalveolar epithelial hyperplasia and increased cell size of alveolar epithelial cells in postnatal SOPten\textsuperscript{flx/flx} mice. To determine whether mice that suffered loss of Pten after birth develop lung tumors, we administered doxycycline to WT and SOPten\textsuperscript{flx/flx} mice at P21 or P84 for 7 days and examined the bronchioalveolar epithelium 9 weeks later. Histological analysis revealed mild bronchiolar and alveolar epithelial hyperplasia in both SOPten\textsuperscript{flx/flx}(P21–P27) and

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>WT(E10–E16)</th>
<th>SOPten\textsuperscript{flx/flx}(E10–E16)</th>
<th>P</th>
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<tr>
<td>pH</td>
<td>7.44 ± 0.04</td>
<td>6.89 ± 0.19</td>
<td>0.0083</td>
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<tr>
<td>PaO\textsubscript{2} (Torr)</td>
<td>106.4 ± 4.6</td>
<td>57.6 ± 3.5</td>
<td>0.001</td>
</tr>
<tr>
<td>PaCO\textsubscript{2} (Torr)</td>
<td>38.4 ± 4.6</td>
<td>72.5 ± 20.8</td>
<td>0.04</td>
</tr>
<tr>
<td>SaO\textsubscript{2} (%)</td>
<td>100.0 ± 0.0</td>
<td>48.7 ± 12.2</td>
<td>0.0003</td>
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</table>

Arterial blood gas analysis in WT(E10–E16) and SOPten\textsuperscript{flx/flx}(E10–E16) mice at P0. Results are expressed as the mean ± SD of 4 mice/group. Statistical differences were determined using the Student’s t test.

Abnormal thickness of the blood-air barrier and impaired alveolar epithelial cell differentiation in SOPten\textsuperscript{flx/flx}(E10–E16) lungs. (A) Transmission electron micrographs of the lung septa of E19.5 embryos shown at low (LM) and high (HM) magnification. WT(E10–E16) lung showed AE-I and AE-II cells plus 2 layers of capillaries (asterisks) separated by mesenchymal cells (M; upper left panel). The AE-II cells contained many lamellar bodies (white arrowheads) and apical microvilli (lower left panel). SPs (arrow) were visible in the saccular spaces (upper left panel). Black arrowheads (left panels) indicate the thin, normal blood-air barrier, composed of AE-I cells and capillary endothelial cells, in the WT lung. In the SOPten\textsuperscript{flx/flx}(E10–E16) lung, the septa were thick with increased mesenchymal cells (upper right panel). Increased numbers of undifferentiated cuboidal epithelial cells of enlarged size were present (CC; upper right panel). The blood-air barrier (red bars) was significantly thicker in SOPten\textsuperscript{flx/flx} lungs than in WT lungs (lower panels). Scale bars: 10 μm (LM); 5 μm (HM). (B) Western blotting of SP-A, -B, -C, -D, AQP5, and CCSP proteins in extracts of whole lungs taken from WT(E10–E16) and SOPten\textsuperscript{flx/flx}(E10–E16) mice at E19.5. Actin was used as a loading control. Data shown are representative of 3 trials. (C) IHC analysis of SP-C, AQP5, CCSP and CGRP. SP-C and AQP5 immunostaining was intense in cuboidal AE-II and flat AE-I cells, respectively, in WT(E10–E16) lungs at E19.5 but dramatically decreased in SOPten\textsuperscript{flx/flx}(E10–E16) lung at E19.5. Scale bars: 50 μm.
SOPten\textsuperscript{flx/flx}(P84–P90) mice (Figure 4A). However, the structure of these alveolar epithelial cells in SOPten\textsuperscript{flx/flx}(P21–P27) and SOPten\textsuperscript{flx/flx}(P84–P90) mice was normal, as determined by transmission electron microscopy (Figure 4B). An increase in the cell size of both AE-I and AE-II cells was also evident in both SOPten\textsuperscript{flx/flx}(P21–P27) and SOPten\textsuperscript{flx/flx}(P84–P90) lungs (Figure 4B). IHC analyses of lung epithelial cell–specific marker proteins showed no significant differences in bronchiolar and alveolar epithelial differentiation in SOPten\textsuperscript{lox/lox}(P21–P27) and SOPten\textsuperscript{lox/lox}(P84–P90) mice compared with controls (Figure 4C).

Spontaneous lung adenocarcinomas and increased susceptibility to urethane-induced lung tumor formation in SOPten\textsuperscript{flx/flx} mice. About 10% of SOPten\textsuperscript{flx/flx}(E10–E16) mice survived until adulthood. Because a reduction or absence of PTEN protein expression has frequently been reported in human non–small cell lung cancers, we monitored development of adenocarcinomas. Strikingly, all SOPten\textsuperscript{flx/flx}(E10–E16) mice showed macroscopic lung tumors (Supplemental Figure 4A, upper right panel). Histological examination revealed that 13 of these tumors were adenocarcinomas (Supplemental Figure 4A, lower left panel), while 1 mutant mouse developed a squamous cell carcinoma (Supplemental Figure 4A, lower right panel). In contrast, spontaneous lung tumor formation was not observed in any WT(E10–E16) mice (Supplemental Figure 4A, upper left panel) during the observation period. Kaplan-Meier analyses showed that the cancer-free survival rates for SOPten\textsuperscript{flx/flx}(E10–E16) mice were significantly lower than for controls (log-rank, \(P < 0.001\); Supplemental Figure 4B).

We next investigated the effect of Pten deficiency on carcinogenesis in the lung after this organ had been fully formed. As was true for SOPten\textsuperscript{flx/flx}(E10–E16) mice, 87% (13/15) of SOPten\textsuperscript{flx/flx}(P21–P27) mice showed spontaneous lung adenocarcinoma formation, whereas no tumors were observed in WT(P21–P27) lungs (\(n = 36\)) during the 40- to 70-week observation period (Figure 5A). These findings indicate that Pten function is essential both prenatally and postnatally for preventing lung tumorigenesis, especially the development of adenocarcinomas.

To examine chemically induced lung carcinogenesis in the absence of Pten, 8- to 10-week-old WT(E10–E16) and SOPten\textsuperscript{flx/flx}(E10–E16) mice were i.p. injected with urethane, a well known initiator of lung carcinomas (31). At 20 weeks after injection, lung tumors had developed in 94% (17/18) of SOPten\textsuperscript{flx/flx}(E10–E16) mice, whereas only 42% (11/26) of WT(E10–E16) animals showed lung tumors (\(P < 0.001\)). The numbers and sizes of the lung tumors were also significantly greater in SOPten\textsuperscript{flx/flx}(E10–E16) mice than in WT(E10–E16) mice (Supplemental Figure 4C, upper panels, and Supplemental Figure 4D). Histological examination demonstrated that 1 SOPten\textsuperscript{flx/flx}(E10–E16) mouse developed a lung adenocarcinoma (Supplemental Figure 4C, lower right panel), whereas all other tumors that formed in both WT and SOPten\textsuperscript{flx/flx} lungs were lung adenomas (Supplemental Figure 4C, lower left panel). Similar results were obtained when the lungs of WT(P21–P27) and SOPten\textsuperscript{flx/flx}(P21–P27) mice were subjected to urethane treatment. The numbers and sizes of the tumors that developed in the lungs of SOPten\textsuperscript{flx/flx}(P21–P27) mice were significantly greater than those forming in WT(P21–P27) lungs (\(P < 0.001\) and \(P < 0.005\), respectively; Figure 5, B and C). Thus, both prenatal and postnatal Pten-deficient mice show increased susceptibility to urethane-induced lung tumorigenesis.

Pten deficiency induces significant increases in numbers of BASCs and side population cells. Our results showed that bronchioalveolar epithelium–specific Pten deficiency almost inevitably leads to lung adenocarcinoma. Since lung adenocarcinomas reportedly originate from BASCs, which are defined as Sca-1–CD34–CD45–CD31– and possess stem cell properties (5), we evaluated the number of BASCs in lungs of WT(E10–E16) and SOPten\textsuperscript{flx/flx}(E10–E16) mice prior to the development of tumors in the latter. Interestingly, there was a 5.2-fold increase in BASCs in 8-week-old SOPten\textsuperscript{flx/flx}(P21–P27) mice compared with WT(P21–P27) animals (\(P < 0.05\), Figure 6A and 6B, right panel). A comparable increase in BASCs was observed in SOPten\textsuperscript{flx/flx}(E10–E16) mice compared with WT(E10–E16) mice (\(P < 0.05\); Figure 6, left panel). We also examined the lungs of WT(P21–P27) and SOPten\textsuperscript{flx/flx}(P21–P27) mice for side population cells, which exhibit Hoechst dye efflux properties commonly observed in various types of stem cells (32). The side population fraction is highly enriched in clonogenic cells of the proximal and distal airway (including BASCs; ref. 33) that express epithelial transcription factors (34). In lungs of 8-week-old SOPten\textsuperscript{flx/flx}(P21–P27) mice, we found a 4.0-fold increase in side population cells compared with controls (\(P < 0.005\); Figure 6, right panel).
Lung side population cells were similarly increased in *SOPten* ^{flx/flx} (E10–E16) mice compared with WT(E10–E16) mice (P < 0.05; Figure 6D, left panel). These results indicate that Pten deficiency leads to an abnormal increase in numbers of BASCs and side population cells.

**Altered cancer-related molecules in SOPten^{flx/flx} lungs.** Several downstream effectors of the Pten/PI3K pathway are reportedly involved in stem cell self renewal and lung carcinogenesis (15, 35–41). We used Western blotting to examine the expression of these molecules in whole lung extracts from 8-week-old WT(P21–P27) and *SOPten* ^{flx/flx} (P21–P27) and WT(P84–P90) and *SOPten* ^{flx/flx} (P84–P90) mice. The increased sizes of AE-I and AE-II cells can be seen in *SOPten* ^{flx/flx} lungs at both time points. Lamellar bodies and apical microvilli, which are signature structures of AE-II cells, are visible within the AE-II cells of both WT and *SOPten* ^{flx/flx} lungs. Scale bars: 5 μm. (C) IHC analysis of SP-C, AQP5, and CCSP expression in lungs of WT(P21–P27) and *SOPten* ^{flx/flx} (P21–P27) and WT(P84–P90) and *SOPten* ^{flx/flx} (P84–P90) mice. Although the numbers of AE-I and AE-II cells were increased in lungs of *SOPten* ^{flx/flx} (P21–P27) and *SOPten* ^{flx/flx} (P84–P90) mice, no significant differences between WT and *SOPten* ^{flx/flx} mice were observed in the staining intensity of SP-C, AQP5, or CCSP at either time point. Scale bars: 50 μm.

Parallel molecular alterations were observed in the lungs of neonatal *SOPten* ^{flx/flx} (E10–E16) mice (data not shown). There were no obvious differences between WT and *SOPten* ^{flx/flx} lungs (either prenatal or postnatal) in the expression of pErk, pRb, p53, p21, or Notch1. Among these molecules, activation of Akt (35), c-Myc (36), Shh (37), and Bcl-2 (38) and inactivation of p27 (39) have been shown to positively regulate stem cell self renewal. Moreover, elevated expression levels of pAkt (15), c-Myc (40), and Bcl-2 (41) have been observed in human lung adenocarcinomas. These findings collectively suggest that loss of Pten mediates alterations in the expression of molecules that promote the expansion of BASCs. This expansion may be an underlying cause of the onset of bronchioalveolar epithelial cell hyperplasia and lung adenocarcinomas observed in our Pten-deficient mutants.
Frequent K-ras mutations in spontaneous adenocarcinomas of SOPten\textsubscript{flox/flox}\textsuperscript{(P21–P27)} lungs. Because activating K-ras mutations are detected in 20%-30% of human lung adenocarcinomas (42), we hypothesized that the Pten deficiency in our mice might elevate the risk of additional oncogenic events such as K-ras mutations. We used direct sequencing of PCR-amplified DNAs to analyze paired samples of a spontaneous adenocarcinoma and the corresponding non-tumorous lung tissue from 6 SOPten\textsubscript{flox/flox}\textsuperscript{(P21–P27)} mice. Interestingly, activating point mutations in codon 61 of K-ras were observed in 2 of these 6 (33%) spontaneous adenocarcinomas. In both cases, the mutation was a transversion of the second nucleotide of codon 61 (CAA to CTA and CAA to CGA) (Figure 7B and data not shown). These types of alterations have been previously reported in spontaneous lung adenocarcinomas of A/J × C3H F1 mice (43). In contrast, no K-ras mutations were detected in the corresponding non-tumorous lung tissue samples obtained from the same SOPten\textsubscript{flox/flox}\textsuperscript{(P21–P27)} mice. Our results thus suggest that the expansion of BASCs driven by the loss of Pten in SOPten\textsubscript{flox/flox}\textsuperscript{(P21–P27)} mice may increase the risk of additional oncogenic mutations in these cells, including alterations of K-ras. The combined effects of Pten deficiency, K-ras mutation, and perhaps other tumorigenic events may further increase the proliferation of BASCs such that they eventually initiate lung adenocarcinoma formation.

Discussion

Pten is essential for lung morphogenesis and distal lung epithelial cell differentiation. The formation of the fetal lung occurs via a process of branching morphogenesis and depends upon complex paracrine signaling between epithelial and mesenchymal cells. This signaling in turn controls transcriptional programs regulating cell behavior during development (20). In this study, we provide what is to our knowledge the first evidence of a critical role for Pten in murine lung development. Targeted inactivation of the Pten gene in the embryonic pulmonary epithelium resulted in alveolar septal and bronchiolar hyperplasia, impairments of branching morphogenesis and distal alveolar epithelial cell differentiation, and, ultimately, failure of lung function.

Spatiotemporal localization of Pten gene expression has been previously reported in developing mice (21). High levels of Pten expression and low levels of Akt activation were observed in terminal sac–stage respiratory epithelium, whereas high levels of Akt activation and low levels of Pten expression occurred in the pseudoglandular stage (21, 44). Taken together with our finding that SOPten\textsubscript{flox/flox}\textsuperscript{(E10–E16)} mice show impairments of both terminal sac formation and alveolar epithelial cell differentiation, these observations demonstrate that Pten has an important role in lung development, especially during the terminal sac stage.

Lungs of SOPten\textsubscript{flox/flox}\textsuperscript{(E10–E16)} mice exhibited septal hyperplasia due not only to increased numbers of alveolar epithelial cells and mesenchymal cells (mainly myofibroblast precursors) but also to an enlargement of individual alveolar epithelial cells. These alterations led to a dramatic thickening of the blood-air barrier and inhibited efficient gas exchange. Because loss of Pten in various tissues activates Akt, which is important for cell proliferation and survival (45) and cell size regulation (46), the observed changes to the activation of Akt and its downstream effectors may account for the hyperproliferation and enlargement of Pten-deficient alveolar epithelial cells. The proliferation
of lung mesenchymal and alveolar epithelial cells is regulated by Pten as well as Shh, a soluble factor (29, 30). Overexpression of Shh in the distal airway epithelium causes increased proliferation of both mesenchymal and alveolar epithelial cells (29), and Shh-null mice show significantly reduced proliferation of these cell types (30). In our study, we detected a dramatic induction of Shh in the alveolar epithelium of SOPten^flox/flox(E10–E16) lungs compared with controls. Because activation of PI3K/Akt signaling upregulates Shh expression (47), the increased activation of the Shh pathway in SOPten^flox/flox(E10–E16) lung may be due to the enhanced PI3K/Akt signaling that occurs in the absence of Pten. The SP-C–Cre construct used to delete Pten in alveolar epithelial cells cannot delete the target gene in mesenchymal cells (23), meaning that Pten was likely intact in mesenchymal cells in our SOPten^flox/flox mice. Indeed, our IHC study demonstrated that upregulation of Shh was confined to the alveolar

Figure 6
Pten deficiency induces significant increases in numbers of BASCs and side population cells. (A) Increased numbers of BASCs in lungs of SOPten^flox/flox(P21–P27) mice. Representative flow cytometric profiles of BASCs in lungs of 8-week-old WT(P21–P27) and SOPten^flox/flox(P21–P27) mice. Upper panels show the percentages of CD34^+CD45^−CD31^− cells in the total lung cell population. Lower histograms show the percentages of Sca-1^− cells in the CD34^+CD45^+CD31^− population. (B) Bar graph representation of the percentages of BASCs in total lung cells of WT(E10–E16) and SOPten^flox/flox(E10–E16) mice and WT(P21–P27) and SOPten^flox/flox(P21–P27) mice. Data are expressed as the mean percentage ± SD for 4 mice/group. *P = 0.011; †P = 0.015. (C) Increased numbers of side population cells in SOPten^flox/flox(P21–P27) lungs. Representative flow cytometric profiles of side population cells in lungs of 8-week-old WT(P21–P27) and SOPten^flox/flox(P21–P27) mice. Side population cells were identified by staining with 5 μg/ml Hoechst 33342 alone (upper panels) or in combination with 50 μM verapamil, which is an inhibitor of ATP-binding cassette transporters and blocks the dye efflux (lower panels). The percentages of side population cells (boxed areas) in the total lung cells are indicated in each panel. (D) Bar graph representation of the percentages of side population cells in total lung cells of WT(E10–E16) and SOPten^flox/flox(E10–E16) and WT(P21–P27) and SOPten^flox/flox(P21–P27) mice. Data are expressed as the mean percentage of side population cells ± SD for 3 mice/group. *P < 0.05; **P = 0.001. For B and D, statistical differences were determined using the Student’s t test. Data shown are representative of at least 4 trials.
epithelium and was not observed in mesenchymal cells. Thus the enhanced proliferation of mesenchymal cells observed in SOPten\textsuperscript{flox/flox}(E10–E16) lungs might be caused by the elevated production of soluble Shh by Pten-deficient alveolar epithelial cells. The increased proliferation of alveolar epithelial cells may also result from enhanced Shh signaling and autocrine production of Shh.

The lungs of SOPten\textsuperscript{flox/flox}(E10–E16) mice at E19.5 showed rare apical microvilli, few lamellar bodies, PAS positivity, and reduced production of SPs. These observations suggest that Pten is involved in the maturation of AE-II epithelium. Because AE-I cells can be generated from AE-II cells (20), AE-I cell differentiation is also impaired in SOPten\textsuperscript{flox/flox}(E10–E16) lungs. Screening of the expression of 20 genes important in distal epithelial cell differentiation revealed a marked increase in Spry2 expression in Pten-deficient alveolar epithelium. Overexpression of Spry2 in the distal airway epithelium leads to decreased SP-C mRNA expression (28), and reduction of Spry2 expression by antisense oligonucleotides results in increased expression of SP-A, -B, and -C in the lung (27). Thus the enhanced expression of Spry2 by the alveolar epithelium of SOPten\textsuperscript{flox/flox}(E10–E16) lungs may be responsible (at least in part) for the observed defect in AE-I cell differentiation. Since insufficient secretion of SPs by AE-II cells is a major cause of respiratory distress syndrome in mammalian neonates (26), the neonatal lethality of SOPten\textsuperscript{flox/flox}(E10–E16) mice may be caused not only by the thickened blood-air barrier but also by impaired AE-II differentiation. Collectively, our data provide novel molecular evidence of a previously unrecognized contribution of Pten to normal lung morphogenesis and alveolar epithelial cell differentiation.

In contrast to SOPten\textsuperscript{flox/flox}(E10–E16) mice, SOPten\textsuperscript{flox/flox}(P21–P27) and SOPten\textsuperscript{flox/flox}(P84–P90) mice examined at 9 weeks after doxycycline administration showed no significant difference in alveolar epithelial differentiation compared with WT(P21–P27) or WT(P84–P90) mice. Specifically, the expression levels of SP-C and AQP5 in SOPten\textsuperscript{flox/flox}(P21–P27) and SOPten\textsuperscript{flox/flox}(P84–P90) lungs were essentially comparable with those in the WT(P21–P27) and WT(P84–P90) controls, respectively, although the mild alveolar epithelial hyperplasia and increased cell size observed in SOPten\textsuperscript{flox/flox}(E10–E16) mice were also seen in both SOPten\textsuperscript{flox/flox}(P21–P27) and SOPten\textsuperscript{flox/flox}(P84–P90) mice. These discrepant effects of Pten deficiency on alveolar epithelial differentiation in SOPten\textsuperscript{flox/flox}(E10–E16) mice versus SOPten\textsuperscript{flox/flox}(P21–P27) and SOPten\textsuperscript{flox/flox}(P84–P90) mice may be explained in 2 ways. First, since the turnover time of the respiratory epithelia of adult rodents is reported to be greater than 16 weeks (48), differentiated epithelial cells that were present prior to doxycycline administration to SOPten\textsuperscript{flox/flox}(P21–P27) or SOPten\textsuperscript{flox/flox}(P84–P90) mice may have survived. Second, since AE-II cells and Clara cells have proliferative capacity in adult mice (2), and AE-I cells can be generated from AE-II cells (20), Pten deficiency in the mouse lung may increase the proliferation and/or survival of these committed cells as well as increase their size.

**Pten deficiency leads to increased BASC numbers and lung adenocarcinomas.** In this study we demonstrated that almost all mice with a bronchioalveolar-specific Pten deficiency spontaneously developed lung adenocarcinomas. In both SOPten\textsuperscript{flox/flox}(P21–P27) and SOPten\textsuperscript{flox/flox}(E10–E16) mice, the lungs showed increased numbers of both BASCs and stem cell–like side population cells. Two lines of evidence suggest that BASCs initiate lung adenocarcinomas. First, lung injury induces an increase in BASCs that correlates positively with both the incidence and size of lung adenocarcinomas (5). Second, the BASC population is expanded in tissues that subsequently develop lung tumors in vivo (5). Cancers of diverse tissues are thought to be maintained by minor subpopulations of cells within the tumor called cancer stem cells (4), and BASC-like cells have been detected in established adenocarcinomas (49). Therefore, BASCs may not only initiate tumors but may also function as cancer stem cells, driving tumor development and progression, similar to cancer stem cells observed in hematopoietic (50), breast (51), and brain (52) malignancies. Because BASCs are increased in the lungs of SOPten\textsuperscript{flox/flox} mice, these animals have a greater risk of acquiring multiple oncogenic mutations and developing cancer. Indeed, the tumors observed in SOPten\textsuperscript{flox/flox} lungs (which show increased BASC numbers) are much more frequent and larger than those in WT lungs and also show frequent mutation of the K-ras oncogene. The facts that Pten deletion leads to stem and/or progenitor cell expansion in the prostate (10), central nervous system (9), and blood cells (11) and promotes the generation of leukemia-initiating cells (11) support our hypothesis.

Significantly, almost all SOPten\textsuperscript{flox/flox} mice in which Pten was deleted post-natally also developed spontaneous adenocarcinomas. Previous studies reported that somatic inactivation of PTEN in human lung tumors makes an important contribution to the progression of established human lung cancers (53). In contrast, our experimental model indicates that postnatal loss of Pten function can actually initiate lung tumors in mice. In
combination, these findings demonstrate that Pten has a key role in preventing lung adenocarcinoma formation and blocking the progression of these malignancies.

To date, the only molecule that has been reported to affect BASC numbers is K-ras. Overexpression of K-ras increases both BASC numbers and the incidence of lung adenocarcinomas in mice (5). Our Western blotting assay revealed no significant differences between WT(P21–P27) and SOPten flox/flox(P21–P27) lungs in the activation of Erk, a downstream effector of K-ras. Therefore, K-ras may not be involved in the process that enhances BASC numbers in non-tumorous regions of SOPten flox/flox(P21–P27) lungs. However, we found that K-ras mutation had occurred in one-third of lung adenocarcinoma samples obtained from SOPten flox/flox(P21–P27) mice, whereas no mutation was detected in the paired non-tumorous lung samples of the same mice. It is possible that the occurrence of a K-ras mutation in Pten-deficient BASCs may further increase BASC proliferation and eventually induce the formation of lung adenocarcinomas.

The onset of skin tumor formation in mice requires Akt signaling in addition to SOS/Ras/ERK signaling (54). Indeed, the MAPK/ERK pathway has been reported to act in synergy with the PI3K pathway to stimulate cyclin D1 transcription in NIH3T3 cells (55). Thus the onset of tumors in SOPten flox/flox mice could be caused principally by BASC hyperproliferation and/or apoptotic resistance induced by PI3K and Akt hyperactivation, with a contribution by deregulated ERK activation. The spatiotemporal relationship between loss of PTEN and mutation of K-ras has not been elucidated in human lung tumors. However, considering that loss of PTEN expression (14, 15) is much more frequent than K-ras mutation (42) in human lung adenocarcinomas, PTEN deficiency (rather than K-ras mutation) may be the primary event driving human lung adenocarcinoma development.

Our study also provides insight into the molecular mechanism underlying the increase in BASC numbers and adenocarcinoma onset in Pten-deficient mice. Loss of Pten in the lung leads not only to activation of Akt but also to elevated expression of c-Myc, Bcl-2, and Shh and decreased expression of p27. These gene expression alterations have been shown to induce stem cell self-renewal and/or progenitor cell expansion and to trigger tumor formation in a variety of organs (15, 35–41). Significantly, c-Myc, Bcl-2, Shh, and p27 are all downstream targets of Akt (47, 56, 57). Our data therefore suggest that the increased BASC numbers and adenocarcinoma formation in SOPten flox/flox mice are likely due to the enhanced Akt activation that occurs in the absence of Pten.

Our study is, to our knowledge, the first report of a functional analysis of Pten in murine bronchioalveolar cells in vivo. We have demonstrated that an intact PI3K/Pten/Akt pathway in bronchioalveolar cells is essential for normal lung morphogenesis and BASC homeostasis as well as for the prevention of lung tumorigenesis. Our results suggest that the PI3K/PTEN/Akt pathway, particularly in BASCs and/or cancer stem cells, may be an attractive therapeutic target for the treatment of lung adenocarcinomas in humans.

Methods

Generation of SOPten flox/flox mice and doxycycline administration. Pten flox/flox mice (129Ola x C57BL6/J F6), generated as previously described (24), were mated to SP-C-rtTA mice (23) that expressed the rtTA gene (23) (which can be activated by doxycycline) under the control of the 3.7-kb human SP-C promoter. The human SP-C promoter selectively directs expression of the transgene in BASCs, in bronchioalveolar epithelial cells of primordial lung buds, and in both developing and mature bronchiolar and AE-II cells (58). Transgenic (tetO)-Cre mice (22), in which Cre expression is activated by rtTA, were mated to SP-C-rtTA/Pten flox/flox (SOPten flox/flox) mice to generate triple transgenic mice. Offspring carrying SP-C-rtTA and (tetO)-Cre plus 2 copies of the floxed Pten allele (SOPten flox/flox) and offspring carrying (tetO)-Cre plus 2 copies of the floxed Pten allele (OPten flox/flox) were used in the analysis as homozygous mutant (SOPten flox/flox) and WT (OPten flox/flox) mice, respectively. SOPten flox/flox and Pten flox/flox mice were also used as WT controls in some experiments because OPten flox/flox, SOPten flox/flox, and Pten flox/flox mice were indistinguishable in pilot experiments examining histology and Pten protein expression levels. To induce expression of the Cre transgene in BASCs, mice were administered doxycycline in utero or postnatally. Specifically, dams bearing pups (E10), 3-week-old mice (P21), and 12-week-old mice (P84) were fed doxycycline (Sigma-Aldrich) in their drinking water (1 mg/ml) for 7 days (23). After this period of doxycycline administration, the mice were designated as E10–E16, P21–P27, or P84–P90 mice, respectively. All animal experiments were approved by the Animal Experiment Review Board of the Akita University School of Medicine.

PCR analyses and Southern and Western blotting. Mouse genotyping was performed by PCR as previously described (59) using genomic DNA isolated from mouse tails. Sequences of the primers specific for the floxed 7 allele (22) and Cre expression (23) were 5′-GACCGAGGGATCTGTGGCTG-3′ and 5′-TTCTCCCATCCACATGTTCTCAG-3′, respectively. PCR products were separated on 1% agarose gel and visualized under ultraviolet light. Southern blot analysis was performed using a 3.7-kb human SP-C promoter fragment as a probe.

Histology and immunohistochemistry. Lung tissue samples were fixed in 4% paraformaldehyde in PBS or Amsterdam’s fixative (methanol/acetone/acidic acid/water, 35:35:5:25 v/v) and embedded in paraffin using standard procedures. Ultrathin sections were stained in uranyl acetate/water, 35:35:5:25 v/v) and embedded in epoxy for electron microscopy according to the standard procedures. Sections (5 μm) were mounted on slides for histological or IHC analysis. IHC analysis was performed using an indirect method with anti-c-Myc (all from Santa Cruz Biotechnology Inc.); CCSP (Upstate); CGRP (Sigma-Aldrich); total Rb, p27, c-Myc, p21, Bcl-2, Shh, Notch1, SP-A, SP-B, and SP-D (all from Chemicon International Inc.); CCSP (Upstate); and actin (Sigma-Aldrich).

Arterial blood gas analysis. Soon after birth, samples of arterial blood (80–100 μl) were collected from the carotid arteries of WT(E10–E16) and SOPten flox/flox(E10–E16) mice at room temperature and immediately analyzed for blood gases on a blood gas analyzer (Radiometer ABL 510; Diamond Diagnostics).

Total cell number determinations in neonatal lungs. Lungs of neonatal mice were minced and treated with 1,000 U dispase (Godoshide) for 15 minutes at 37°C and filtered using a Cell Strainer (Becton Dickinson—Discovery Technologies). Whole lungs were obtained from WT(E10–E16) mice at room temperature and immediately analyzed by Western blotting using antibodies recognizing the following proteins: Pten (Cascade Bioscience), phosphorylated Akt (Ser473), total Akt, phosphorylated Erk (Thr202/Tyr204), total Erk, and phosphorylated Rb (Ser807/811) (all from Cell Signaling Technology); total Rb, c-Myc, Bcl-2, Shh, Notch1, SP-A, SP-C, and AQPS (all from Santa Cruz Biotechnology Inc.); p53 (DAKO); SP-B and SP-D (all from Chemicon International Inc.); CCSP (Upstate); and α-SMA (Sigma-Aldrich).

Arterial blood gas analysis. Soon after birth, samples of arterial blood (80–100 μl) were collected from the carotid arteries of WT(E10–E16) and SOPten flox/flox(E10–E16) mice at room temperature and immediately analyzed for blood gases on a blood gas analyzer (Radiometer ABL 510; Diamond Diagnostics).

Total cell number determinations in neonatal lungs. Lungs of neonatal mice were minced and treated with 1,000 U dispase (Godoshide) for 15 minutes at 37°C and filtered using a Cell Strainer (Becton Dickinson—Discovery Labware). Dissociated lung cells were washed and resuspended in PBS, and the total cell number per lung was determined by Giemsa nuclear staining.

Histology and immunohistochemistry. Lungs were fixed in either 4% paraformaldehyde in PBS or Amsterdam’s fixative (methanol/acetone/acidic acid/water, 35:35:5:25 v/v) and embedded in paraffin using standard procedures. Sections (5 μm) were mounted on slides for histological or IHC analysis. IHC analysis was performed using an indirect method with antibodies recognizing the following proteins: SP-C, AQPS, Spry2, Shh, and GFAP (all from Santa Cruz Biotechnology Inc.); CCSP (Upstate); CGRP (Sigma-Aldrich); CD31 (Pharmingen); and α-SMA and desmin (DAKO).

Electron microscopy. Lung tissues were washed with washing buffer (3.5% sucrose in 0.1 M sodium cacodylate buffer at pH 7.3), postfixed in buffered osmium tetroxide, and embedded in epoxy for electron microscopy according to the standard procedures. Ultrathin sections were stained in uranyl acetate plus lead citrate and examined using a 100CX electron microscope operated at 60 kV (JEOL Ltd.).
RT-PCR. Total RNA from lung tissues of E19.5 WT(E10–E16) and SOPTen+/−/floxed(E10–E16) embryos was extracted using TRIzol (Invitrogen) according to manufacturer’s protocol. Total RNA (5 μg) was reverse-transcribed using the Advantage RT-for-PCR kit (Clontech). Sequences of specific primers are listed in Supplemental Table 1.

Cell proliferation and apoptosis. Cell proliferation was assessed using BrdU incorporation. Timed pregnant WT(E10–E16) and SOPTen+/−/floxed(E10–E16) mice at E19.5 were injected i.p. with 50 mg/kg body weight of BrdU (Sigma-Aldrich) in sterile PBS. After 2 hours, lungs of embryos were collected and fixed in 4% paraformaldehyde. Anti-BrdU staining was performed as described in ref. 60. For apoptosis, the frequency of apoptotic cells in the lungs of E19.5 embryos was determined by the TUNEL assay using the In Situ Cell Death Detection Kit (Roche Diagnostics GmbH) according to the manufacturer’s protocol. Sections were counterstained with DAPI.

Detection of K-ras mutations. PCR-amplified DNA samples from spontaneous adenocarcinomas and the corresponding non-tumorous lung tissues from 6 SOPTen+/−/floxed(P21−P27) mice were analyzed to detect K-ras mutations as described previously (43). Briefly, direct sequence analysis was performed to examine the first exon (spans the region containing codons 12 and 13) and second exon (spans the region containing codon 61) of the K-ras oncogene. The primers used to amplify the DNAs of first and second K-ras exons as well as the primers for these DNA sequences are listed in Supplemental Table 1.

Statistics. Statistical differences were determined using the 2-tailed Student’s t test or Welch’s t test. A P value of less than 0.05 was considered statistically significant.

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