I went to the woods because
I wished to live deliberately,
to front only the essential facts of life,
and see if I could not learn what it had to teach,
and not, when I came to die, discover that I had not lived …
I did not wish to live what was not life,
living is so dear;
nor did I wish to practice resignation, unless it was quite necessary.
I wanted to live deep and suck out all the marrow of life,
to live so sturdily and Spartan-like as to put to rout all that was not life,
to cut a broad swath and shave close,
to drive life into a corner, and reduce it to its lowest terms..."

Henry David Thoreau

CONTENTS

ABSTRACT

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.

Objective: To test the role of *Pten* in lung development and injury, we conditionally deleted *Pten* throughout the lung epithelium by crossing $Pten^{flox/flox}$ with *Nkx2.1-cre* driver mice and throughout the lung mesenchyme by crossing $Pten^{flox/flox}$ with *Nkx2.1-cre* driver or *Dermo1-cre* driver. The resulting $Pten^{Nkx2.1\text{-}cre}$ mutants were analyzed for lung defects and response to injury.

Results: $Pten^{Nkx2.1\text{-}cre}$ embryonic lungs showed airway epithelial hyperplasia with no branching abnormalities. In vitro culture of mutant lungs also showed an altered responsed to TGF-β when in vivo In adult mice, $Pten^{Nkx2.1\text{-}cre}$ lungs exhibit increased progenitor cell pools comprised of basal cells in the trachea, CGRP/CC10 double-positive neuroendocrine cells in the bronchi and CC10/SpC double positive cells in the bronchioalveolar duct junction (BADJ). *Pten* deletion impacted differentiation of various lung epithelial cell lineages, with decreased number of terminally differentiated cells. Over time, $Pten^{Nxk2.1\text{-}cre}$ epithelial cells residing in the BADJ underwent proliferation, and formed uniform masses, supporting the concept that the cells residing in this distal niche may also be the source of pro-carcinogenic "stem" cells. Finally, increased progenitor cells in all the lung compartments conferred an overall selective advantage to naphthalene injury compared to wild type control mice.

$Pten^{Dermo1cre}$ embryonic lungs, moreover, showed normal lung development but increased collagen1 and extracellular matrix production.

Conclusions: Pten has a pivotal role in lung stem cell homeostasis, cell differentiation and consequently resistance to lung injury in the epithelium, Further studies are necessary to clarify the real role of *Pten* in lung mesenchyme.

ABSTRACT

Rationale: Pten e' un gene coinvolto nell'omeostasi delle cellule staminali e nella formazione di tumori. Nei topi, *Pten* inizia ad essere presente 7 giorni dopo il concepimento. *Pten* ha un ruolo critico nello sviluppo embrionale: gli embrioni di topo $Pten^{-/-}$, infatti, muoiono molto presto durante la gestazione.
Scopo dello studio: Studiare il ruolo di *Pten* nello sviluppo polmonare, eliminando *Pten* nell'epitelio polmonare, incrociando $Pten^{flox/flox}$ con topi portatori di *Nkx2.1-cre*; *Pten* e' stato anche eliminato dal mesenchima polmonare incrociando $Pten^{flox/flox}$ con topi *Dermo1-cre*. I risultanti $Pten^{Nkx2.1cre}$ sono stati analizzati alla ricerca di difetti nello sviluppo polmonare.
Risultati: I polmoni $Pten^{Nkx2.1\text{-}cre}$ hanno evidenziato in vitro una alterata risposta al TGF-β. In vivo non presentavano nessuna alterazione nel branching bensi una iperplasia polmonare nelle vie aerre. Nei topi adulti, I polmoni $Pten^{Nkx2.1\text{-}cre}$ presentavano un aumentato pool di cellule progenitori in tutti i distretti: nella trachea, le cellule basali, nei bronchi le cellule neuroepiteliali, positive per CGRP/CC10 ed infine, nella giunzione tra gli alveoli e I bronchi terminali (BADJ), le cellule positive per Spc/CC10. L'assenza di *Pten* ha un impatto nella differenziazione cellulare, con un diminuito aumento delle cellule all'ultimo stadio di differenziazione. Nel tempo, le cellule epiteliali $Pten^{Nxk2.1\text{-}cre}$ residenti a livello del BADJ proliferano e formano delle masse di tipo tumorale; questi dati supportano l'idea che le cellule presenti in questa niche possano essere l'origine delle cosidette "pro-carcinogenic stem cells". L'aumento delle cellule progenitrici, inoltre, conferisce un selettivo vantaggio dopo danno polmonare. I topi con *Pten* eliminato nell'epitelio, invece, non evidenziavano ne uno sviluppo polmonare alterato ne una alterata differenziazione delle cellule mesenchimali; tuttavia, dimostravano un aumentata deposizione di collagene1 e di matrice extracellulare. *Conclusioni: Pten* ha un ruolo importante nell'omeostasi delle cellule progenitori del polmone, nella differenziazione epiteliale polmonare e nella resistenza dopo danno. Ulteriori studi sono necessary per chiarire l'esatto ruolo di Pten nel mesenchima polmonare.

INTRODUCTION

The lung is an important organ for survival, being the one responsible for the oxigenation of the blood and, therefore, of all the body.

From birth, several insults can damage and jeopardize its function: infant respiratory distress syndrome (RDS) and Bronchopulmonary dysplasia, (BPD) are severe complications of premature birth, affecting about 8000-10.000 infants every years in the United States (Ehrenkranz RA et al, 2005).

So far, no therapies are available to treat or prevent these conditions and several more affecting the lung and, for this reason, it is mandatory to find new therapeutical approaches.

Residential Stem cells are multipotent source of multiple cell lineages. These cells are critical for development and growth through childhood and are hypothesized to be the source of the frequently limited tissue regeneration and repair after injury.

Unlike tumor cells and embryonic stem cells, adult stem cells are not immortal, and they decrease in number with increasing age. The naturally limited replacement capacity of such endogenous stem cell pools, though efficient and functional through young adult life, has been associated with the inability to repair damage that accumulates to a critical point late in life. This may occur via simple exhaustion of the stem cell pool or arise as a consequence of inherited or acquired mutations that impede proper stem cell function. It is speculated that these events could be reversed via stimulation or rehabilitation of the endogenous stem cell pool or by introduction of exogenous stem cells to the debilitated organ to reverse the effects of aging and/or disease. Thus, there has been ever-increasing recognition of the potential role stem cells could play in regenerative medicine. Recent studies have shown that the failure to regenerate and repair that inevitably occurs with aging may be due to endogenous stem cell failure. Manipulation of endogenous lung progenitors and delivery of exogenous cells are two

potential therapeutic approaches currently being explored in animal models of lung injury.

The first possibility, using endogenous lung progenitors cells, presents the advantage to overcome ethical issues and avoid possible collateral effects such as tumor formation, associated with exogenous stem cells therapy.

For this reason, we focused our work on a well known stem cells regulator such as phosphatase and tensin homologue (PTEN) and analyzed its role in lung progenitor cells homeostasis

1. LUNG MORPHOGENESIS.

The basic design of the mammalian respiratory system, referred to here as the trachea and lung, is that of a tree of epithelial tubules in which air is cleaned, humidified and delivered to numerous alveolar units closely apposed to blood vessels, where the circulating blood is oxygenated.

The respiratory system arises from the ventral foregut endoderm. Although the lung development is a continuum, for simplicity it can be viewed as occurring in 4 phases (Table 1): embryonic-pseudoglandular stage (E9-E16.6), canalicular stage (E16.5-E17.5), saccular (E17.5-PN5) and alveolar (PN5-PN30) (Minoo et al, 2000).

TABLE 1. *The five structural epochs of lung formation*

Phase	Events	Mouse	Human
Embryonic	Formation of the lung buds and major bronchi, division of tracheal-esophageal tube	E9–11.5	3–7 wk
Pseudoglandular	Proliferation of bronchial branches, acinar tubules and buds; vasculogenesis and innervation	E11.5–16.5	5–17 wk
Canalicular	Organization of the pulmonary vascular bed, pulmonary acinus, and increasing innervation	E16.5–17.5	16–26 wk
Saccular	Dilation of peripheral airspaces, differentiation of the respiratory epithelium and increasing vascularity of the saccules, surfactant synthesis	E17.5–PN5	24–38 wk
Alveolar	Growth and septation of the alveoli, maturation of the pulmonary vascular system	PN5–28	38 wk to maturity

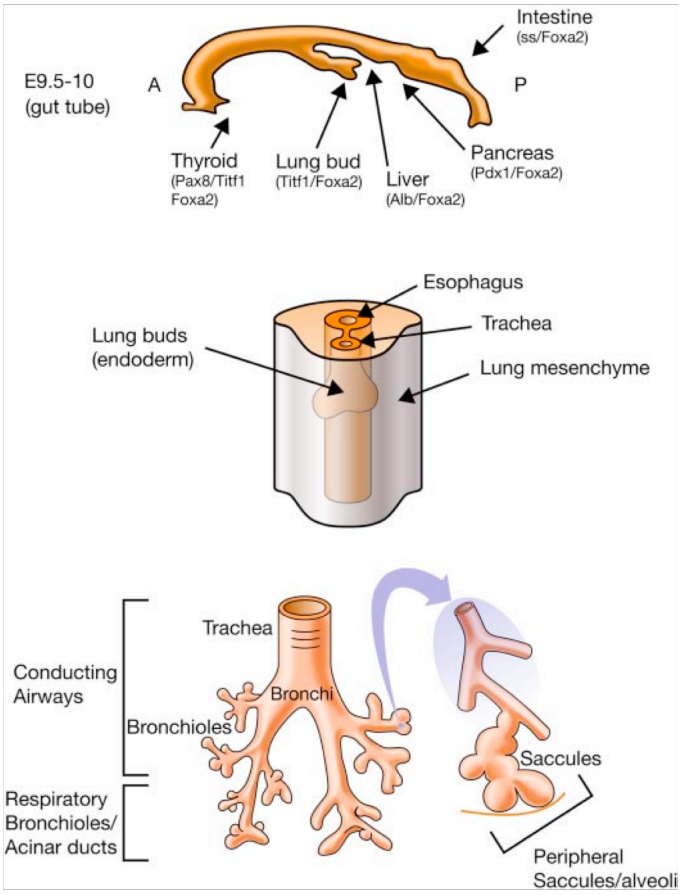
E, embryonic; PN, postnatal.

Maeda et al, 2007.

The first, **the pseudoglandular stage**, is the specification of the lung primordium, in the mice from E9.5 to E16.5, during the pseudoglandular stage. During this phase, lineage analysis suggests that the progenitor cells of the trachea and proximal lung differ in origin from those that will form the distal

region of the lung (Perl et al., 2002). Precisely when respiratory cell fate is established in the foregut endoderm is still unclear. However, respiratory progenitors are first visualized by in situ hybridization as a group of *Nkx2.1*-expressing endodermal cells in the prospective lung/tracheal region of the foregut, at E9 (Minoo et al., 1999). *Nkx2.1* transcripts have been reported by RT-PCR to be present in the foregut as early as E8-8.5 (eight-somite stage) (Serls et al., 2005). Although *Nkx2.1* is the earliest known marker of the presumptive respiratory region, *Nkx2.1*-null mutant mice do have lungs (Minoo et al., 1999), even if they are highly abnormal with a proximalization of the terminal cystic structures. Besides *Nkx2.1*, no other early marker is currently available. The surfactant-associated protein C (*Sftpc*) gene is the most specific marker of lung epithelial cell lineage, but its expression is consistently detected only by E10-10.5, after the primary buds form (Wert et al., 1993).

.



10. **FIG. 1. Patterning of the foregut endoderm: lung bud formation and branching morphogenesis. *Top*: mouse endoderm is depicted at approximately E9.5–10. Transcription factors (Pax8, Titf1, Pdx1, Foxa2) and markers (Alb, albumin; ss, somatostatin) identify cells that contribute to organ formation along the anterior-posterior axis (A-P). TTF-1 (Titf-1 gene) is expressed at sites of lung and thyroid formation, with the latter in cells expressing both PAX8 and TTF-1. *Middle*: lung buds and trachea at E9.5–10, as the early**

buds evaginate into the mesenchyme. *Bottom*: conducting and peripheral regions of the lung at approximately E12. Later in morphogenesis (E17–18) peripheral saccules are formed (*bottom right inset*). Alveolarization occurs in the postnatal period.
(Maeda et al, 2007).

The second stage is called **canalicular stage**, and it happened from E16.5 to E17.5, when the distal endoderm begins to form terminal sacs. Before E15 in the mouse fetus, epithelial cells lining the trachea, bronchi, and bronchioles are relatively undifferentiated as defined by the expression of cell-specific markers used to identify epithelial cell types later in development. In contrast, the adult conducting airways are lined by relatively diverse cell types, expressing different markers. Transcriptional mechanisms controlling epithelial cell differentiation in the conducting airways during lung morphogenesis and repair remain less completely understood; however, TTF-1, NF-1, GATA-6, and other transcription factors, play roles in cell specific differentiation and gene expression in the conducting airways (Maeda et al 2007). Concentrations of these transcription factors vary among distinct cell types along the cephalo-caudal axis of the conducting airways where differentiation is influenced by their concentration, activation, and interactions with other transcription factors (Fig.2).

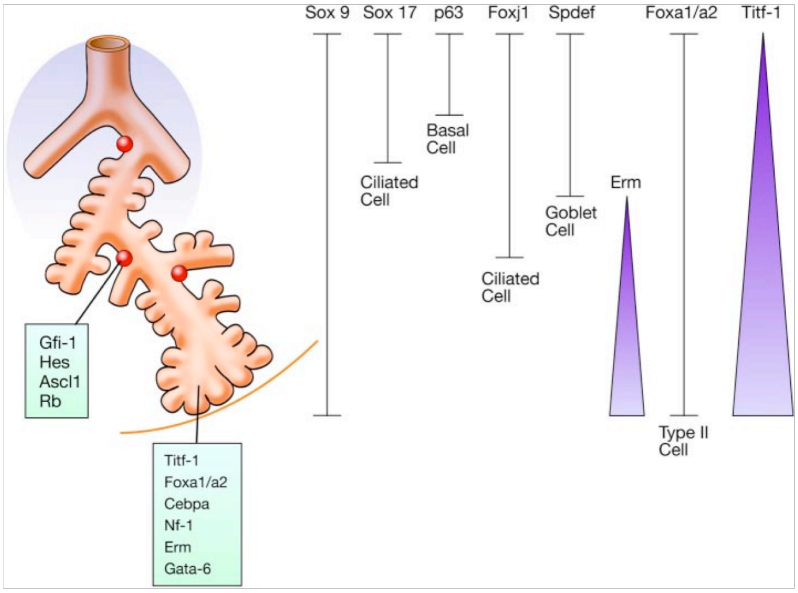


FIG. 2. Distribution of transcription factors in respiratory epithelial cells. Conducting and peripheral airways are depicted (*left*). The distribution

of transcription factors varies among distinct cell types along the cephalocaudal axis of the lung. GFI-1, HES, MASH, and RB influence differentiation/growth of neuroendocrine cells (circles). Alveolar type II cells, but not type I cells, express TTF-1, FOXA1/2, C/EBP, NF-1, ERM, and GATA-6. TTF-1, SOX family members, p63, FOXJ1, SPDEF, and other transcription factors vary in concentration along the airways where they influence epithelial cell differentiation and gene expression. (Maeda et al, 2007)

The third period is the **saccular stage**, from E17.5 to PN5, characterized by thinning of the mesenchyme, increase in the number of terminal scas, vascularization and differentiation of the endoderm into type I and type II cells. This period of lung morphogenesis represents a particularly vulnerable time in mammalian development, marking the transition from a fluid-filled to air-filled lung upon which survival depends following birth. Supportive structures of the peripheral air saccules become more gracile as type I epithelial cells become increasingly squamous, providing close apposition between pulmonary blood vessels and the respiratory epithelium. Peripheral lung tissues thin as lung saccules dilate and pulmonary capillaries come into increasingly close contact with the epithelial lining where gas exchange is facilitated. At the time of birth, pulmonary vascular resistance falls, pulmonary blood flow increases, lung fluid is resorbed, and pulmonary surfactant is secreted into the peripheral saccules of the lung, the latter reducing surface tension that prevents alveolar collapse once the lung is filled with air (De Felice et al, 2003; Dave et al, 2006; Bassers et al, 2006; Wan et al, 2006).

Finally, after birth (PN5-PN30), during the **alveolar stage**, alveologenesis and differentiation of distal epithelial cell types take places and the terminal sacs develop into mature alveolar ducts and alveoli. Formation of the highly septated and alveolarized structures comprising the alveolar gas exchange area occurs primarily in the postnatal period in mice, while in humans begins before birth. Recent gene targeting experiments have identified several transcription factors influencing alveolarization, but cellular processes, signaling mechanisms, and transcription factors controlling alveolarization are relatively poorly understood (Liu et al, 2003; Wert et al, 2002).

During all these stages, signals from the epithelium influence **lung**

mesenchyme development maintaining a balance of differentiated and proliferating multipotent progenitors while the lung grows (Weaver et al., 2003). The lung mesenchyme originates from the lateral plate mesoderm and gives rise to multiple components of the lung, including its connective tissue, endothelial cell precursors, the smooth muscle that surrounds airways and blood vessels, the cartilage of the trachea, the lymphatics, and the mesothelial cells that cover the outer surface of the lung, the pleura. The lung vasculature forms, in part, by migration of blood vessels from the aortic arches and from the left atrium to the lung (angiogenesis). Blood vessels also develop by vasculogenesis in the lung mesenchyme near developing epithelial buds; a rudimentary capillary network initially forms and expands, and later connects to the larger vessels to give rise to the lung vasculature (Wood et al., 1997; Gebb and Shannon, 2000). Transcription factors of multiple families are expressed in various cells during lung morphogenesis. While some transcription factors are highly restricted to cell type, for example, in the mesenchyme or epithelium, others are expressed in multiple tissue compartments in a distribution pattern that may also change during development (Pepicelli et al., 1998; Shu et al., 2002; Del Moral et al., 2006). Through all these stages, all the different **epithelial cells** lining the lung tubules become increasingly differentiated along the cephalo-caudal axis associated with expression of cell type specific markers. The proximal lung includes ciliated cells (ß-tubulin^{pos}), 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 form the most distal compartment of the lung and are composed of alveolar type I (T1-α^{pos}) and type II (SpC^{pos}) cells. **β-Catenin,** an evolutionarily conserved signaling system utilized by many cell types throughout organogenesis, plays a critical role in establishing this proximal-distal cell fate in the respiratory epithelium. It is very important for regulating branching morphogenesis and airway epithelial differentiation (Huelsken J et al, 2000; Mucenski ML et al, 2003; Okubo T et al, 2004; Tebar M, et al, 2001;

Shu W et al, 2005).

Overall, the studies so far suggest that the major events in early lung morphogenesis are controlled by a relatively limited group of molecules. The lack of early markers of lung progenitor cells represents a clear gap of knowledge in the field. Because *Sftpc* expression cannot be identified prior to the emergence of primary buds, *Nkx2.1* is the only early marker currently available for these cells. A confounding issue is that this gene is also expressed by the thyroid. Crucial will be the development of tools for targeting genes to these early progenitors in the foregut in future functional studies. A rather more complex problem is the understanding of how the coordinates that set up the three-dimensional pattern of morphogens, such as Fgf10, are established in the lung. Finally, there is the debated issue of stem cells. What are these cells? Where are their niches in the developing lung? How can they be identified? Tackling these issues will provide insights into the molecular and cellular mechanisms by which the lung develops.

2. STEM/PROGENITOR CELLS IN THE LUNG.

2.1 Defining stem cells.

Throughout adult life, multicellular organisms must generate new cells to maintain the structure and function of their tissues. In young animals, tissue damage can usually be repaired quickly, but this natural capacity may fail after repeated challenges and with age. Diseases such as cancer may usurp and exploit the mechanisms by which the body normally rebuilds itself. These considerations drive us to understand the mechanisms by which adult organs normally achieve tissue homeostasis and repair. The emerging picture is that different organs use different strategies to renew themselves, and that more diversity and flexibility underpin these renewal processes than previously imagined. Some organs, such as hair follicles, blood and gut, which constantly renew themselves throughout life, contain adult stem cells that are morphologically unspecialized, have a relatively low rate of division and are

topologically restricted to localized regions known as `niches' that tightly regulate their behavior (Fuchs et al., 2004; Lanza, 2006). These `dedicated' stem cells (Box 1) undergo long-term self-renewal. They also produce a population of transit amplifying (TA) daughter cellls that have a high rate of proliferation, can self-renew over the short term and give rise to precursors of all or many of the differentiated cell types of the organ. These concepts are now well established (Fig.3).

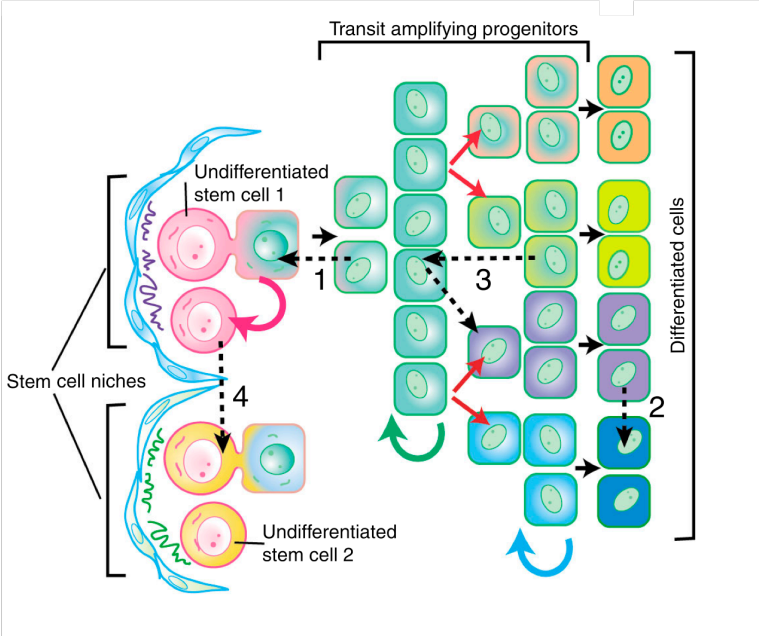


Figure 3. Classical stem cell hierarchy. Model of the `classical' hierarchy of undifferentiated epithelial stem cell, transit amplifying (TA) progenitor cells and mature postmitotic differentiated cells. Cell fate choices are indicated by red arrows. In this model, the stem cell in its `niche' and different TA cell subclasses can self-renew (curved arrows). Stem cells self-renew infrequently and TA cells more rapidly. Early TA cells may be able to replace stem cells if the niche is depleted (dashed arrow 1). The niche probably consists of several cell types and associated molecules, including blood vessels and nerves. `Transdifferentiation' of one well-defined differentiated cell type into another could occur directly, without cell division (dashed arrow 2) or might also involve reversion or de-differentiation between distinct TA progenitor populations (dashed arrows 3). Rarely, stem cells switch from one tissue-specific lineage to another (dashed arrow 4) in a process called metaplasia or transdetermination (see Box 1). Adapted, with permission, from Watt and Hogan (Watt and Hogan, 2000).

However, recent research has emphasized that the classical hierarchy of the

tissue-specific stem cell, TA cells and differentiated cells is not always rigid and irreversible (Raff, 2003). For example, the commitment of cells to a specific fate may occur gradually, so that if stem cells are ablated, some early TA cells may enter the empty niche and function as stem cells (Kai and Spradling, 2004; Potten, 2004). TA cells may also be able to change their fate to give rise to cells of another tissue type when exposed to appropriate signals. This process is known as `transdifferentiation' - a term that needs careful use according to its context (see Box 2). In contrast to rapidly renewing organs such as the skin and gut, some organs apparently maintain themselves without the aid of an undifferentiated stem cell population. Evidence for this concept comes from recent experiments in the liver, where turnover and regeneration after hepatectomy involves the division of differentiated hepatocytes. However, if hepatocyte proliferation is inhibited, interlobular bile duct cells can replenish the hepatocyte population (Alison et al., 2004). Such observations have engendered the concept of `facultative' stem cells - normally quiescent differentiated cells that can act as stem cells after injury, perhaps by recapitulating processes that are active during development (see Box 1).

The adult lung is a vital and complex organ that normally turns over very slowly. The epithelial cells that line the airways are constantly exposed to potential toxic agents and pathogens in the environment, and they must therefore be able to respond quickly and effectively to both cellular damage and to the local production of immune cytokines. Over the years, several experimental protocols have been developed in mice that mimic the injuries and rapid repair processes elicited in the lung by environmental challenges. The picture that is emerging from these models is that different regions of the respiratory system - the trachea and large airways, and the distal bronchioles and alveoli - use different kinds of stem cells and strategies for maintenance and repair. Moreover, there is evidence that differentiated epithelial cell types are able to proliferate and transdifferentiate in response to some conditions. However, the precise mechanisms involved in any of these processes are still very unclear.

Adapted from Rawlisg et al, 2005.

Box 1. A glossary

Dedicated stem cell A relatively undifferentiated cell present in the adult organ, usually in localized niches. It normally divides infrequently; is capable of both long-term (`lifetime') self-renewal and of giving rise to daughter cells that differentiate into one or more specialized cell type; and it functions in both tissue homeostasis and repair.

Facultative stem cell Differentiated cell that is normally quiescent but responds to injury by dividing and self-renewing, and giving rise to progeny that differentiate into one or more cell types.

Metaplasia Strictly, the process by which a stem or progenitor cell of one tissue switches to become a progenitor of cells of another tissue type.

Post-mitotic differentiated cell A cell that can no longer divide and must be replenished during normal turnover or injury.

Progenitor cell Either a cell in the developing organ, usually multipotent, that is the source of an initial population of adult cells before turnover begins, or, more loosely, a cell that gives rise to another cell. Cell lineage relationships during development may not necessarily reflect those that occur during repair.

Self-renewing differentiated cell Differentiated cell that divides and self-renews over the long term. Functions in both normal tissue homeostasis and in response to injury.

Transdifferentiation See Box 2.

Transit amplifying (TA) cell An intermediate between a dedicated stem cell and its final differentiated progeny. Can proliferate, self-renew over the short term and give rise to one or more differentiated cell type.

Box 2. Transdifferentiation

This refers to the transformation of one well-defined type of fully differentiated cell into another well-defined type. In this review, we refer to `direct transdifferentiation' as the transformation from one phenotype to another without an obligatory round of cell proliferation. `Transdifferentiation with proliferation' requires at least one intervening round of cell proliferation. The direct mechanism is more likely to involve the transient existence of a cell that co-expresses differentiation markers of both old and new phenotypes. By contrast, the mechanism that involves proliferation is more likely to involve the transient existence of a `de-differentiated' cell, which has a pattern of gene expression that is different from either the initial or final cell type. Some stem cell researchers have applied the term `transdifferentiation' to `the ability of a particular cell of one tissue...including stem or progenitor cells, to differentiate into a cell type characteristic of another tissue'. This much looser definition really describes a phenomenon that is known as `transdetermination' when it occurs in

embryos and as `metaplasia' when it occurs in adults.

2.2 Endogenous Progenitor cells in the lung.

In adult mammals, the lung epithelium undergoes slow homeostatic turnover, resulting in the replacement of most of the epithelium after approximately 4 months in rats (Blenkinsopp et al, Exp cell res 1967).

The epithelium of the lung is a major target of insults and is organized into functional compartments along its proximal-distal axis

Both homeostatic turnover and regeneration after injury are thought to involve endogenous lung progenitor cells. The progenitor niches within the lung are poorly characterized but recent studies have identified several stem cell niches, containing multipotent and lineage-restricted progenitor cells (Englehardt et al., 1995) and located in distinct positions along the proximal–distal axis of the airways (Fig.4) (Giangreco et al, Am J Path, 2002; Rawlins et al, development, 2006; Kim et al, Cell, 2005). 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” (Schoch et al., 2004; Avril-Delplanque et al., 2005; Liu et al., 1994). A subpopulation of NE cells expressing both CGRP and CC10 may also have progenitor cell properties (Hong et al, 2001). In the distal lung, differentiated alveolar type II epithelial cells are likely facultative progenitor cells (Aso et al, 1976; Isakson et al, 2001). 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) (Kim et al., 2005). Rarity of progenitor cells represents a major technical block to the badly needed characterization of their functional properties. BACS represent one of these regional progenitor cell population and are located in the bronchioalveolar duct junction (BADJ) at the terminal end of distal bronchioles (Giangreco et al, KIM). BASCs are thought to regenerate both bronchiolar and alveolar epithelium during homeostatic turnover and in response to injury.

The transcriptional and signaling pathways required for the differentiation and expansion in BASCs are largely unknown. Given the proposed requirement for BASCs and other progenitor cells in normal homeostatic turnover in the lung as well as in regeneration and repair after injury, characterization of the regulatory mechanisms controlling the expansion and differentiation these cells could have a profound impact on our understanding and treatment of lung disease.

2.3 Endogenous Mesenchymal Progenitors cells.

Until recently, the early lung mesenchyme was recognized to exert inductive properties on the early lung epithelium to initiate branching morphogenesis. It has recently emerged that the peripheral mesenchyme that expresses Fgf10 also serves as a progenitor cell population for peripheral airway smooth muscle (De langhe et al, 2006). Thus, lineage tracing studies with Fgf10-lacz reveal that airway smooth muscle progenitors begin as Fgf10-expressing cells that, as the airway grows outwards, become distributed along the elongating peripheral airway. Transdifferentiation of these progenitors to express – smooth muscle actin fibers occurs under the control of SHH and BMP4, which are expressed proximal to the very tip of the airway. Thus, the population size and placement of peripheral airway smooth muscle progenitors appears to occur very early in development. Another population of airway smooth muscle progenitors was recently shown to arise in the proximal mesenchyme and progress peripherally.

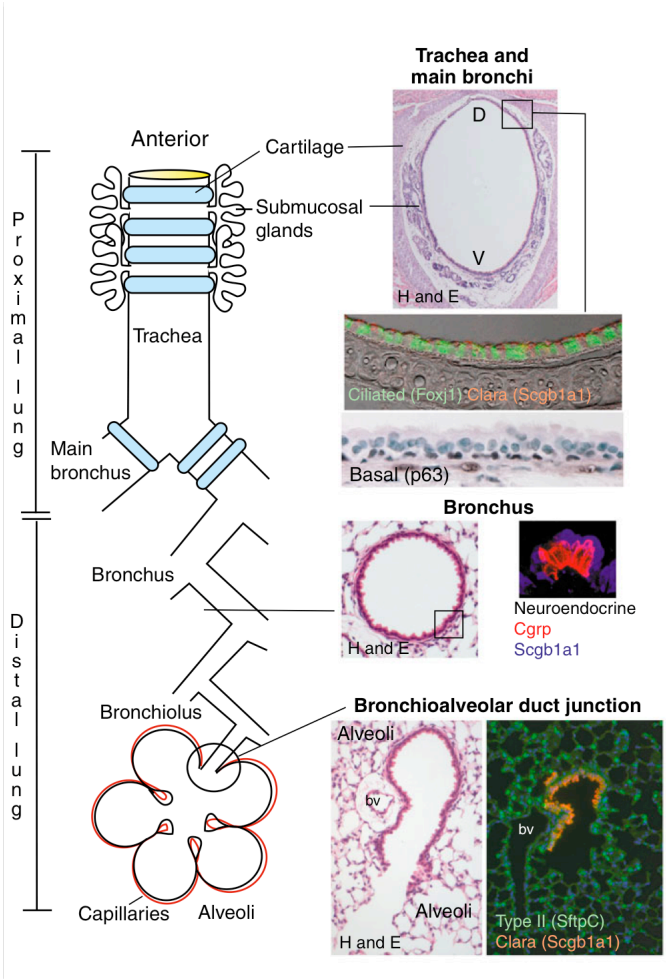


Fig. 4. A schematic of the main cell types along the proximodistal (rostrocaudal) axis of the mouse lung. Not all of the cartilage elements associated with the trachea and main bronchi are shown. Submucosal glands are present only in the upper part of the trachea in the mouse. The pseudostratified epithelium of the proximal airways contains mostly ciliated cells, which express the forkhead transcription factor Foxj1, Clara-like secretory cells [detected with antibody to Scgb1a1 (red)] and basal cells, detected with antibody to the transcription factor p63 (black). The bronchi also contain ciliated and Clara cells. They have many more neuroendocrine cells than the trachea, often in clusters at airway branch points, known as neuroendocrine bodies (NEBs), shown here by staining with antibody to calcitonin-gene related peptide (Cgrp, red). Each narrow bronchiolus opens into alveoli through a bronchioalveolar duct. This junction region is usually associated with a blood vessel (bv). The alveoli contain type II cells, which secrete large amounts of surfactant proteins [detected with antibody to surfactant protein C (Sftpc)]. The thin, flattened type I cells line the alveoli and are closely apposed by capillaries. Photographic images were all provided by E.R. from her own research material. (Rawlins et al, 2006).

3. Phosphatase Tensin Homologue (PTEN).

In 1997, three laboratories independently reported a tumor suppressor located on human chromosome 10q23. Sequence analysis demonstrated that this gene encodes a phosphatase with homology to tensin and auxilin. This gene was named PTEN for phosphatase and tensin homologue deleted on chromosome 10 (or MMAC1/TEP1) (Stiles et al., 2004). Later studies demonstrated that PTEN is a negative regulator of a major cell growth and survival signaling pathway, namely the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway (Downes et al, 2007; Stiles et al, 2004). PTEN antagonizes the function of PI3K by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3) (Fig.5), a key product of PI3K responsible for the activation of downstream target molecules including AKT, an oncogenic protein (Downes et al., 2007). Many studies using either primary tumor tissues or established tumor cell lines demonstrated high frequencies of *PTEN* mutation/deletion in various human cancers, including brain, bladder, breast, prostate, and endometrial cancers (Ali et al,1999; Aveyard et al, 1999; Dahia, 2000; Dreher et al., 2004; Rasheed et al, 1997), marking *PTEN* the second most frequently mutated human tumor suppressor gene (Stokoe, 2001). Therefore, loss of *PTEN* has become an important marker for ensuring the sensitivity of chemotherapies targeted at PI3K/AKT signaling pathway (Neshat et al., 2001). *PTEN* mutation was also found to be responsible for at least 3 autosomal dominant tumor predisposition syndromes, such as Cowden's disease (where the patients suffer from hamartomas in multiple organs with a tendency of malignant transformation) (Liaw et al, 1997; Nelen et al, 1997), Bannayan–Zonana syndrome (BZS) and Lhermitte–Duclos disease (LDD) (Arch et al, 1997; Lida et al, 1998,; Koch et al, 1999; Marsh et al, 1999). Deletion of *Pten* in mouse models revealed that PTEN is critical for animal development. *Pten* null embryos die early during embryogenesis (Di Cristofano et al, 1998; Podsypanina et al, 1999; Stambolic et al, 1998; Suzuki et al, 1998) and thus much of our current knowledge regarding the functions of PTEN in development is acquired from animals with tissue-specific *Pten* deletion using the Cre-loxP system.

3.1 *Pten*-controlled signaling pathways and development.

Studies on the molecular mechanism of PTEN function revealed that the signaling pathways controlled by the PTEN tumor suppressor are vital for both cell growth and animal development (Vivanco and Sawyers, 2002). PTEN functions as a phosphatase with both lipid and protein phosphatase activities in vitro (Li and SU, 1997; Maehama and Dixon, 1998; Myers et al, 1998). While its in vivo protein phosphatase activity remains to be further investigated, PTEN's lipid phosphatase activity has been demonstrated both in vitro and in vivo. As a lipid phosphatase, PTEN dephosphorylates phosphotidylinosital-3,4,5-triphosphate (PIP_3), a product of phosphotidylinosital-3-kinase (PI3Kinase) (Maehama and Dixon, 1998; Stambolic et al, 1998; Sun et al, 1999). By dephosphorylating PIP_3, PTEN inhibits the growth factor signals transduced through PI3Kinase, thus has a broad impact on cell growth, cell migration, cell death, and cell differentiation, processes involved in normal development (Fig.5).

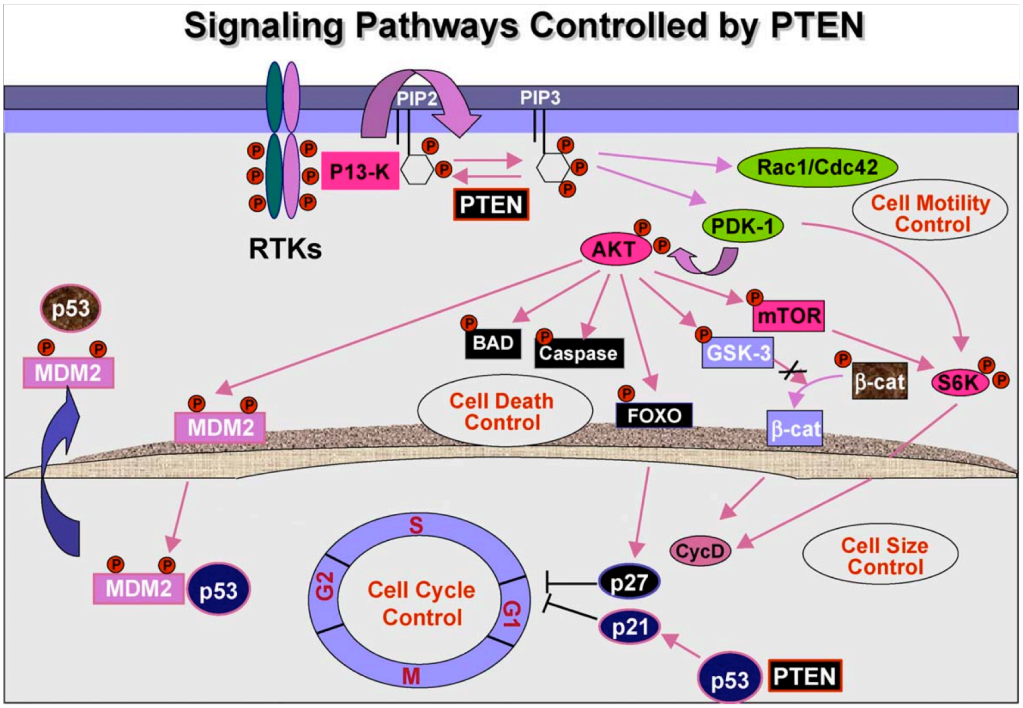


Figure 5; Stiles et al, 2004.

PTEN deficiency leads to accumulation of PIP_3 that in turn activates several signaling molecules including the phosphotidylinositol-dependent kinases (PDKs), the serine/threonine kinases AKT/PKB, S6 kinase, and mTOR, as well as small GTPases Rac1 and Cdc42 (Anderson et al, 1998; LIliental et al, 2000; Pene et al, 2000; Stiles et al, 2002; Sun et al, 1999; Wu et al, 1998). Among these downstream signaling molecules, AKT is the best characterized one (Anderson et al, 1998; Sun et al, 1999; Wu et al, 1998). A number of substrates have been identified for AKT kinase, including caspase 3 and 9 (Cardone et al., 1998), metabolic enzyme glycogen synthase kinase (Cross et al., 1995), Phosphorylation of these molecules leads to changes in their subcellular localization, activities, or half lives, which in turn controls cell metabolism, cell death, cell cycle progression, and cell differentiation (for review, see Vivanco and Sawyers, 2002).

The PTEN/PI3 kinase/AKT signaling pathway also interacts with other signaling pathways known to be essential for normal development, including the TGF-β/Smad pathway and the Wnt/β-catenin pathway. TGF-β regulates many cellular processes that are crucial in normal development. PTEN, also called TEP1 for TGF-β-regulated and epithelial cell-enriched phosphatase (Li and Sun, 1997), is rapidly downregulated by TGF-β in keratinocytes and pancreas (Ebert et al, 2002; Li and Sun, 1997). Conversely, PI3 kinase/AKT phosphorylates SMAD3, a receptor-regulated SMAD, and inhibits SMAD3-dependent TGF-β signaling (Song et al., 2003). Similar to the TGF-β pathway, the Wnt signaling pathway is also conserved in various organisms from worms to mammals, and plays important roles in development, cellular proliferation, and differentiation. In mammals, the Wnt signal transduction pathway is involved in many differentiation events during embryonic development (for review, see Polakis, 2000). Activation of the Wnt downstream molecule β-catenin leads to tumor formation of various origins (Polakis, 2000). The cytosolic pool of β-catenin has a short half-life in the absence of Wnt signaling. Its turnover is controlled by the action of a multi-protein complex (for a review, see (Kikuchi, 2000), including at least Axin, APC, PP2A, GBP, and GSK-3β. GSK-3β kinase phosphorylates β-catenin on specific serine and threonine residues at its N-terminus, targeting it to

ubiquitin-mediated degradation (Munemitsu et al, 1996; Yost et al, 1996). AKT kinase phosphorylates and inhibits GSK-3β, leading to β-catenin nuclear translocation and activation (Pap and Cooper, 1998; Yost et al, 1996). In addition to the above signaling pathways, recent studies have pointed out the role of PTEN in regulating the expression of homeobox genes, such as NKX3.1 (Wang et al., 2003) and hepatic nuclear factors (Wolfrum et al., 2003). Taken together, PTEN can modulate animal development by multiple mechanisms.

3.2 PTEN expression and function in early development

Pten expression can be detected as early as embryonic stem cell stage, which is derived from embryonic day 3.5 blastocysts (Sun et al., 1999). In situ hybridization and immunohistochemistry analyses indicate that *Pten* is expressed in both extraembryonic and embryonic tissues (Luukko et al, 1999; Podsypanina et al, 1999). Within the embryo proper, *Pten* is expressed ubiquitously during the early stage of embryonic development (E7–11) but becomes more restricted in the later stage (E15–19) in tissues and organs (Podsypanina et al., 1999). *Pten* is highly expressed in the central nervous system, liver, heart, skin, and gastrointestinal tract, similar to the expression patterns observed during human development (Gimm et al., 2000). At the cellular level, PTEN is present in both cytosol and nucleus (Freeman et al, 2003; Gimm et al, 2000; Li and Sun, 1997). Our recent study indicates that the subcellular localization of PTEN may be developmentally regulated and differential nuclear and cytoplasmic expression of PTEN may reflect its role during different stages of development and cellular functions (Wang and Wu, unpublished observations).

To study the biological functions of PTEN, independent groups have generated *Pten* mutant animals by deleting different regions of the *Pten* locus (Di Cristofano et al, 1998; Podsypanina et al, 1999; Suzuki et al, 1998). Animals heterozygous for *Pten* developed normally but homozygotes died early during embryogenesis, bearing different onsets and phenotypes. While all groups agreed that PTEN are essential for normal embryonic development, they reached different conclusions on the exact embryonic function of PTEN.

Di Cristofano et al. (1998) could not recover null embryos post E7.5 and observed differentiation defects in *Pten*$^{-/-}$ ES cell-derived embryoid bodies. *Pten* null ES cells also failed to contribute to the formation of the chimeric organism. Thus, they concluded that PTEN is required for the differentiation and organization of three germ layers (Di Cristofano et al, 1998; Suzuki et al, 1998) obtained gastrulated null embryos at E7.5 with severely expanded and abnormally patterned cephalic and caudal regions at E8.5. In light of the ubiquitous expression nature of *Pten* gene during early embryogenesis, one should not be surprised if *Pten* null embryos are dead due to abnormal development of multiple organs and tissues.

3.3 PTEN regulates stem cell function

Among many key molecules that are crucial for normal development and tumorigenesis, Wnt, Shh, Notch, as well as their controlled signaling pathways are known to have important roles in regulating stem cell self-renewal, proliferation, and differentiation (Reya et al., 2001). Because *Pten* is highly expressed in ES cells, the functions of PTEN in ES cells have been studied independently by different groups. Di Cristofano et al. showed that *Pten* null ES cells have enhanced anchorage-independent growth property. However, under regular adherent conditions *Pten* null ES cells behaved similar to WT controls in their growth rate and cell cycle distribution (Di Cristofano et al., 1998). Sun et al showed that *Pten−/−* ES cells exhibited an increased growth rate under normal growth condition and could proliferate and survive even in the absence of serum. Importantly, deletion of *Akt-1*, the major *Akt* family member in *Pten* null ES cells, completely reversed the growth advantage phenotype seen in *Pten−/−* cells (Stiles et al., 2002), further supporting the essential role of AKT in PTEN-controlled ES cell proliferation and survival.

By specifically deleting *Pten* in the brain during mid-embryonic development, Groszer et al generated mutant mice in which the brain size as well as weight is doubled, similar to macrocephaly found in humans with inherited *PTEN* deletions/mutations (Groszer et al., 2001). Further studies indicated that the enlarged brain results from increased cell proliferation, decreased cell death,

and enlarged cell size. The histoarchitecture of the mutant brain also appears abnormal. However, cell fate commitments of the progenitors were largely undisturbed. The in vitro analysis indicates that there are more stem cells in the mutant brain, and these stem cells are undergoing more self-renewal divisions. Moreover, PTEN-deficient neural stem/progenitor cells have a greater proliferation capacity, which is due, at least in part, to a shortened cell cycle time (Groszer et al., 2001).

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 (He et al., 2007). In the hemopoietic system, PTEN is required to maintain haemopoietic stem cells (HSCs) in a quiescent state and absence of PTEN drives the entry of HSC into cell cycle generating leukemic stem cells (Yilmaz et al, 2006). The role of PTEN in regulating stem cell self-renewal and proliferation is further supported by the generation of *Pten* null primordial germ cells (PGCs) (Kimura et al, 2003;Moe-Behrens et al, 2003).

3.4 PTEN and cell fate determination

Even though PTEN plays important roles in stem cell self-renewal and proliferation, *Pten* deletion does not change the overall cell differentiation program. Humans with *PTEN* germline mutations develop hamartomas in tissues derived from all three germ layers (Liaw et al, 1997;Marsh et al, 1998; Nelen et al, 1997). These focal hyperplastic or dysplastic lesions are formed by overgrowth of tissue elements normally present at these sites, indicating that *PTEN*-deficient cells in a variety of tissues remain responsive to exogenous and endogenous differentiation cues. Concordantly, it has been shown that PTEN is dispensable for fate determination of forebrain cortical (Groszer et al., 2001) and cerebellar (Marino et al., 2002) progenitor cells in vivo and in vitro. Similarly, overexpression of *Akt* or deletion of *dPTEN* in *Drosophila* did not affect cell-fate determination either (Gao et al, 2000; Huang et al, 1999; Verdu et al, 1999).

AIM OF THE STUDY

Deletion of Pten in mouse revealed a critical role for this gene in development. *Pten* null embryos die at E7.5. Thus, much of the knowledge regarding the role of *Pten* in development of various organs has emerged from organ-specific deletions of *Pten*. *Pten*'s role in stem cell self-renewal and proliferation and cell differentiation has been already described in different organs. For exemple, tissue-specific deletion of *Pten* in the brain causes a phenotype similar to macrocephaly in humans due to increased number of stem cells (Groszer et al, 2001). Loss of *Pten* in the intestinal stem cells initiates polyposis, a condition characterized by precancerous neoplastic increase in the number of crypts which contain intestinal stem cells (He et al., 2007).

At the beginning of our study, no data on pten role lung development were available. Therefore, we decided to look at the role of *Pten* in lung morphogenesis and its role in governing lung progenitor cells homesostasis: we used an in vitro system such as lung endodermal explant culture (Xing et al, 2008) and an in vivo system to study epithelial cellular lineage after *Pten* deletion (Tiozzo et al, 2009, submitted). Finally, we deleted *Pten* in the mesenchymal compartment to study the role of *Pten* in the mesenchymal lineage formation (work in progress). As this study was underway two reports outlined the results of epithelial-specific deletion of *Pten* on lung morphogenesis (Yanagi et al, 2007; Dave et al, 2007). In both previous studies *Pten* deletion was achieved using the *SPC-rtTA;Tet(O)-cre* line. Our data confirms and add new data on the role of *Pten* in lung development and lung progenitor cells homeostasis.

MATERIAL AND METHODS

a. Lung culture and lung endodermal explant culture

Whole embryonic lungs were dissected at gestational stage E11.5. In each Grobstein Falcon dish, two to three lungs were placed on filters (Millipore, Bedford, MA) that were placed on top of a stainless steel grid. The filters were in close contact with BGJb (GIBCO) growth medium supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml), with 10 or 50 ng/ml recombinant human TGFβ1 peptide (R&D systems) or same amount of Bovine Serum Albumin (BSA) as control. Heparin beads (Sigma) were incubated with recombinant human TGFβ1 (10 µl of a 50 ng/µl solution; R&D Systems, MN) or in BSA (control) at 37 °C for 2 h, grafted onto lung explants and cultured for 48 h or 96 h. The lungs were incubated under optimal humidity in 95% air/5% CO_2. At indicated times, the lung explants were either homogenized in Trizol (GIBCO) for RNA isolation or fixed in 4% Paraformaldehyde (PFA). For mesenchyme-free endodremal lung explant culture, distal lung tips were isolated as previously described (Bellusci et al., 1997). Briefly, lungs from mutant or control embryos were dissected at E12.5 and treated with Dispase (50 U/ml, BD Biosciences) at 4 °C for 20 min. Epithelial buds of distal lung tips were then isolated by removing mesenchyme with tungsten needles and embedded into growth factor-reduced Matrigel (Fisher Scientific), diluted 1:1 in culture medium (50% DMEM: 50% Ham's F12, 0.05 U/ml penicillin, 0.05 mg/ml streptomycin). After polymerization of the Matrigel at 37 °C, explants were covered with culture medium with or without FGF and TGFβs and cultured at 37 °C at 5% CO_2 for various lengths of time as indicated.

b. Proliferation assay on the lung endoderm explants

Subsequent to culturing, endodermal explants were treated with bromodeoxyurdine (BrdU) reagent at 1 µl per 400 µl medium for 3 h. The explants were fixed, dehydrated and paraffin embedded. The sections were then re-hydrated and labeled by BrdU staining kit (Zymed Laboratories Inc.) and Hematoxylin/Eosin staining. The sections were then photographed. The *BrdU* positive cells as well as the total number of cells (Hematoxylin/Eosin positive cells) in the explants per section were counted manually using photomicrograph of tissue taken at 40 on a Zeiss Microscope. The percent of labeled cells was calculated. To compare the proliferation in FGF10 or FGF10+TGFβ1 treated endodermal explants, 678 cells/5 sections from FGF10 treatment and 399 cells/4 sections from FGF10+TGFβ1 treatment were analyzed. To compare the proliferation in wild-type and $Pten^{\Delta/\Delta}$ endodermal explants after FGF10 or FGF10+TGFβ1 treatments, 2651 cells/7 sections and 734 cells/7 sections from wild-type explants and 1343 cells/8 sections and 1035 cells/8 sections from $Pten^{Nkx2.1\text{-}cre}$ explants were analyzed.

c. 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 (Xu et al, 2008). The *Nkx2.1-cre* transgenic mice are fertile and show no obvious abnormalities.

d. Generation of $Pten^{Nkx2.1cre}$ mice

$Pten^{flox/flox}$ females (BALB) were mated with *Nkx2.1-cre* male mice (C57BL6 background). We backcrossed the mice for 5 generations to obtain mice carrying *Nkx2.-cre;* $Pten^{flox/flox}$ (hereforth referred to as $Pten^{Nkx2.1\text{-}cre}$) in a pure BALB background. $Pten^{flox/flox}$ mice were used as control.

Genotyping of the *Nkx2.1-cre* mice (Xu et al, 2008) and of $Pten^{flox}$, $Pten^{\Delta}$ and $Pten^{wt}$ alleles was carried out as previously described (Lesche et al, 2002).

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

e. Naphthalene treatments.

Naphthalene treatments were carried out as previously described (Hong et al, 2001).

Briefly, naphthalene (Sigma, St. Luise, MO) was dissolved in corn oil (Sigma, St. Luise, MO) in a final concentration of 30 mg/ml. *Pten*$^{Nkx2.1\text{-}cre}$ and *Pten*$^{flox/flox}$ animals received 250 mg of Naphthalene/kg body weight i.p. Control animals received an equivalent of corn oil volume carrier. Mice were recovered in filtered air and sacrificed by infusion of 100 mg/Kg Pentobarbital after three days or seven days. The lungs were collected for further analysis.

Group of 4 animals for each experiment and time point were studied.

f. Tissue collection

Embryonic lungs from control and mutants embryos were collected at E15.5 and E18.5. Adult mice (at least 3 *Pten*$^{Nkx2.1\text{-}cre}$ and 3 *Pten*$^{flox/flox}$) were euthanized by CO_2 administration at different stages: at 60 days, 90 days and 6 months.

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.

g. Immunohistochemistry analysis.

Sections were cleared with 2 changes of xylene, hydrated with a successive graded Ethanol series to water.

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

For NKX2.1, PTEN, P-AKT, β-catenin and HES-1 antigen retrieval was performed by boiling the samples for 20 minutes in Na-citrate buffer (10 mM ph6.0) and incubating the samples with NKX2.1 (1:1500, Seven Hills), PTEN

(1:100, Cell Signaling), P-AKT (1:50, Cell Signaling), β-catenin TOTAL (1:100, BD Bioscience) and ACTIVE (1:100, Millipore), Collagen 1 (1:500, Abcam), PECAM (1:50, LAbvision) and HES-1 (1:50, Santa Cruz) overnight. The signals were visualized with the Histostatin Rabbit Primary Kit (Zymed-Invitrogen) as recommended by the manufactor.

Incubation of antibodies for K14 (1:200, Labvision), CC10 (1:100, Santa Cruz), P63 (1:500, Santa Cruz), SPC (1:500, Seven Hills), CGRP (1:100, Abcam), ß-tubulin IV (1:50, Biogenex), T1-α (Hybridoma Bank), E-cadherin (1:200, BD), α-SMA (1:200, Sigma), N-myc (1:100, Santa Cruz) was performed in TBS with 3% of Bovine serum albumine and 0.1% triton overnight at 4°C degree.

Secondary antibodies were from Jackson Immunoresearch; vectashield with DAPI was the mounting medium.

PAS staining and Masson staining were performed according to the manufacturer's protocol (respectively Sigma 395B and HT-15).

The Photomicrographs were taken using Leica DMRA fluorescence microscope with a Hamamatsu Digital Camera CCD camera and Zeiss Axioplan, Germany.

The lung sections where were performed the double staining for CC10/SpC were examinated with a LSM 510 Confocal / Multiphoton.

h. Cell proliferation analysis.

Cells proliferation was assessed using Ki67 staining on 3 months old lungs.

The tissues were cleared with 2 changes of xylene, hydrated with a different graded Ethanol series to water. Antigen retrieval was performed by boiling the sample for 12 minutes in Na-citrate buffer (10 mM ph6.0) and incubating the sample with Ki67 (1:200, Labvision) antibodies for 10 minutes at RT. Signals were visualized with the Histostatin Rabbit Primary Kit (Zymed-Invitrogen) following the manufactorer's instructions. Slides were counterstained with Hematoxilyn.

The total number of cells and the number of Ki67 positive cells in the airways epithelium were scored in 10 photomicrographs (80x magnification) in random

portions of 3 different sections in 3 mutants and 3 controls. The significance in proliferation between control and mutant lungs was evaluated by T-test.

i. Cell death analysis

The number of apoptotic cells in 3 months old lungs was determined by TUNEL assay using the In Situ Cell Death Detection Kit (Roche Diagnostic Gmb) according to the manufacturer's protocol. Sections were counterstained with DAPI.

The sections were photographed using a fluorescence microscopy Leica DMRA fluorescence microscope.

j. Protein extraction and western blot

Total protein extracts were prepared from 3 weeks old *Pten*$^{flox/flox}$ and *Pten*$^{Nkx2.1\text{-}cre}$ lungs with RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma), separated on SDS-PAGE gel and then blotted to PVDF (Polyvinylidene Difluoride) membrane (Millipore). p-AKT was detected with an antibodies purchased from Cell signalling (p-AKT) at the concentration suggested by the manufacturer.

k. RNA extractions and Quantitative RT-PCR

RNA was extracted from lungs of transgenic mice and wild-type littermate controls at specified postnatal stages using a Qiagen RNAeasy kit as described by the manufacturer. Total RNA (1 µg) was then reverse-transcribed into cDNA using the Superscript II reverse transcriptase (Invitrogen) for further realtime PCR analysis.

Real time PCR using primers to detect murine *Surfactant protein C* (*Spc*), murine *aquaporin-5* (*Aqp5*), murine *clara cell secretory protein* (*CC10*), murine *calcitonin gene-related peptide (CGRP*), murine *tubulin IV*, murine *Caspase 3*, murine *Hes1, murine Pten and Erg1* and housekeeping gene mouse *ß-actin* (ACTB) was performed on a ABI PRISM 7700 Sequence Detection System using Pre-Developed TaqMan Assay Reagents (Applied Biosystems, Foster City, CA). Quantitative analysis of gene expression was performed using the comparative CT (ΔCT) method, in which CT is the threshold cycle number (the minimum number of cycles needed before the

product can be detected) (Londhe et al., 2005). The arithmetic formula for the ΔCT method is the difference in threshold cycles for a target (i.e. CC10) and an endogenous reference (i.e. housekeeping gene *ß-actin*). The amount of target normalized to an endogenous reference (i.e. CC10 in transgenic animals) and relative to a calibration normalized to an endogenous reference (i.e. CC10 in wild-type controls) is given by 2-ΔΔCT. The calculation of 2-ΔΔCT then gives a relative value when comparing the target with the calibrator, which we designate in this context as the fold increase of transgenic animals to wild-type controls of the target mRNA relative quantification.

I. FACS sorting.

Mice were euthanized with CO_2, the lungs were isolated and perfused with 10ml PBS through intracardiac route. The lungs were then washed in Milteni solution and the cells were incubated with 0.001% DNAase (Sigma) and 0.01% Collagenase (Roche) for 15 minutes at 37°C. Cells were filtered through 40 µl filters, centrifuged at 8000 rpm for 5 minute then respuspended in red blood cell lysis buffer for 15 minutes at 37ºC for 2 times.
The cells were washed in Milteni solution, separated, resuspended in Milteni solution at a final concentration of $10X10^6$/500 µl and blocked for 10 minutes at 4ºC with 10 µl of FC block (BD Pharmingen). After 2 washed in Milteni solution, they were incubated for 15 minutes at 4ºC with 20 µl of the following antibodies: Lys6A (Sca-1)-Cy7, CD31 and CD45 Biotinilated, CD34Fluor647, secondary antibodies PE-Texas Red Streptavidine (all from BD Bioscience).
Analysis was performed in a FACSAria Cytometer (BD bioscience) and the data were analized by FACSDiva software version.

RESULTS

1. *Pten* and lung morphogenesis: a new role of *Pten* in TGFβ-induced inhibition of lung endodermal cell proliferation.

Although an indirect link between diminished *Pten* and Transforming growth factor betaβ (TGFβ) was discovered In human idiopatic pulmonary fibrosis, the in vivo relationship between TGFβ signaling, *Pten* and proliferation remains unkown.

For this reason, we assessed the direct impact of TGFβ on isolated mesenchyme-free embryonic lung endodermal explants: the results demonstrate that all 3 isoforms of TGFβ inhibit lung endodermal morphogenesis and that the inihibitory impact of TGFβ is only partly dependent on SMAds, but requires funcional TβRII activity. Since the TGFβ-induced inhibition of endodermal morhogenesis is associated with inhibition of cell proliferation and considering the increased *Pten* mRNA in the endodermal explants treated with TGFβ (Figure 1), we hypothesized that this inhibitory activity may be mediated througth increased *Pten.* To test this hypothesis, we first validated the PCR results by precise measurement of mRNA for Pten and its transcriptional regulatro EGR1 (Okamura et al, 2005). Real-time PCR analysis showed a 4-fold increase in *Pten* mRNA in endodermal explants treated with TGFβ (Figure 2, panel A). Treatment with TGFβ also increased *Egr1* mRNA level by nearly 14 fold (Figure 2, panel B).

To confirm the functional involvement of Pten, the response to TGFβ of lung endodermal explants from conditionally deleted *Pten Nkx2.1-cre* driven recombinatioin was examined (Figure 3).

Deletion of one copy of *Pten* gene (*Pten* $^{\Delta/+}$) in the endoderm was sufficient to cause major changes in response to FGF10 alone. In contrast to the control (*Pten* $^{flox/flox}$) which showed normal morphogenesis when treated wih FGF10,

the *Pten* $^{\Delta/+}$ endoderm presents only marginal budding (Figure 3, panel G), when the homozygous deletion of *Pten* (*Pten*$^{Nkx2.1\text{-}cre}$) entirely abrogated budding altogether (Figure 3, panel K).

This observation suggested that even reduced *Pten* interferes with normal processes involved in morphogenesis (haploinsufficiency) at least in the mesenchyme-free endodermal explant culture model. Importantly however, both heterozygous and homozygous deletion of *Pten* overcame the TGFβ-induced inhibition of cell proliferation as assessed by size of the explant and BrdU labeling index measurements (Figure 4).

This finding demonstrates that the PTEN/AKT pathway plays a key role in the TGFβ-induced inhibition of lung endodermal cell proliferation.

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

Nkx2.1 Cre-recombinase Driver mouse line.

Based on our previous results, we decided to look at the potential role of *Pten* in lung morphogenesis in vivo, using the *Nkx2.1-cre* mouse line to delete *Pten* in the lung epithelium. *Nkx2.1-cre* mouse line is a novel transgenic cre mouse line generated by inserting a modified bacterial artificial chromosome (BAC) in which the second exon of *Nkx2.1* is replaced by the cre recombinase (Xu et al, 2008). 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 (Figure 5).

LacZ activity was virtually absent in the WT lungs (Figure 5C). In E10.5 *ROSA26R-LacZ* $^{Nkx2.1\text{-}cre}$ embryos, LacZ activity was limited to the primordial lung and brain (Figure 5A, B, arrows). At E13.5, it was possible to detect Lac-Z activity in the lung epithelium, brain and thyroid (Figure 5D-E; arrows) in the *ROSA26R-LacZ*$^{Nkx2.1\text{-}cre}$ 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 (Figure 5E-G). In E15.5 and adult lungs (Figure 5H-K), 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. *Pten* deletion was detected by

PCR analysis of lung DNA and confirmed via Immunohistochemistry. Analysis of PTEN protein in *Pten*$^{Nkx2.1\text{-}cre}$ lungs by immunohistochemistry showed absence of PTEN protein in nearly 100% of epithelial cells with only rare positive staining in the mutant lungs (Figure 6 E and F; arrows). The PTEN negative epithelial cells in the mutant lungs were positive for NKX2.1, indicating their lung epithelial cell identity (Figure 2G, H). We confirmed the deletion of *Pten* with PCR using DNA from lung tissue and 2 different sets of primers. Our results indicate the presence of the $\Delta 5$ allele that confirms the deletion (Lesche te al, 2002) (Figure 6I). At this stage of development, there were no detectable abnormalities in branching morphogenesis of the embryonic mutant versus control (*Pten*$^{flox/flox}$) lungs (Figure 6, compare A and C to B and D). Therefore, epithelial deletion of *Pten* during the pseudoglandular stage does not appear to interfere with lung branching. However, in the proximal lung epithelium, progressive epithelial hyperplasia extending from the trachea to the small bronchioles (Figure 7A-F) was detected in the mutant embryos of all embryonic stages examined.

The epithelial cells positive for E-cadherin (Figure 7E, F) displayed a papillae-like structure with the apical side of the cells facing the airway lumen. The hyperplastic epithelium showed evidence of increased cell proliferation, as documented by Ki67 immunostaining (Figure 7G-M). In addition, analysis by TUNEL revealed decreased apoptosis in the mutant lungs when compared to controls (Figure 7K, L). Further quantification of apoptosis, using Real Time PCR for *Caspase 3* (Figure 7N), confirmed the TUNEL results. This analysis showed a statistically significant decrease in *Caspase 3* mRNA in mutant (n=3) versus wild type lungs (n=3, $P<0.05$). Therefore, early epithelial deletion of *Pten* causes airway hyperplasia that is detectable from early stages of lung development and in the adult mice, due to increased cell proliferation and decreased apoptosis.

Deletion of *Pten* results in expansion of epithelial cell populations in multiple progenitor cell niches.

When compared to controls, $Pten^{Nkx2.1\text{-}cre}$ 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 and Keratin14 were significantly more numerous (0.5%±0.08 versus 0.28%±0.02, n=3, $p<0.05$; Figure 8, trachea, compare I and J). More distally, in the bronchi, the neuroepithelial bodies (NEB), identifiable by CGRP/CC10 overlapping expression, were also increased in number in the $Pten^{Nkx2.1\text{-}cre}$ versus control lungs. Of note, although immunohistochemistry is not a quantitative technique, the NEB clusters were not only more numerous (Figure 5I), but showed stronger immunoreactivity (Figure 8 bronchi, G, H). Real Time PCR data confirmed our Immunohistochemistry data, showing a nearly 80 fold increase in CGRP expression in mutant compared to control lungs (Figure 8 bronchi, J). 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 to controls, $Pten^{Nkx2.1\text{-}cre}$ lungs also showed an expansion of progenitor cells occupying the BADJ region (Figure 9, compare A & C to B &D, respectively). Many of the $Pten^{Nkx2.1\text{-}cre}$ cells were distinctly larger in size (Figure 9C, D, arrows). We used immunohistochemistry to determine whether any of the over-expanded cells in the BADJ were double positive for CC10 and SpC, a characteristic previously associated with putative progenitor cells in this region (Kim et al, 2005). While in the control lungs these cells are extremely rare (Figure 9,E and H), double staining for anti-CC10 and anti-SpC antibodies detected increased number of CC10/SpC double positive cells in the mutant lungs (1.8%±4 versus 0.3%±0, n=3, $p<0.05$; Figure 9, compare I and J to H; quantification analysis, Figure 9 O). The CC10/SpC positive cells were more convincingly revealed by confocal microscopy (Figure 9K-N). Using FACS 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 3 fold increased in *Pten*$^{Nkx2.1\text{-}cre}$ compared to the control lungs (9.5% versus 2.8%) (Figure 9P, Q). 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 cells form putative progenitor cell masses in the BADJ.

Then, we examined the behavior of the *Pten*$^{Nkx2.1\text{-}cre}$ cells residing within the BADJ in the mutant lungs over time. *Pten*$^{Nkx2.1\text{-}cre}$ progenitor cells undergo proliferation as a function of time and within approximately 8 weeks of postnatal life, a "mass" consisting of *Pten*$^{Nkx2.1\text{-}cre}$ epithelial cells around the BADJ area is detected in some, but not all mutant lungs (Figure 10A, B). These masses are slow growing, benign 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) (Figure 10D, E). E-cadherin immunostaining showed that within the mass, the cells are organized into duct-like structures reminiscent of the pseudoglandular stage of early lung development and a distinct property of lung endodermal progenitor cells (Figure 10C). Importantly, *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*$^{Nkx2.1\text{-}cre}$ mass (Figure 10F).
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* $^{Nkx2.1\text{-}cre}$ 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 (He at al, 2007). Under normal physiological conditions, PTEN inhibits the stabilization of β-catenin by increasing the activity of GSK3. We therefore examined whether lung epithelial-specific

deletion of *Pten* leads to increased phospho-AKT and β-catenin. Immunohistochemical staining of *Pten*$^{Nkx2.1\text{-}cre}$ lungs showed increased level of phospho-AKT as well as total and active β-catenin (data not shown). Western Blot analysis revealed a difference at the protein level for phospho-AKT in mutant versus the wild type lung. Finally, nuclear localized N-myc a downstream target of β-catenin activation was readily detectable in *Pten*-depleted epithelial cells (Figure 10, F), as well as a decrease of APC (data not shown).

These data indicate that β-catenin signaling has been functionally activated in *Pten*$^{Nkx2.1\text{-}cre}$ lungs.

Impact of *Pten* deletion on epithelial cell lineage determination.

Expression of a number of cell markers was examined by immunofluorecence and Real Time PCR in *Pten*$^{Nkx2.1\text{-}cre}$ versus *Pten*$^{flox/flox}$ (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, immunostaining for CC10 revealed a markedly increased number of Clara cells in the mutant lungs (Figure 11A, B). The increase in CC10 positive cells was associated with a decrease in the number of ciliated cells, thought to be their terminally differentiated progeny, as revealed by β-tubulin staining (Figure 11C, D, arrows). In the distal compartments, SpC, a Type II cell marker, was increased (Figure 11E, F), whereas T1-α, a Type I cell marker was decreased in *Pten*$^{Nkx2.1\text{-}cre}$ lungs (Figure 11G, H), indicating a block in transition from precursor to terminally differentiated cell types. The Immunohistochemical 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 *hairy and enhancer of split 1* (HES1), known to be involved in cell determination in lung, particularly in the Clara cell lineage (Ito et al, 2000). Both Immunohistochemistry and Real Time

PCR showed increased *HES-1* in the mutant compared to the control lungs (Figure11, I and J). 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. These cells are CC10-positive in the bronchi but CC10-negative and negative for any of the known lung epithelial markers in the trachea (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 $Pten^{Nkx2.1\text{-}cre}$ and control mice to naphthalene, a simple and well-defined model of lung injury. Corn oil instead of naphthalene was used as control.
In $Pten^{fl/fl}$ control mice, naphthalene injury was detected in the trachea (Figure 12, A and D) and in the distal compartments (Figure 12G and J) when compared to the corn oil controls. In the wild type animals, peritoneally administered naphthalene denuded entirely the tracheal epithelium after three days (Figure 12B) with a partial re-epithelitation after 7 days (Figure12C). In the distal airway, naphthalene caused epithelial cell death within 72 hours (Figure 12H) followed by re-epithelization of the airways by presumably the $P450^{neg}$ variant of Clara cells (Stripp et al, 1995). After 7 days of injury, the epithelium was in part restored (Figure 12I). In contrast, after naphthalene administration, the proximal airway epithelium of $Pten^{Nkx2.1\text{-}cre}$ lungs at 3 and 7 days post injury appeared to remain intact with no signs of injury (Figure 12E, F). At the bronchial level, injury was reduced (Figure 12, compare K to H) and repair enhanced (Figure 12, compare L to I) in the $Pten^{Nkx2.1\text{-}cre}$ lungs, in comparison with the control group. These results suggest that the $Pten^{Nkx2.1\text{-}cre}$ airway epithelium has a significantly increased level of resistance to naphthalene and confers a better capacity to recover after injury. The mechanism underlying this selective resistance is being currently investigated.

3. Role of *Pten* in mesenchymal lineage formation (work in progress).

We deleted *Pten* in the lung mesenchymal cells in a vivo system:
in absence of a lung specific mesenchymal cre line, we deleted *Pten* with a *dermo1-cre* line, that acts in all the mesodermal derived tissues during embryogenesis.
The *Pten* ^{Dermo1cre} mice, generated in a pure Bl57 background, were born alive with expected mendelian ratio but they were not able to survive for more than few days.
The reason of the death is still to be determined, as it is possible to involve all the organs where the mesenchymal compartment is essential for their own correct function.
Histological analysis, performed on different gestational ages, didn't show any particular defects on lung morphogenesis except for a hypercellularity in the mesenchyme, due to an increase of proliferation, as expected. (Figure 13, compare A,D,G to C, F, J). There were not changes in the expression of different markers for mesenchymal derived cells, such α-SMA and PECAM (Figure 14) showing the *Pten* deletion doesn't appear to affect mesenchymal differentiation.
However, there was an increase of collagen 1 deposition (showed by the MASSON trichrome staining and by specific staining for Collagen 1) and extracellular matrix (PAS staining), confirming the role of *Pten* in collagen and extracellular matrix production.
Based on these preliminary results and on previous publications (White et al, 2006), we hypothesized that *Pten* can have a role in myofibroblasts differentiation after injury.
Therefore, we performed bleomycine injury, a very well known model of lung injury.
In the heterozygous animals at 21 days after injury, the collagene deposition and the fibrosys were worse compared to the control (data not shown). The

mechanism underlying this selective disadvantage is being currently investigated.

DISCUSSION

1. *Pten* mediates the effect of TGFβ-induced inhibition of cell proliferation

With our first work, on the lung endodermal explands, we found involvement of PTEN, in TGFβ-induced inhibition of lung endodermal cell proliferation. Direct evidence for the latter was derived by examining the response of $Pten^{Nkx2.1\text{-}cre}$ endodermal explants to TGFβ. Deletion of *Pten* restored cell proliferation in explants treated with combination of FGF10 and TGFβ. Initial identification of *Pten* was based on its decreased expression in response to TGFβ in a human keratinocyte cell line, HaCaT (Li et al., 1997). In contrast to the latter, we found increased *Pten* and its upstream transcription factor *Egr1* in embryonic lung endoderm, treated with TGFβ. The reason for this discrepancy remains unknown, but it may simply reflect differences between normal, embryonic tissue used in the current study, compared to a transformed cell line in the previous work (Li et al., 1997). However, this finding correlates well with the known function of *Pten* as a regulator of cell proliferation through the PTEN/AKT pathway. PTEN antagonizes PI3K, which is activated downstream of tyrosine kinase receptors, a major growth factor signaling pathway (For Reviews, Goberdhan et al., 2003 and Stiles et al., 2004). Thus, increased expression of *Pten* is expected to block cell proliferation as we have found in endodermal explants treated with TGFβ and this is, to our knowledge this is the first demonstration of the role of PTEN in TGFβ-induced inhibition of lung endodermal cell proliferation.

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

The results of our second work confirm that *Pten* affects the proliferation in vivo, like in vitro and add new information on the role of *Pten* in lung development,

Furthermore, *Pten* does not affect lung branching morphogenesis, but impacts 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 (Yanagi et al, 2007; dave et al, 2007). In both studies *Pten* deletion was achieved using the *SPC-rtTA;Tet(O)-cre* line.

Our findings are partially consistent with both reports: Yanagi et al (2007) who induced *Pten* deletion in the distal lung epithelium from E10 to E16 and found delayed lung branching along with impaired epithelial cell differentiation and neonatal lethality in 90% of mice, due likely to respiratory insufficiency. By contrast, Dave et al (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.

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 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 BALB 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 endogeneous *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*$^{Nkx2.1\text{-}cre}$ neonates experienced any respiratory distress and any sporadic death within the first two 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 to 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.

3. 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 bronchio-alveolar duct junction BADJ. In *Pten*$^{Nkx2.1\text{-}cre}$ 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 neuroepithelial bodies (NEB) and SpC/CC10 double positive cells in the BADJ were also increased (Figure 5 and 6). Yanagi et al. and Dave et al. also described an increased of NEB and BADJ cells, but an increase in progenitor cells in the *Pten*$^{Nkx2.1cre}$ trachea (area not affected by *SpcrtTA;Tet(O)*cre driver line) reveals an additional role for *Pten* which had gone unnoticed by the previous studies (Yanagi et al, 2007; Dave et al, 2007).

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 number 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 (T1α positive). Both the Clara cells and the type II cells are considered to be progenitors 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 non-endocrine epithelial cell fate (Ito et al, 2000). Interestingly, in our study we did not observe a decrease in the neuroepithelial bodies, which is inconsistent with previous reports where *Hes-1* inhibited neuroendocrine differentiation. Further studies are necessary to clarify the mechanisms underlying the impact of *Pten* in lung epithelial cell determination.

4. PTEN may control cell fate and progenitor cell homeostasis through β-catenin.

Absence of *Pten* in the cells leads to PIP_3 accumulation which in turn leads to over-activation 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 (Stiles et al, 2004). 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 $Pten^{Nkx2.1\text{-}cre}$ lungs (C.Li, Personal Communication). 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.

5. Absence of *Pten* at the BADJ leads to generation of benign 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 (Lahad et al, 2005). 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 hemopoietic 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 (Bonnet et al, 1997).

Different candidate genes are suggested as regulator for the proliferative capacity of these cells. One of these is *Pten*, having a role both in restricting the activation of hematopoietic stem cells as well as preventing leukemogenesis (Zhang et al, 2006).

In our model it appears that, in absence of PTEN, the CC10/SpC double positive cells, considered as progenitor cells in the lung, in time give rise to slow growing benign 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.

6. *Pten*$^{Nkx2.1\text{-}cre}$ airway epithelium exhibits relative resistance to Naphthalene injury.

Since mutant lungs showed an increase in the number of progenitors 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 (NAPH) is converted by the P450 (CytP4502F2) enzyme into its toxic derivatives, 1, 2-epoxide (jerina et al, 1971), a diepoxide (Stillweel et al, 1982), and quinines (Hesse et al, 1979; d'Arcy et al, 1984). A rare population of variant Clara cells (Clara^{v} cells) is thought to lack CytP4502F2 enzyme activity and hence, resistant to Naphthalene killing. Clara^{v} cells are thought 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 NAPH killing. If expansion of the cells in the airway epithelium of *Pten*$^{Nkx2.1\text{-}cre}$ 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.

7. Mesenchimal deletion of *Pten* does not affect lung mesenchymal cells differention but it worsens experimental pulmonary fibrosis.

A lethal disease of the lung, idiopathic pulmonary fibrosis (IPF) is a fibroproliferative disorder of the lung characterized by progressive and unrelenting ECM secretion and remodeling by a-SMA-expressing myofibroblasts which ultimately cause lung dysfunction and death (Mutsaers et al, Int J Bioch Cell Biol 1997). In IPF usual interstitial pneumonia (UIP), persistence of myofibroblasts is believed to contribute to the development of fibrosis. It has been established that myofibroblast differentiation occurs through combinatorial signals involving transforming growth factor-β (TGF-β) and integrin signaling (Serini et al, 1998). Conversely, mechanisms that inhibit or reverse myofibroblast differentiation are less well defined. Currently, no effective therapies exist to quell ongoing fibrosis in IPF/UIP. Thus, further investigation into the mechanisms that regulate myofibroblast differentiation may yield better insights into potential therapeutic options for patients with IPF/UIP. Nho et al previously observed diminished PTEN expression in fibroblasts isolated from lungs of patients with pulmonary fibrosis (Nho et al, 2006), and have hypotesized that this may account for the increased migratory capacity of these cells (Suganuma et al, 1995). White et al., 2006 recently showed that in human lung IPF specimens, myofibroblasts within fibroblastic foci showed diminished PTEN (White et al, 2006). In an experimental model of Bleomycin-induced fibrosis, inhibition of PTEN worsened fibrosis. Also in Pten(-/-) fibroblasts, a-SMA, proliferation and

collagen synthesis is increased. Treatment of wild type and not Pten(-/-) fibroblasts with TGF-b increased alpha-SMA, proliferation and collagen. Importantly reconstitution of Pten in Pten(-/-) cells decreased a-SMA production, proliferation and collagen production.

Previous reports showed a selective clonal expansion in subpopulations of fibroblasts derived from patients with fibroproliferative diseases (Jelaska et al, Springer Semin Immunopathol 1999). This clonal expansion, although not malignant *per se*, is reminiscent of malignant cells. Features of myofibroblasts in fibroproliferative disorders include a relative resistance to apoptosis (Desmouliere et al, Int J Biochem cell Biol, 1997), increased migration/invasion of tissue (White ES, et al, 2003) and increased proliferation (Zhang S et al, 1999), all characteristics of malignant cells. It has been well established that PTEN negatively regulates cell survival (Stambolic V et al, 1998) and fibroblast proliferation (Paramio JM et al, 1999). It is been previously shown that PTEN is suppressed in lung fibroblasts isolated from patients with IPF, which may account for increased migratory/invasive behavior (White ES et al, 2003). It was therefore propose that phenotypic behavior of myofibroblasts in fibroproliferative disorders is similar to that of malignant cells in cancers in which PTEN is mutated, deleted, or otherwise inactivated. Thus, it is likely that other features of myofibroblasts not still evaluated, such as contractility or secretion of other ECM proteins, may be orchestrated through the activity of PTEN. These observations clearly indicate the critical role of *Pten* in regulating myofibroblast proliferation, a-SMA and collagen production, suggesting a key role for *Pten* in IPF. Since further investigation into this area will likely shed more light on the spectrum of phenotypic behaviors modulated by PTEN in the lung mesenchyme.

Our preliminary data are therefore controversial: the absence of an impact on myofibroblast differentiation and on lung development is to puy in contrast with the previous reports (White et al, 2006). However, the worsening of the fibrosis after bleomycine injury is consistent with the literature.

It is possible to suppose that deletion of *Pten* has a different impact if it happens in cultured fibroblasts or in vivo.

Moreover, further investigations are necessary to address the real role of *Pten* on mesenchymal progenitor cells homeostasis: the worsening of the damage

after injury, in fact, together with a normal mesenchymal cell differentiation, can be explaned by an altered mesenchymal progenitor pool that, under stress conditions, does not behave normally; a better understanding of the mechanisms and the pathways altered is currently under investigation

In summary, our studies show that TGFβ, via stimulation of the transcription factor EGR1 and its target gene, *Pten* has a profound inhibitory effect on endodermal cell proliferation. Inhibition of cell proliferation undoubtedly slows, if not block morphogenesis. These data suggested a role of *Pten* during lung development that was investigated in vivo: absence of *Pten* in the epithelial or in the mesenchyme compartment does not affect lung branching morphogenesis in vivo. Deletion of *Pten* increased several progenitor pools in both proximal and distal epithelial lung compartments. In addition, absence of *Pten* inhibited only cell differentiation of specialized epithelial cell types. We also showed 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 and lung mesenchyme injury.

FINAL CONCLUSIONS

With our work, we demonstrated a role of *Pten* in lung stem cells homeostasis, in lung injury and repair. Actually, we are trying to determine the role of *Pten* in lung mesenchymal progenitor homeostasis and behaviour.

A better understanding of the mechanisms regulating lung progenitors cell homeostasis will be useful, not only in order to understand the pathogenesis of chronic pulmonary disease (like asthma or Idiopatic Pulmonary Fibrosis) but, also to address innovative therapeutic solutions like “manipulating the residential lung progenitors cell” in treating lung injury.

If these new informations can be translated in innovative therapeutic approaches is still to be determinated. In order for therapy to succeed, significant hurdles will need to be overcomed.

BIBLIOGRAPHY:

1. Ali I.U., L.M. Schriml and M. Dean, Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity, *J. Natl. Cancer Inst.* 91 (1999), pp. 1922–1932.

2. Alison M. R., Vig, P., Russo, F., Bigger, B. W., Amofah, E., Themis, M. and Forbes, S. (2004). Hepatic stem cells: from inside and outside the liver? *Cell Prolif.* 37, 1-21.

3. Anderson K.E., J. Coadwell, L.R. Stephens and P.T. Hawkins, Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B, *Curr. Biol.* 8 (1998), pp. 684–691.

4. Arch E.M., B.K. Goodman, R.A. Van Wesep, D. Liaw, K. Clarke, R. Parsons, V.A. McKusick, M.T. Geraghty, D.J. Marsh, P.L. Dahia, Z. Zheng, R.J. Gorlin, C. Eng, J. Li, S.I. Wang, S. Bose, K.M. Call, H.C. Tsou and M. Peacocke, Deletion of PTEN in a patient with Bannayan–Riley–Ruvalcaba syndrome suggests allelism with Cowden disease, *Am. J. Med. Genet.* 71 (1997), pp. 489–493.

5. Aso, Y., Yoneda, K. and Kikkawa, Y. (1976). Morphologic and biochemical study of pulmonary changes induced by bleomycin in mice. *Lab. Invest.* 35,558 -568.

6. Aveyard J.S., A. Skilleter, T. Habuchi and M.A. Knowles, Somatic mutation of PTEN in bladder carcinoma, *Br. J. Cancer* 80 (1999), pp. 904–908.

7. Avril-Delplanque, A., Casal, I., Castillon, N., Hinnrasky, J., Puchelle, E. and Peault, B. (2005). Aquaporin-3 expression in human fetal airway epithelial progenitor cells. *Stem Cells* 23,992 -1001.

8. Batenburg JJ, Elfring RH. Pre-translational regulation by glucocorticoid of fatty acid and phosphatidylcholine synthesis in type II cells from fetal rat lung. *FEBS Lett* 307: 164–168, 1992.

9. Basseres DS, Levantini E, Ji H, Monti S, Elf S, Dayaram T, Fenyus M, Kocher O, Golub T, Wong KK, Halmos B, Tenen DG. Respiratory failure due to differentiation arrest and expansion of alveolar cells following lung-specific loss of the transcription factor C/EBPalpha in mice. *Mol Cell Biol* 26: 1109–1123, 2006.

10. Bellusci S. Bellusci, J. Grindley, H. Emoto, N. Itoh and B.L. Hogan,

Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung, *Development* 124 (23) (1997), pp. 4867–4878.

11. Blenkinsopp, W. K. (1967). Proliferation of respiratory tract epithelium in the rat. *Exp. Cell Res.* 46,144 -154

12. Bonnet D, Dick JE.Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997 Jul;3(7):730-7.

13. Bort, R., Martinez-Barbera, J. P., Beddington, R. S. and Zaret, K. S. (2004). Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. *Development* 131,797 -806.

14. Cardone M.H., N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch and J.C. Reed, Regulation of cell death protease caspase-9 by phosphorylation, *Science* 282 (1998), pp. 1318–1321.

15. Cardoso WV, Lü J. Regulation of early lung morphogenesis: questions, facts and controversies. Development. 2006 May;133(9):1611-24.

16. Cross D.A., D.R. Alessi, P. Cohen, M. Andjelkovich and B.A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B, *Nature* 378 (1995), pp. 785–789.

17. Dahia P.L., PTEN, a unique tumor suppressor gene, Endocr. Relat. Cancer 7 (2000), pp. 115–129.

18. Davé V, Childs T, Xu Y, Ikegami M, Besnard V, Maeda Y, Wert SE, Neilson JR, Crabtree GR, Whitsett JA. Calcineurin/Nfat signaling is required for perinatal lung maturation and function. *J Clin Invest* 116: 2597–2609, 2006.

19. DeFelice M, Silberschmidt D, DiLauro R, Xu Y, Wert SE, Weaver TE, Bachurski CJ, Clark JC, Whitsett JA. TTF-1 phosphorylation is required for peripheral lung morphogenesis, perinatal survival, tissue-specific gene expression. *J Biol Chem* 278: 35574–35583, 2003.

20. Davé V, Childs T, Whitsett JA. Nuclear factor of activated T cells regulates transcription of the surfactant protein D gene (Sftpd) via direct interaction with thyroid transcription factor-1 in lung epithelial cells. *J Biol Chem* 279: 34578–34588, 2004.

21. Davé V, Wert SE, Tanner T, Thitoff AR, Loudy DE, Whitsett JA. Conditional deletion of Pten causes bronchiolar hyperplasia. Am J Respir Cell Mol Biol. 2008 Mar;38(3):337-45.

22. De Langhe SP, Carraro G, Warburton D, Hajihosseini MK, Bellusci S. Levels of mesenchymal FGFR2 signaling modulate smooth muscle progenitor cell commitment in the lung. Dev Biol. 2006 Nov 1;299(1):52-62.

23. Del Moral, P. M., Sala, F. G., Tefft, D., Shi, W., Keshet, E., Bellusci, S. and Warburton, D. (2006). VEGF-A signaling through Flk-1 is a critical facilitator of early embryonic lung epithelial to endothelial crosstalk and branching morphogenesis. *Dev. Biol.* 290,177 -188

24. Desmoulière A, Badid C, Bochaton-Piallat ML, Gabbiani G. Apoptosis during wound healing, fibrocontractive diseases and vascular wall injury. Int J Biochem Cell Biol. 1997 Jan;29(1):19-30.

25. Di Cristofano A., B. Pesce, C. Cordon-Cardo and P.P. Pandolfi, Pten is essential for embryonic development and tumour suppression, *Nat. Genet.* 19 (1998), pp. 348–355.

26. Downes C.P., S. Ross, H. Maccario, N. Perera, L. Davidson and N.R. Leslie, Stimulation of PI 3-kinase signaling via inhibition of the tumor suppressor phosphatase, PTEN, *Adv Enzyme Regul* 47 (2007), pp. 184–194.

27. Dreher T., H. Zentgraf, U. Abel, A. Kappeler, M.S. Michel, U. Bleyl and R. Grobholz, Reduction of PTEN and p27(kip1) expression correlates with tumor grade in prostate cancer. Analysis in radical prostatectomy specimens and needle biopsies, *Virchows Arch.* 29 (2004), p. 29.

28. Ebert M.P., G. Fei, L. Schandl, C. Mawrin, K. Dietzmann, P. Herrera, H. Friess, T.M. Gress and P. Malfertheiner, Reduced PTEN expression in the pancreas overexpressing transforming growth factor-beta 1, *Br. J. Cancer* 86 (2002), pp. 257–262.

29. Ehrenkranz RA, Walsh MC, Vohr BR, Jobe AH, Wright LL, Fanaroff AA, Wrage LA,Poole K; National Institutes of Child Health and Human Development Neonatal Research Network.Validation of the National Institutes of Health consensus definition of bronchopulmonary dysplasia.
Pediatrics. 2005 Dec;116(6):1353-60.

30. Engelhardt, J. F., Schlossberg, H., Yankaskas, J. R. and Dudus, L. (1995). Progenitor cells of the adult human airway involved in submucosal gland development. *Development* 121,2031 -2046.

31. Freeman D.J., A.G. Li, G. Wei, H.H. Li, N. Kertesz, R. Lesche, A.D. Whale, H. Martinez-Diaz, N. Rozengurt, R.D. Cardiff, X. Liu and H. Wu, PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms, *Cancer Cell* 3 (2003), pp. 117–130.

32. Fuchs, E., Tumbar, T. and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. *Cell* 116,769 -778.

33. Gao X., T.P. Neufeld and D. Pan, Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways,

Dev. Biol. 221 (2000), pp. 404–418.

34. Gebb, S. A. and Shannon, J. M. (2000). Tissue interactions mediate early events in pulmonary vasculogenesis. *Dev. Dyn.* 217,159 -169.

35. Giangreco, A., Reynolds, S. D. and Stripp, B. R. (2002). Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. *Am. J. Pathol.* 161,173 -182.

36. Gimm O., T. Attie-Bitach, J.A. Lees, M. Vekemans and C. Eng, Expression of the PTEN tumour suppressor protein during human development, *Hum. Mol. Genet.* 9 (2000), pp. 1633–1639.

37. Goberdhan and Wilson, 2003 D.C. Goberdhan and C. Wilson, PTEN: tumour suppressor, multifunctional growth regulator and more, *Hum. Mol. Genet.* 12 (2003), pp. R239–R248 Spec No 2.

38. Groszer M., R. Erickson, D.D. Scripture-Adams, R. Lesche, A. Trumpp, J.A. Zack, H.I. Kornblum, X. Liu and H. Wu, Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo, *Science* 294 (2001), pp. 2186–2189.

39. He XC, Yin T, Grindley JC, Tian Q, Sato T, Tao WA, Dirisina R, Porter-Westpfahl KS, Hembree M, Johnson T, Wiedemann LM, Barrett TA, Hood L, Wu H, Li L. PTEN-deficient intestinal stem cells initiate intestinal polyposis. Nat Genet. 2007 Feb;39(2):189-98.

40. Hesse S, Mezger M. Involvement of phenolic metabolites in the irreversible protein-binding of aromatic hydrocarbons: reactive metabolites of [14C]naphthalene and [14C]1-naphthol formed by rat liver microsomes. Mol Pharmacol. 1979 Sep;16(2):667-75.

41. Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR. Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. Am J Respir Cell Mol Biol. 2001 Jun;24(6):671-81.

42. H. Huang, C.J. Potter, W. Tao, D.M. Li, W. Brogiolo, E. Hafen, H. Sun and T. Xu, PTEN affects cell size, cell proliferation and apoptosis during *Drosophila* eye development, *Development* 126 (1999), pp. 5365–5372.

43. Huelsken J, Vogel R, Brinkmann V, Erdmann B, Birchmeier C, Birchmeier W. Requirement for beta-catenin in anterior-posterior axis formation in mice. J Cell Biol 148: 567–578, 2000.

44. Isakson BE, Lubman RL, Seedorf GJ, Boitano S. Modulation of pulmonary alveolar type II cell phenotype and communication by extracellular matrix and KGF. Am J Physiol Cell Physiol. 2001 Oct;281(4):C1291-9.

45. Ito T, Udaka N, Ikeda M, Yazawa T, Kageyama R, Kitamura H. Significance of proneural basic helix-loop-helix transcription factors in neuroendocrine differentiation of fetal lung epithelial cells and lung carcinoma cells. Histol Histopathol. 2001 Jan;16(1):335-43.

46. Jelaska A, Strehlow D, Korn JH. Fibroblast heterogeneity in physiological conditions and fibrotic disease. Springer Semin Immunopathol. 1999;21(4):385-95.

47. Jerina DM, Daly JW, Jeffrey AM, Gibson DT.Cis-1,2-dihydroxy-1,2-dihydronaphthalene: a bacterial metabolite from naphthalene. Arch Biochem Biophys. 1971 Jan;142(1):394-6.

48. Kai, T. and Spradling, A. (2004). Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. *Nature* 428,564 -569.

49. Kikuchi A., Regulation of beta-catenin signaling in the Wnt pathway, *Biochem. Biophys. Res. Commun.* 268 (2000), pp. 243–248.

50. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer, Cell 2005; 121: 823-835.

51. Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzalez, F. J. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* 10,60 -69.

52. Kimura T., A. Suzuki, Y. Fujita, K. Yomogida, H. Lomeli, N. Asada, M. Ikeuchi, A. Nagy, T.W. Mak and T. Nakano, Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production, *Development* 130 (2003), pp. 1691–1700.

53. Koch R., M. Scholz, M.R. Nelen, K. Schwechheimer, J.T. Epplen and A.G. Harders, Lhermitte–Duclos disease as a component of Cowden's syndrome. Case report and review of the literature, *J. Neurosurg.* 90 (1999), pp. 776–779.

54. Lahad JP, Mills GB, Coombes KR. Stem cell-ness: a "magic marker" for cancer. J Clin Invest. 2005 Jun;115(6):1463-7.

55. Lanza, R. (2006). *Essentials of Stem Cell Biology*. New York: Academic Press.

56. Lesche R, Groszer M, Gao J, Wang Y, Messing A, Sun H, Liu X, Wu H. Cre/loxP-mediated inactivation of the murine Pten tumor suppressor gene.Genesis. 2002 Feb;32(2):148-9.

57. Li D.M. and H. Sun, TEP1, encoded by a candidate tumor suppressor

locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta, *Cancer Res.* 57 (11) (1997), pp. 2124–2129.

58. Liaw D., D.J. Marsh, J. Li, P.L. Dahia, S.I. Wang, Z. Zheng, S. Bose, K.M. Call, H.C. Tsou, M. Peacocke, C. Eng and R. Parsons, Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome, *Nat. Genet.* 16 (1997), pp. 64–67.

59. Iida S., Y. Tanaka, H. Fujii, S. Hayashi, M. Kimura, T. Nagareda and K. Moriwaki, A heterozygous frameshift mutation of the PTEN/MMAC1 gene in a patient with Lhermitte–Duclos disease—only the mutated allele was expressed in the cerebellar tumor, *Int. J. Mol. Med.* 1 (1998), pp. 925–929.

60. Li D.M. and H. Sun, TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta, *Cancer Res.* 57 (1997), pp. 2124–2129.

61. Liaw D., D.J. Marsh, J. Li, P.L. Dahia, S.I. Wang, Z. Zheng, S. Bose, K.M. Call, H.C. Tsou, M. Peacocke, C. Eng and R. Parsons, Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome, *Nat. Genet.* 16 (1997), pp. 64–67.

62. Liliental J., S.Y. Moon, R. Lesche, R. Mamillapalli, D. Li, Y. Zheng, H. Sun and H. Wu, Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases, *Curr. Biol.* 10 (2000), pp. 401–404.

63. Liu C, Ikegami M, Stahlman MT, Dey CR, Whitsett JA. Inhibition of alveolarization and altered pulmonary mechanics in mice expressing GATA-6. *Am J Physiol Lung Cell Mol Physiol* 285: L1246–L1254, 2003.

64. Liu, J. Y., Nettesheim, P. and Randell, S. H. (1994). Growth and differentiation of tracheal epithelial progenitor cells. *Am. J. Physiol.* 266,L296 - L307.

65. Londhe VA, Belperio JA, Keane MP, Burdick MD, Xue YY, Strieter RM.CXCR2/CXCR2 ligand biological axis impairs alveologenesis during dsRNA-induced lung inflammation in mice. Pediatr Res. 2005 Nov;58(5):919-26.

66. Luukko K., A. Ylikorkala, M. Tiainen and T.P. Makela, Expression of LKB1 and PTEN tumor suppressor genes during mouse embryonic development, *Mech. Dev.* 83 (1999), pp. 187–190.

67. Maehama T. and J.E. Dixon, The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate, *J. Biol. Chem.* 273 (1998), pp. 13375–13378.

68. Matsumoto, K., Yoshitomi, H., Rossant, J. and Zaret, K. S. (2001). Liver organogenesis promoted by endothelial cells prior to vascular function.

Science 294,559 -563.

69. Maeda Y, Davé V, Whitsett JA.Transcriptional control of lung morphogenesis. Physiol Rev. 2007 Jan;87(1):219-44.

70. Marino S., P. Krimpenfort, C. Leung, H.A. van der Korput, J. Trapman, I. Camenisch, A. Berns and S. Brandner, PTEN is essential for cell migration but not for fate determination and tumourigenesis in the cerebellum, *Development* 129 (2002), pp. 3513–3522.

71. Marsh et al., 1998 D.J. Marsh, P.L. Dahia, S. Caron, J.B. Kum, I.M. Frayling, I.P. Tomlinson, K.S. Hughes, R.A. Eeles, S.V. Hodgson, V.A. Murday, R. Houlston and C. Eng, Germline PTEN mutations in Cowden syndrome-like families, *J. Med. Genet.* 35 (1998), pp. 881–885.

72. Minoo, P., Su, G., Drum, H., Bringas, P. and Kimura, S. (1999). Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos. *Dev. Biol.* 209, 60-71.

73. Minoo P.Transcriptional regulation of lung development: emergence of specificity. Respir Res. 2000;1(2):109-15.

74. Moe-Behrens G.H., F.G. Klinger, W. Eskild, T. Grotmol, T.B. Haugen and M. De Felici, Akt/PTEN signaling mediates estrogen-dependent proliferation of primordial germ cells in vitro, *Mol. Endocrinol.* 17 (2003), pp. 2630–2638.

75. Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S. and Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* 12,3579 -3590.

76. Mucenski ML, Wert SE, Nation JM, Loudy DE, Huelsken J, Birchmeier W, Morrisey EE, Whitsett JA. beta-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis. J Biol Chem 278: 40231–40238, 2003.

77. Munemitsu S., I. Albert, B. Rubinfeld and P. Polakis, Deletion of an amino-terminal sequence beta-catenin in vivo and promotes hyperphosporylation of the adenomatous polyposis coli tumor suppressor protein, *Mol. Cell. Biol.* 16 (1996), pp. 4088–4094.

78. Mutsaers SE, Bishop JE, McGrouther G, Laurent GJ. Mechanisms of tissue repair: from wound healing to fibrosis.Int J Biochem Cell Biol. 1997 Jan;29(1):5-17.

79. Myers M.P., I. Pass, I.H. Batty, J. Van der Kaay, J.P. Stolarov, B.A. Hemmings, M.H. Wigler, C.P. Downes and N.K. Tonks, The lipid phosphatase activity of PTEN is critical for its tumor suppressor function, *Proc. Natl. Acad.*

Sci. U. S. A. 95 (1998), pp. 13513–13518.

80. Nelen M.R., W.C. van Staveren, E.A. Peeters, M.B. Hassel, R.J. Gorlin, H. Hamm, C.F. Lindboe, J.P. Fryns, R.H. Sijmons, D.G. Woods, E.C. Mariman, G.W. Padberg and H. Kremer, Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease, *Hum. Mol. Genet.* 6 (1997), pp. 1383–1387.

81. Neshat M.S., I.K. Mellinghoff, C. Tran, B. Stiles, G. Thomas, R. Petersen, P. Frost, J.J. Gibbons, H. Wu and C.L. Sawyers, Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001), pp. 10314–10319.

82. Nho RS, Xia H, Diebold D, Kahm J, Kleidon J, White E, Henke CA. PTEN regulates fibroblast elimination during collagen matrix contraction. J Biol Chem. 2006 Nov 3;281(44):33291-301.

83. Okamura H., K. Yoshida, H. Morimoto and T. Haneji, PTEN expression elicited by EGR-1 transcription factor in calyculin A-induced apoptotic cells, *J. Cell. Biochem.* 94 (1) (2005), pp. 117–125.

84. Okubo T, Hogan BL. Hyperactive Wnt signaling changes the developmental potential of embryonic lung endoderm. J Biol 3: 11, 2004.

85. Pap M. and G.M. Cooper, Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway, *J. Biol. Chem.* 273 (1998), pp. 19929–19932.

86. Paramio JM, Navarro M, Segrelles C, Gomez-Casero E, Jorcano JL. PTEN tumour suppressor is linked to the cell cycle control through the retinoblastoma protein. *Oncogene* 1999;18:7462–7468.

87. Pene F., Y.E. Claessens, O. Muller, F. Viguie, P. Mayeux, F. Dreyfus, C. Lacombe and D. Bouscary, Role of the phosphatidylinositol 3-kinase/Akt and mTOR/P70S6-kinase pathways in the proliferation and apoptosis in multiple myeloma, *Oncogene* 21 (2002), pp. 6587–6597.

88. Pepicelli, C. V., Lewis, P. M. and McMahon, A. P. (1998). Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Curr. Biol.* 8,1083 -1086.

89. Perl, A. K., Wert, S. E., Nagy, A., Lobe, C. G. and Whitsett, J. A. (2002). Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc. Natl. Acad. Sci. USA* 99,10482 -10487.

90. Podsypanina K., L.H. Ellenson, A. Nemes, J. Gu, M. Tamura, K.M. Yamada, C. Cordon-Cardo, G. Catoretti, P.E. Fisher and R. Parsons, Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999), pp. 1563–1568.

91. Polakis P., Wnt signaling and cancer, *Genes Dev.* 14 (2000), pp. 1837–1851.

92. Potten, C. S. (2004). Development of epithelial stem cell concepts. In *Handbook of Stem Cells: Vol. 2 Adult and Fetal Stems Cell* (ed. R. Lanza), pp.1 -12. New York: Academic Press.

93. Raff, M. (2003). Adult stem cell plasticity: fact or artifact? *Annu. Rev. Cell Dev. Biol.* 19, 1-22.

94. Rasheed et al., 1997 B.K. Rasheed, T.T. Stenzel, R.E. McLendon, R. Parsons, A.H. Friedman, H.S. Friedman, D.D. Bigner and S.H. Bigner, PTEN gene mutations are seen in high-grade but not in low-grade gliomas, *Cancer Res.* 57 (1997), pp. 4187–4190.

95. Rawlins, E. L. and Hogan, B. L. (2005). Intercellular growth factor signaling and the development of mouse tracheal submucosal glands. *Dev. Dyn.* 233,1378 -1385.

96. Rawlins EL, Hogan BL. Epithelial stem cells of the lung: privileged few or opportunities for many? Development. 2006 Jul;133(13):2455-65.

97. T. Reya, S.J. Morrison, M.F. Clarke and I.L. Weissman, Stem cells, cancer, and cancer stem cells, *Nature* 414 (2001), pp. 105–111.

98. Rossant, J., Zirngibl, R., Cado, D., Shago, M. and Giguere, V. (1991). Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* 5,1333 -1344.

99. Schoch, K. G., Lori, A., Burns, K. A., Eldred, T., Olsen, J. C. and Randell, S. H. (2004). A subset of mouse tracheal epithelial basal cells generates large colonies in vitro. *Am. J. Physiol. Lung Cell Mol. Physiol.* 286,L631 -L642.

100. Serini G, Bochaton-Piallat ML, Ropraz P, Geinoz A, Borsi L, Zardi L, Gabbiani G. The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. J Cell Biol 1998;142:873–881.

101. Serls, A. E., Doherty, S., Parvatiyar, P., Wells, J. M. and Deutsch, G. H. (2005). Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* 132,35 -47.

102. Shu, W., Jiang, Y. Q., Lu, M. M. and Morrisey, E. E. (2002). Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* 129,4831 -4842.

103. Shu W, Guttentag S, Wang Z, Andl T, Ballard P, Lu MM, Piccolo S,

Birchmeier W, Whitsett JA, Millar SE, Morrisey EE. Wnt/beta-catenin signaling acts upstream of N-myc, BMP4, FGF signaling to regulate proximal-distal patterning in the lung. Dev Biol 283: 226–239, 2005.

104. Song K., S.C. Cornelius, M. Reiss and D. Danielpour, Insulin-like growth factor-I inhibits transcriptional responses of transforming growth factor-beta by phosphatidylinositol 3-kinase/Akt-dependent suppression of the activation of Smad3 but not Smad2, *J. Biol. Chem.* 278 (2003), pp. 38342–38351.

105. Stafford, D. and Prince, V. E. (2002). Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. *Curr. Biol.* 12,1215 -1220.

106. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998;95:29–39.

107. Stiles B., V. Gilman, N. Khanzenzon, R. Lesche, A. Li, R. Qiao, X. Liu and H. Wu, Essential role of AKT-1/protein kinase B alpha in PTEN-controlled tumorigenesis, *Mol. Cell. Biol.* 22 (2002), pp. 3842–3851.

108. Stiles B., M. Groszer, S. Wang, J. Jiao and H. Wu, PTENless means more, *Dev. Biol.* 273 (2) (2004), pp. 175–184 15.

109. Stillwell WG, Horning MG, Griffin GW, Tsang WS. Identification and synthesis of the isomeric tetrahydroxytetrahydronaphthalene metabolites excreted in rat urine. Drug Metab Dispos. 1982 Jan-Feb;10(1):11-4.

110. Stokoe D., PTEN, *Curr. Biol.* 11 2001, p. R502

111. Stripp B, R, Maxson K, Mera R, Singh G.Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. Am J Physiol. 1995 Dec;269(6 Pt 1):L791-9.

112. Suganuma H, Sato A, Tamura R, Chida K. Enhanced migration of fibro-blasts derived from lungs with fibrotic lesions. Thorax 1995;50:984–989.

113. Sun H., R. Lesche, D.M. Li, J. Liliental, H. Zhang, J. Gao, N. Gavrilova, B. Mueller, X. Liu and H. Wu, PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999), pp. 6199–6204.

114. A. Suzuki, J.L. de la Pompa, V. Stambolic, A.J. Elia, T. Sasaki, I. del Barco Barrantes, A. Ho, A. Wakeham, A. Itie, W. Khoo, M. Fukumoto and T.W. Mak, High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice, *Curr. Biol.* 8 (1998), pp. 1169–1178.

115. Suzuki A., S. Itami, M. Ohishi, K. Hamada, T. Inoue, N. Komazawa, H. Senoo, T. Sasaki, J. Takeda, M. Manabe, T.W. Mak and T. Nakano, Keratinocyte-specific Pten deficiency results in epidermal hyperplasia, accelerated hair follicle morphogenesis and tumor formation, *Cancer Res.* 63 (2003), pp. 674–681.

116. Tebar M, Destree O, de Vree WJ, Ten Have-Opbroek AA. Expression of Tcf/Lef and sFrp and localization of beta-catenin in the developing mouse lung. Mech Dev 109: 437–440, 2001.

117. Verdu J., M.A. Buratovich, E.L. Wilder and M.J. Birnbaum, Cell-autonomous regulation of cell and organ growth in *Drosophila* by Akt/PKB, *Nat. Cell Biol.* 1 (1999), pp. 500–506.

118. Vivanco I. and C.L. Sawyers, The phosphatidylinositol 3-Kinase AKT pathway in human cancer, *Nat. Rev., Cancer* 2 (2002), pp. 489–501.

119. Wan, H., Dingle, S., Xu, Y., Besnard, V., Kaestner, K. H., Ang, S. L., Wert, S., Stahlman, M. T. and Whitsett, J. A. (2005). Compensatory Roles of Foxa1 and Foxa2 during Lung Morphogenesis. *J. Biol. Chem.* 280,13809 - 13816.

120. Wan H, Xu Y, Ikegami M, Stahlman MT, Kaestner KH, Ang SL, Whitsett JA. Foxa2 is required for transition to air breathing at birth. *Proc Natl Acad Sci USA* 101: 14449–14454, 2004.

121. Wang S., J. Gao, Q. Lei, N. Rozengurt, C. Pritchard, J. Jiao, G.V. Thomas, G. Li, P. Roy-Burman, P.S. Nelson, X. Liu and H. Wu, Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer, *Cancer Cell* 4 (2003), pp. 209–221.

122. Warburton D, Perin L, Defilippo R, Bellusci S, Shi W, Driscoll B. Stem/progenitor cells in lung development, injury repair, and regeneration. Proc Am Thorac Soc. 2008 Aug 15;5(6):703-6.

123. Wells, J. M. and Melton, D. A. (1999). Vertebrate endoderm development. *Annu. Rev. Cell Dev. Biol.* 15,393 -410.

124. Weaver, M., Batts, L. and Hogan, B. L. (2003). Tissue interactions pattern the mesenchyme of the embryonic mouse lung. *Dev. Biol.* 258,169 - 184.

125. Wert, S. E., Glasser, S. W., Korfhagen, T. R. and Whitsett, J. A. (1993). Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev. Biol.* 156,426 -44.

126. Wert SE, Dey CR, Blair PA, Kimura S, Whitsett JA. Increased expression of thyroid transcription factor-1 (TTF-1) in respiratory epithelial cells inhibits alveolarization and causes pulmonary inflammation. *Dev Biol* 242: 75–87, 2002.

127. White ES, Atrasz RG, Hu B, Phan SH, Stambolic V, Mak TW, Hogaboam CM, Flaherty KR, Martinez FJ, Kontos CD, Toews GB. Negative regulation of myofibroblast differentiation by PTEN (Phosphatase and Tensin Homolog Deleted on chromosome 10). Am J Respir Crit Care Med. 2006 Jan 1;173(1):112-21.

128. White ES, Thannickal VJ, Carskadon SL, Dickie EG, Livant DL, Markwart S, Toews GB, Arenberg DA. Integrin {alpha}4{beta}1 regulates migration across basement membranes by lung fibroblasts: a role for phosphatase and tensin homologue deleted on chromosome 10. Am J Respir Crit Care Med 2003;168:436–442.

129. Wolfrum C., D. Besser, E. Luca and M. Stoffel, Insulin regulates the activity of forkhead transcription factor Hnf-3beta/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003), pp. 11624–11629.

130. Wood, H. B., May, G., Healy, L., Enver, T. and Morriss-Kay, G. M. (1997). CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. *Blood* 90,2300 -2311.

131. Wu X., K. Senechal, M.S. Neshat, Y.E. Whang and C.L. Sawyers, The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998), pp. 15587–15591.

132. Xing Y, Li C, Hu L, Tiozzo C, Li M, Chai Y, Bellusci S, Anderson S, Minoo P. Mechanisms of TGFbeta inhibition of LUNG endodermal morphogenesis: the role of TbetaRII, Smads, Nkx2.1 and Pten. Dev Biol. 2008 Aug 15;320(2):340-50.

133. Xu Q, Tam M, Anderson SA. Fate mapping Nkx2.1-lineage cells in the mouse telencephalon. J Comp Neurol. 2008 Jan 1;506(1):16-29.

134. Yanagi S, Kishimoto H, Kawahara K, Sasaki T, Sasaki M, Nishio M, Yajima N, Hamada K, Horie Y, Kubo H, Whitsett JA, Mak TW, Nakano T, Nakazato M, Suzuki A. Pten controls lung morphogenesis, bronchioalveolar stem cells, and onset of lung adenocarcinomas in mice. J Clin Invest. 2007 Oct;117(10):2929-40.

135. Yost C., M. Torres, J.R. Miller, E. Huang, D. Kimelman and R.T. Moon, The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3, *Genes Dev.* 10 (1996), pp. 1443–1454

136. Zhang J, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, Haug JS, Rupp D, Porter-Westpfahl KS, Wiedemann LM, Wu H, Li L. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukemia

prevention. Nature. 2006 May 25;441(7092):518-22.

137. Zhang S, Smartt H, Holgate ST, Roche WR. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma. Lab Invest 1999;79:395–405.

AKNOWLEDGEMENTS

This adventure, a Phd program in the States wouldn't be possible without all the people who were with me, in person or in their spirit:
My mom, my dad and my sister, always my strongest supporters.
Dr.Minoo, that thaught me from the beginning how to do science.
Saverio, who showed me that even after years of science, it is possible to be still excited about a restult or a new experiment.
Roberta, Buri, Matteo, Alberto and all the crowd from Mestre, who are all the times ready for a " goodbye dinner" or a " welcome home spritz".
la Buldini a quadrattini, la Nico, la Andrew and Gea, that even thoughworking in different Hospitals, all told me " my hospital is looking for neonatologist, why you don't apply for a position? Come on, so we can work together!!".
Prof Chiandetti, my mentor for the medical school, the residency, the fellowship and now for my PhD, that convinced me to keep going when I was about to give up.
Dr. Pettenazzo, who introduced me to the "magical world of the intensive care".
Max, for our phonecalls in the middle of the night, the mails, the texts and the chats trying to help each other in this adventure: I wouldn't be able to finish it without you Max!.
Laura, for the adventures and the disadventures shared together and for all the suggestions and advice at work….and for the thesis: THANK YOU!!!!!!.
Gianni and Stjin and their " mentorship": I am the first of thousand of Phd students that will work for you guys: you are the Best!!!; Annina that with Janwang and Eugene were my " rifugio peccatorum" during my difficult times; Giuliani, my favorite capitan, despite everything, we did it…sailing together to Catalina: great adventure!.
Stefano and Igor, will always be " my compter guys"… (please, learn the Mac now) how can I do without your help??.

Frederique, my English would be still incomprehensible without you….even if you were not able to remove my Italian accent " federike!!!".

Bruce and his beautiful pictures when I was " just a little bit blue".

Betsy, my first real american friend, thanks for accepting me into your home without even knowing me.

Ben, and he knows for what: for his help to understand this culture better, the way to think like an American, to live like an american, to prepare an american breakfast, to vote in the elections…and his help to understan better myself and what I really want from my life.

Denise and Mia, thank you for sharing with me the time at work: the fun and the difficulties shared are always great!!.

Elsie, who accepted me in this building even without social security number..thank you to give me a roof over my head.

Linda and the Minoo's family, that make me feel all the time part of the family.

Ved and all his advices about science, work…and men!.

Veronika, for the beers together on the boat.

Isgar, my collegue forever.

La Rombi and her Sardegna.

Chris, Jennifer and Martin and their "surprise birthday party".

finally, thanks to Obama, to make this country again a great country: I am proud to be here in this storical time!

FIGURES

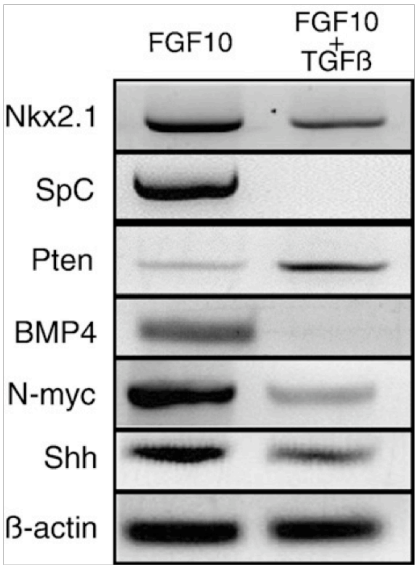


Figure1: TGFβ treatment of lung endodermal explants decreases *N-myc* and *Shh* and increases *Pten* expression.

Analysis of gene expression in endodermal explants treated with TGFβ. RNA isolated from E12.5 wild-type endodermal explants treated with FGF10 or FGF10 + TGFβ for 2 days was analyzed by semi-quantitative RT-PCR. mRNA for SpC and Bmp4 became undetectable, whereas *Nkx2.1*, N-myc and Shh mRNA were reduced. *Pten* mRNA was increased.

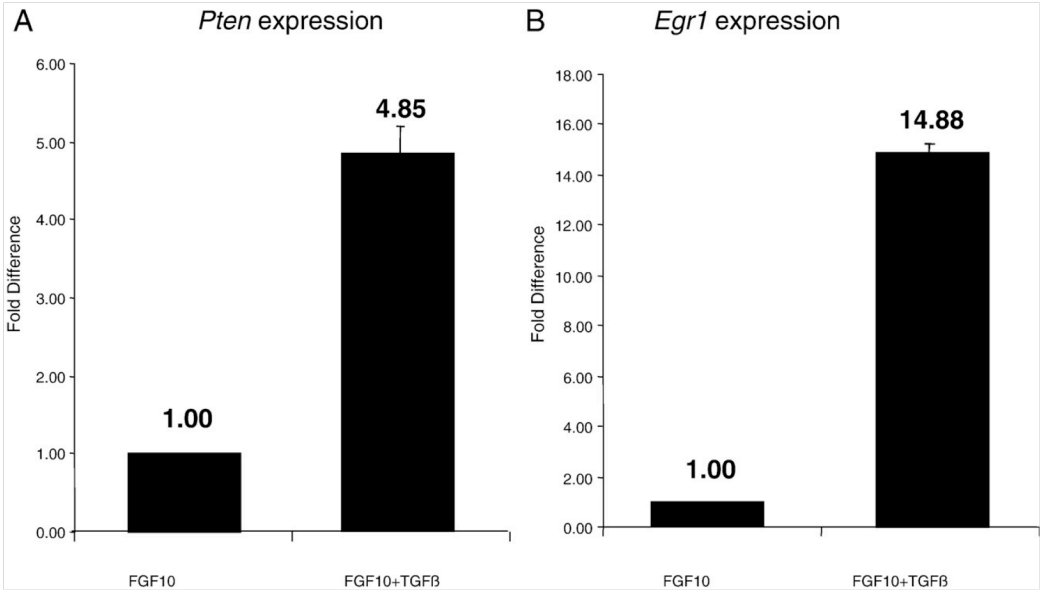


Figure 2: *Pten* and hits substrate *Egr1 mRNA* increases in endodermal explants after TGFβ treatment.

Real-time PCR quantification of *Pten* and Egr1 mRNA in endodermal explants. RNA from endodermal explants treated with FGF10 alone or FGF10 + TGFβ, after 2 days in culture was analyzed for *Pten* (Panel A) and *Egr1* (Panel B) gene expression by Real-time PCR. Both *Pten* and Egr1 mRNA were increased.

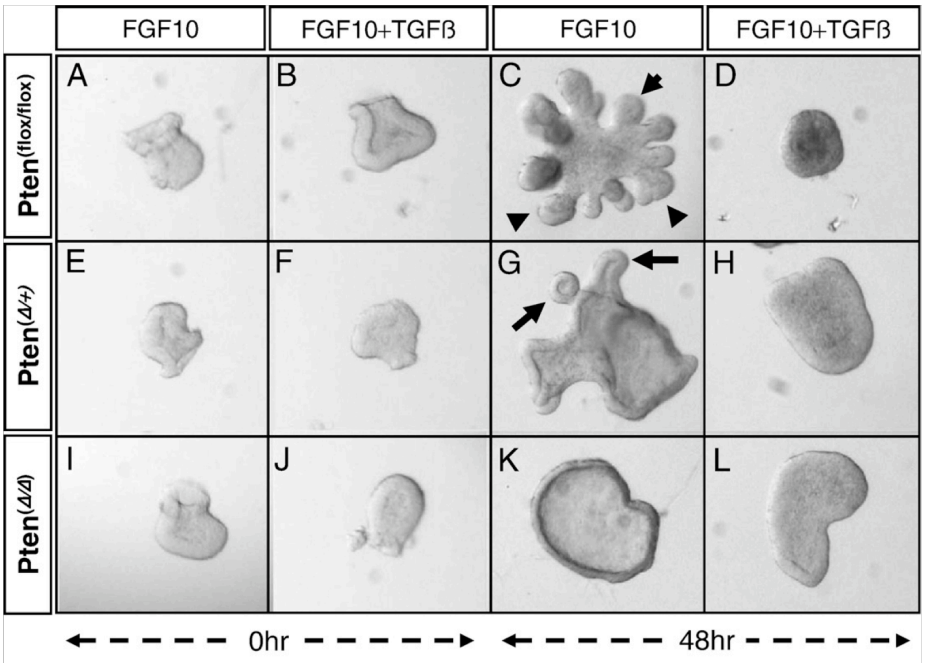


Figure 3: *Pten* mediates the effect of TGFβ-induced inhibition of cell proliferation.

Pten mediates the effect of TGFβ-induced inhibition of cell proliferation. Endodermal explants from E12.5 $Pten^{Nkx2.1\text{-}cre}$ lungs were treated with FGF10 (400 ng/ml) alone or FGF10 (*400 ng/ml*) + TGFβ (*10 ng/ml*). Explants at time 0 h are shown in Panels A, B, E, F, I and J. Culturing for 48 h showed robust morphogenesis of wild-type explants in presence of FGF10 alone (Panel C) and profound inhibition in response to TGFβ (Panel D). Compared to wild-type explants, both $Pten^{Nkx2.1\text{-}cre/flox}$ (Panel G) and $Pten^{Nkx2.1\text{-}cre}$ (Panel K) endodermal explants showed significant decrease in FGF10-induced morphogenesis. TGFβ did not inhibit the growth of either $Pten^{Nkx2.1\text{-}cre/fkox}$ or $Pten^{Nkx2.1\text{-}cre}$ explants (Panels H and L, respectively). (Arrows & and arrowheads show sites of budding).

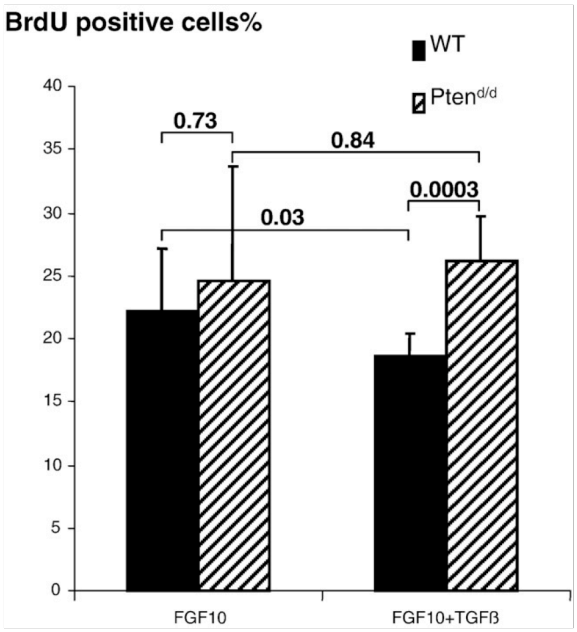


Figure 4: TGFβ does not inhibit *Pten*$^{Nkx2.1\text{-}cre}$ endodermal cell proliferation.

Endodermal explants were treated with BrdU for 3 h. Immunohistochemistry was used to identify the BrdU positive cells, which were counted on multiple random fields. Percentages of BrdU positive cells in total cells are shown. *P* values are given above bars that indicate comparisons between samples..

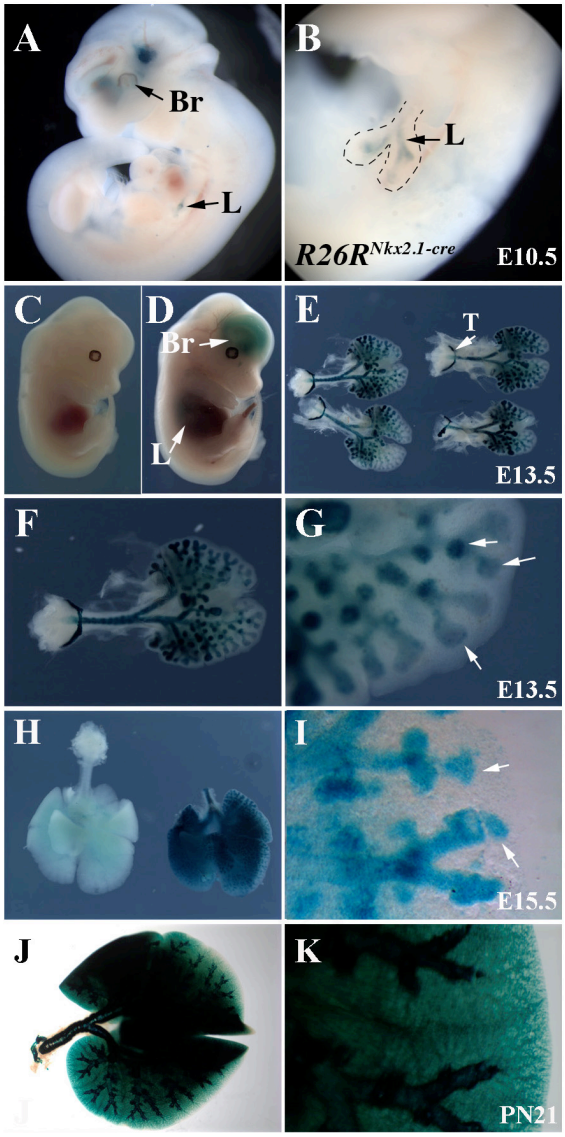


Figure 5: *Nkx2.1-cre* expression during lung development

A-K): Detection of cre induced β-galactoside activity at different embryonic stages. (**A, B**): E10.5 whole mount β-galactoside staining of *Rosa26R*$^{Nkx2.1\text{-}cre}$ detecting activity at the level of the brain and the lung primordial (panel A, arrow. B=brain, L=lung). Notice the strongest staining at the airway level (panel B, arrows).

(**C, D**): β-galactoside staining of WT and *RosaR26*$^{Nkx2.1\text{-}cre}$ embryos at E13.5: the control does not present any staining (C) while the *Rosa26R*$^{Nkx2.1\text{-}cre}$ (D) shows staining at the level of the brain (panel D, arrow, B= brain), thyroid (panel E arrow, T= thyroid) and lung (panel D, arrow. L=lung).
(**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-galactoside activity compared with the distal compartment (K, higher magnification of J).

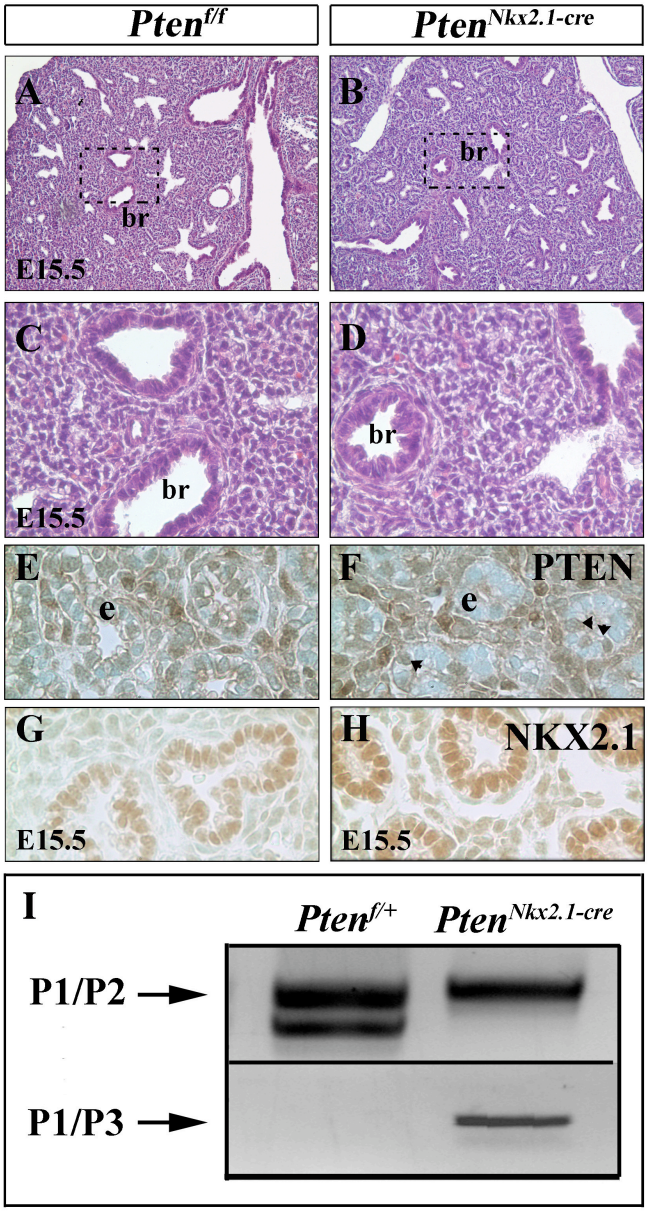


Figure 6: Deletion of *Pten* does not affect branching morphogenesis during lung development.

(**A-D**): H&E staining of lung sections of controls (n=4, A and C) and mutant *Pten*$^{Nkx2.1\text{-}cre}$ (n=4, B and D) at E15.5, detecting no differences in branching between the 2 groups. Magnification panel A and B, 10x; panel C and D, 20x.

(**E-H**): Lung sections were stained with PTEN antibodies and NKX2.1 antibodies (magnification 40x); in the control, the cells expressing NKX2.1 (G) also expressed PTEN (E). In the mutant, these cells (H) did not present PTEN staining, except for very few cells (F, arrows).

(**I**): Tissue specific deletion of *Pten* was also proved by PCR analysis.

Primers for recombination analysis were designed as described on Lesche et al (2002). P1/P2 amplified the floxed and the wt allele, when the P1/P3 amplified the flanked-exon 5 (Δ5).

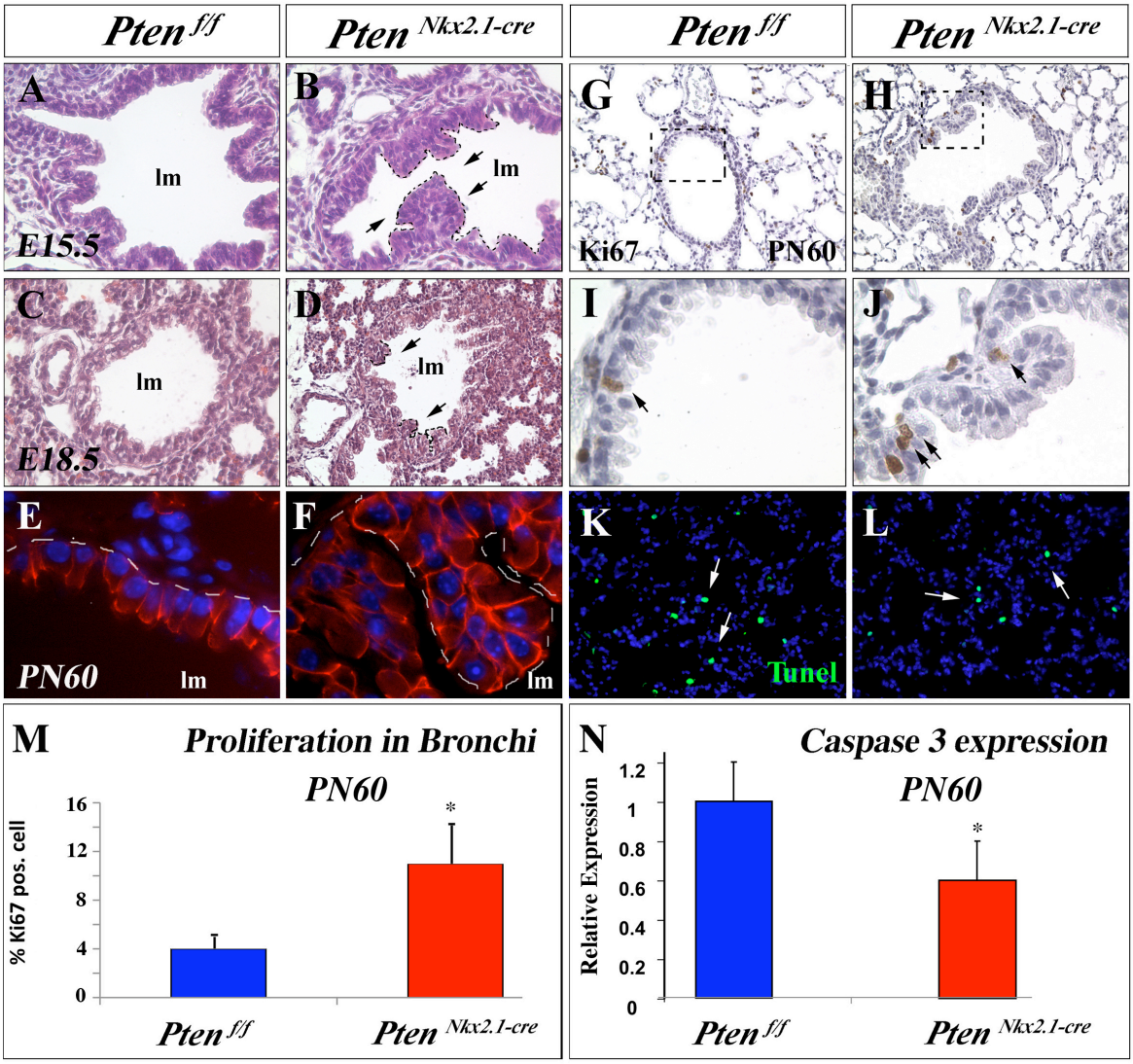


Figure 7: Absence of *Pten* leads to bronchiolar hyperplasia secondary to an increase in proliferation rate and to a decrease in apoptosis. (

A-D): Histological analysis through H&E staining of lungs from WT 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* $^{Nkx2.1cre}$ mice compared with the control (panel G and H, magnification 20x; panel I and J, magnification 80x;).
(**M**): Quantification revealed a statistical significant differences between the 2 groups, (n=4 mice per genotype), * P<0.01 using the standard T test (panel M).
(**K-L**): Tunnel assay in the mutant and control lungs.

(**N**): Real Time PCR for *Caspase3*, with a statistically significant decrease in mutant compared to control lungs..

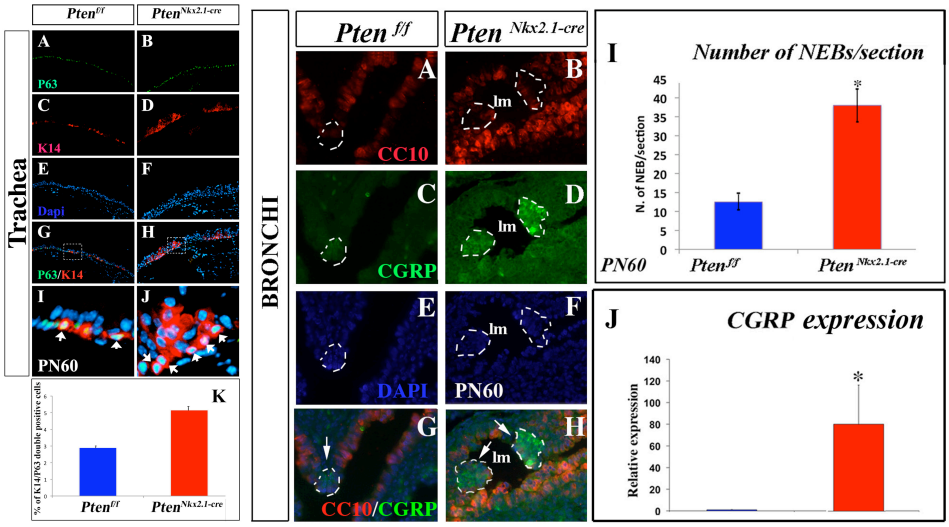


Figure 8: Deletion of *Pten* increases number of basal cells in the *Pten*$^{Nkx2.1\text{-}cre}$ lungs And the neuroepithelial bodies in the bronchi.

TRACHEA:

(**A-J**): Lung sections from mutant *Pten*$^{Nkx2.1\text{-}cre}$ and control littermates at 2 months of age were stained for P63 (green, panel A and B) and Keratin14 (red, panel C and D). Increase number of double positive cells over the P63 positive cells was detected in the mutant lungs compared to control lungs (low magnification, panel G and H; high magnification, panel I and J).

(**K**): Quantification analysis was performed using T-test from 4 mice in each group, * P<0.01.

BRONCHI:

(**A-H**): Immunofluorescence for CC10 (red, panel A and B) and CGRP (green, panel C and D) in 8 weeks old mutant and control lung sections.

Increase size, number and brightness in the NE bodies (panel G and H, arrows) were observed in the *Pten*$^{Nkx2.1\text{-}cre}$ lungs compared to the controls.

(**I**): Quantification analysis. The average and standard deviation from 4 mice were compared using the T-test, * *P*< 0.01.

(**J**): Relative expression of *CGRP* mRNA in the *Pten*$^{Nkx2.1\text{-}cre}$ and control mice, confirming the statistically significant increase of the *CGRP* expression in the mutant compared to the control (data from 3 different animals, p<0.01)

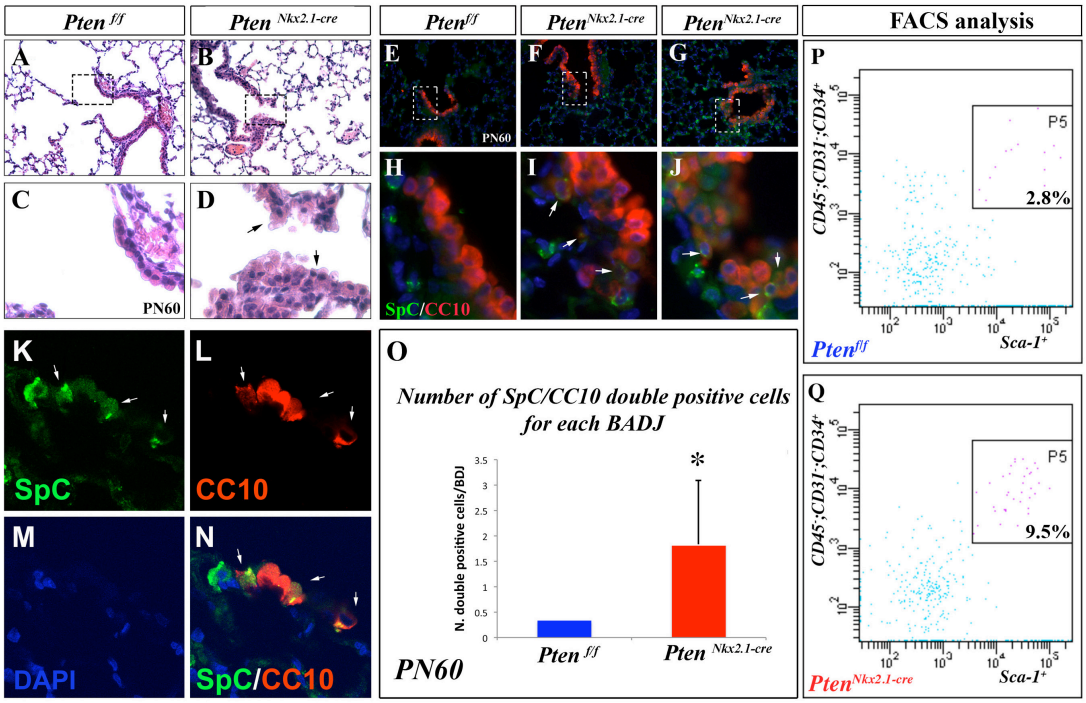


Figure 9: Deletion of *Pten* increases the double positive cells CC10/SpC in the BADJ.

(**A, D**): H&E staining showed in the mutant an increase of cells at the BADJ level (compare A to B, lower magnification 20x; C to D, higher magnification, 40x) and these cells were also enlarged compared to the cells in the control (D, arrows).
(**E-J):** Immunofluorescence for SpC and CC10 in controls (n=3) and mutant *Pten*$^{Nkx2.1cre}$ (n=3) animals at PN60: the mutants presented an increase number of double positive cells (panel E, F, G: lower magnification, 20x; panel H, I and J, higher magnification, 80X).
(**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 4 mice from each group using the T-test, * P<0.01.
(**P-Q**): FACS analysis of control (n=3) and mutants (n=3) lungs detecting $CD45^{-}CD31^{-}CD34^{+}Sca\text{-}1^{+}$ cells. (P5).

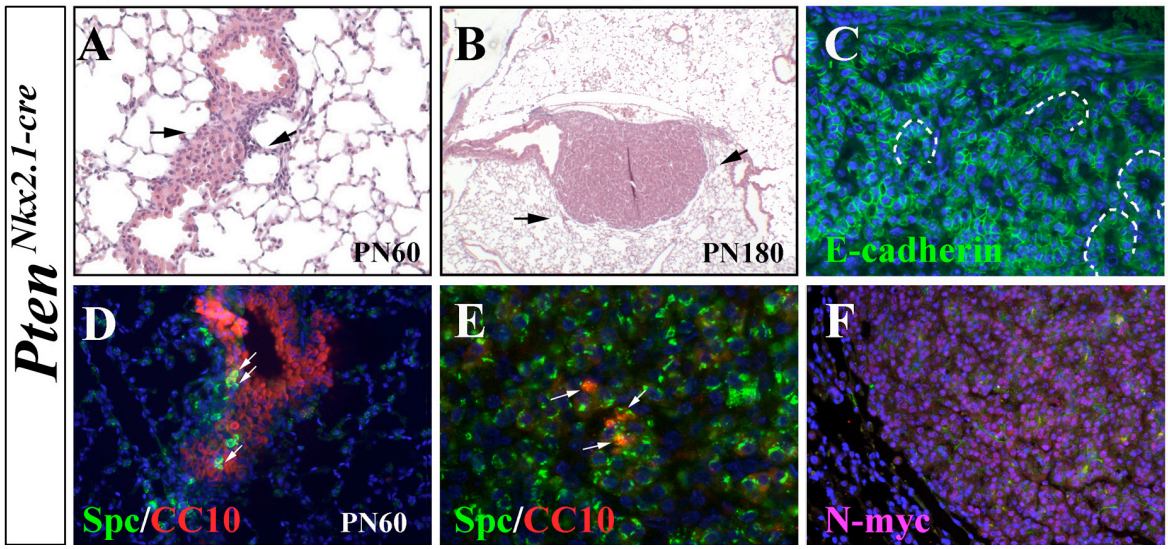


Figure 10: The BADJ cells proliferate inside the parenchyma and give rise to benign masses.

(**A, B**): H&E staining on PN180 *Pten*$^{Nkx2.1\text{-}cre}$ lung showing that over time the cells in the BADJ grow in the parenchyma and give rise to a tumor like 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 the tumoral cells..

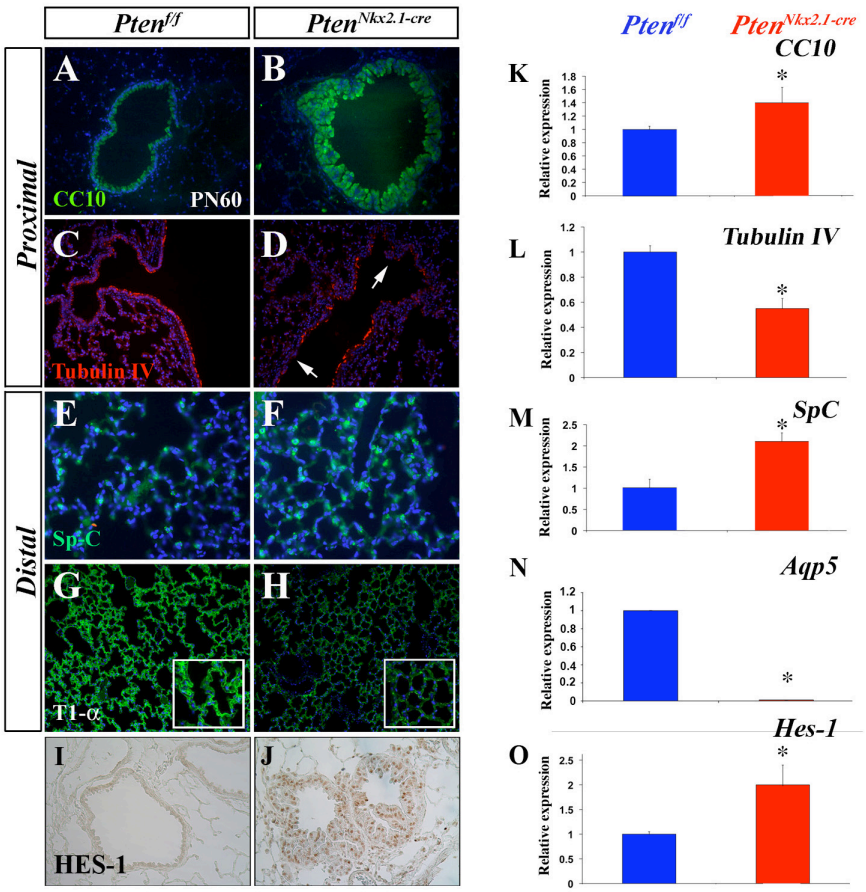


Figure 11: Absence of *Pten* impairs cell fate.
(**A-H**): Immunofluorescence for CC10 (A and B), β-tubulin (C and D), SpC (E and F) and T-1α (G and H) from 2 months old control and mutant lungs.
(**A, B**): In the mutant, an increase of Clara cells (CC10 positive) is detected compared with the control (B).
(**C, D**): β-tubulin staining showed a reduction in the ciliated cell number in the mutant (C, arrows) compared to the control (D).
(**E, F**): Alveolar type II cells (SpC positive) cells were increased in the *Pten*$^{Nkx2.1\text{-}cre}$ (E)
(**G, H**): Decrease alveolar type I cells (T-1 alpha positive) was observed in the mutant.
(**I-J**): Immmunohistochemistry for HES-1 in 2 months old control and mutant lung.
(**K-N**): Related expression, as determined by Real Time PCR of *CC10* (I), *Tubulin IV* (J), *SpC* (**K**), *Aqp-5* (L) mRNAs in *Pten*$^{Nkx2.1\text{-}cre}$ and control mice, confirmed the lack of differentiation in the mutant lung (data from 3 different mice in each group).
(**O-P**): Related expression of *Hes-1* mRNA in *Pten*$^{Nkx2.1\text{-}cre}$ and control mice confirmed the increase of *HES-1* expression in the mutant.

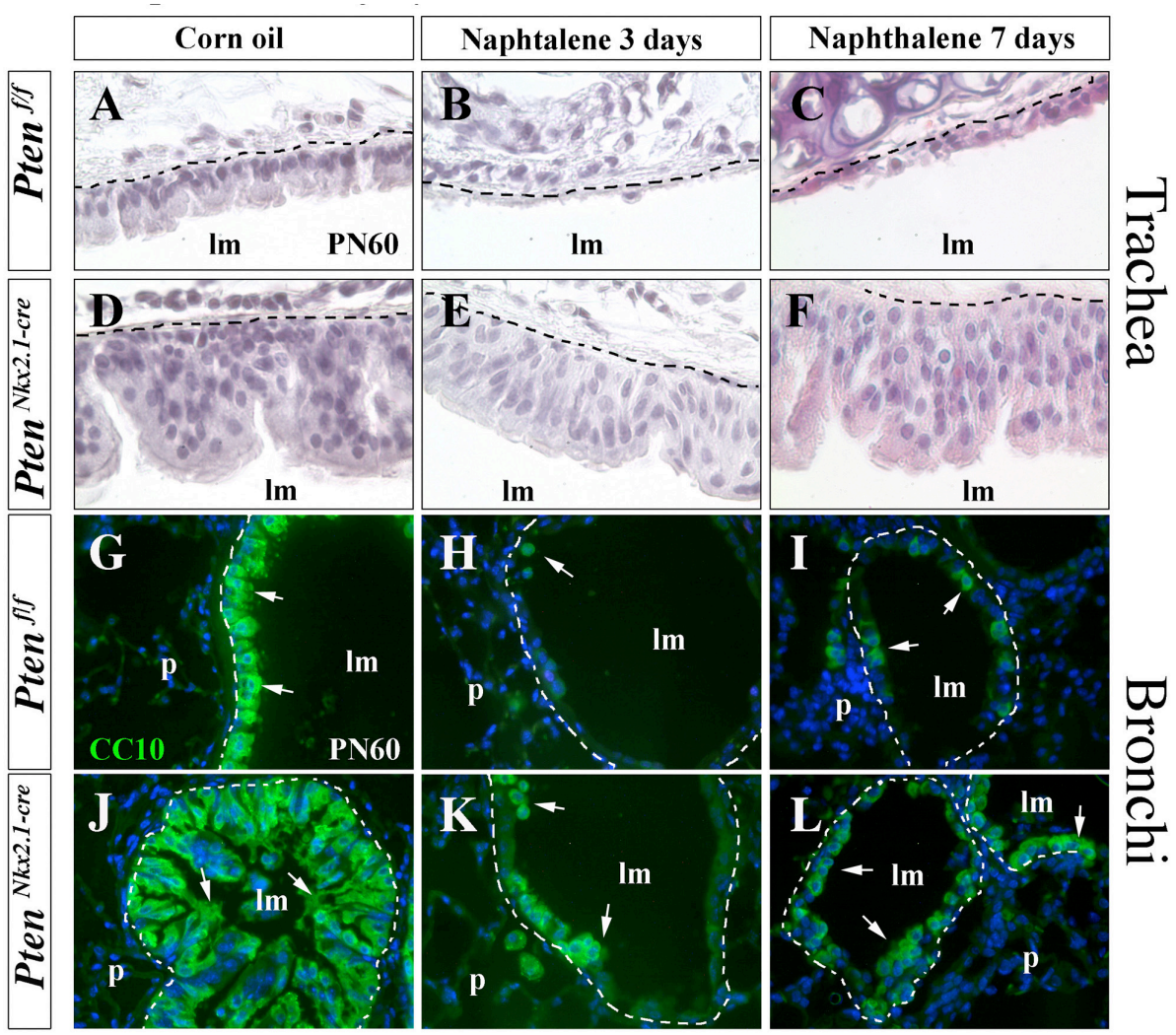


Figure 12: Absence of *Pten* protects the airways from naphthalene injury.
(**A-F**): H&E staining of a control and mutant trachea after corn oil or naphthalene injection.
(**A, D**): Corn oil administration did not affect the tracheal structure in the control (A) or in the mutant (D).
(**B, E**): Three days after naphthalene administration, tracheal epithelium in the control was completely denuded (B) whereas the *Pten*$^{Nkx2.1\text{-}cre}$ 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*$^{f/f}$ trachea showed a re-epithelization (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 to the mutant (K).
(**I, L**): after 7days, the *Pten*$^{Nkx2.1\text{-}cre}$ (L) presented more cells compared to the control (I). Data from 4 different animals for each group

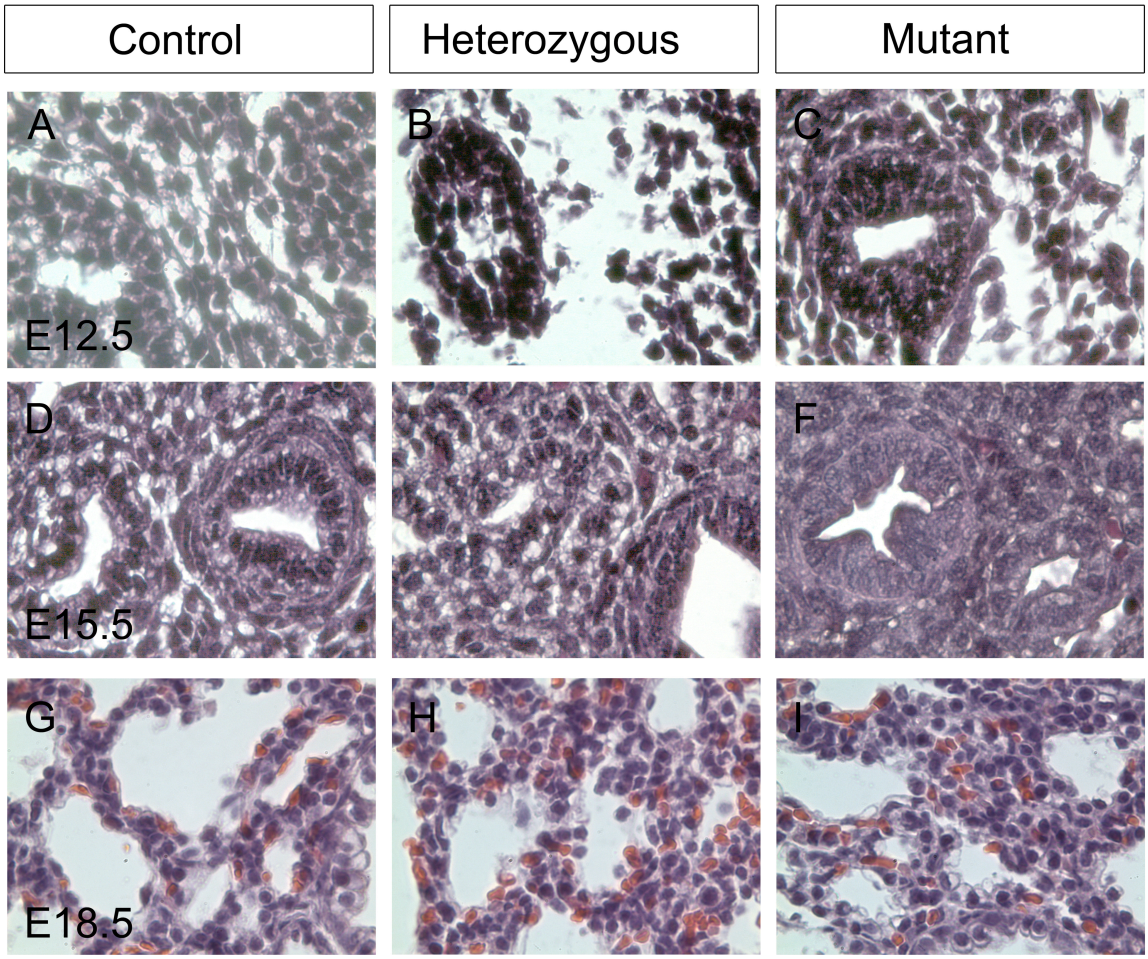


Figure 13: mesenchymal deletion does not seems to affect lung morphogenesys.

(**A-j**): H&E staining of lung sections of controls (n=4, A,D and G), heterozygous (n=4, B, and H), and mutant *Pten*$^{Nkx2.1\text{-}cre}$ (n=4,C, F and J) at E12.5, E15.5 and E18.5, detecting no differences in branching between the 2 groups. Magnification panel A-F, 40x; panel G-J, 20x.

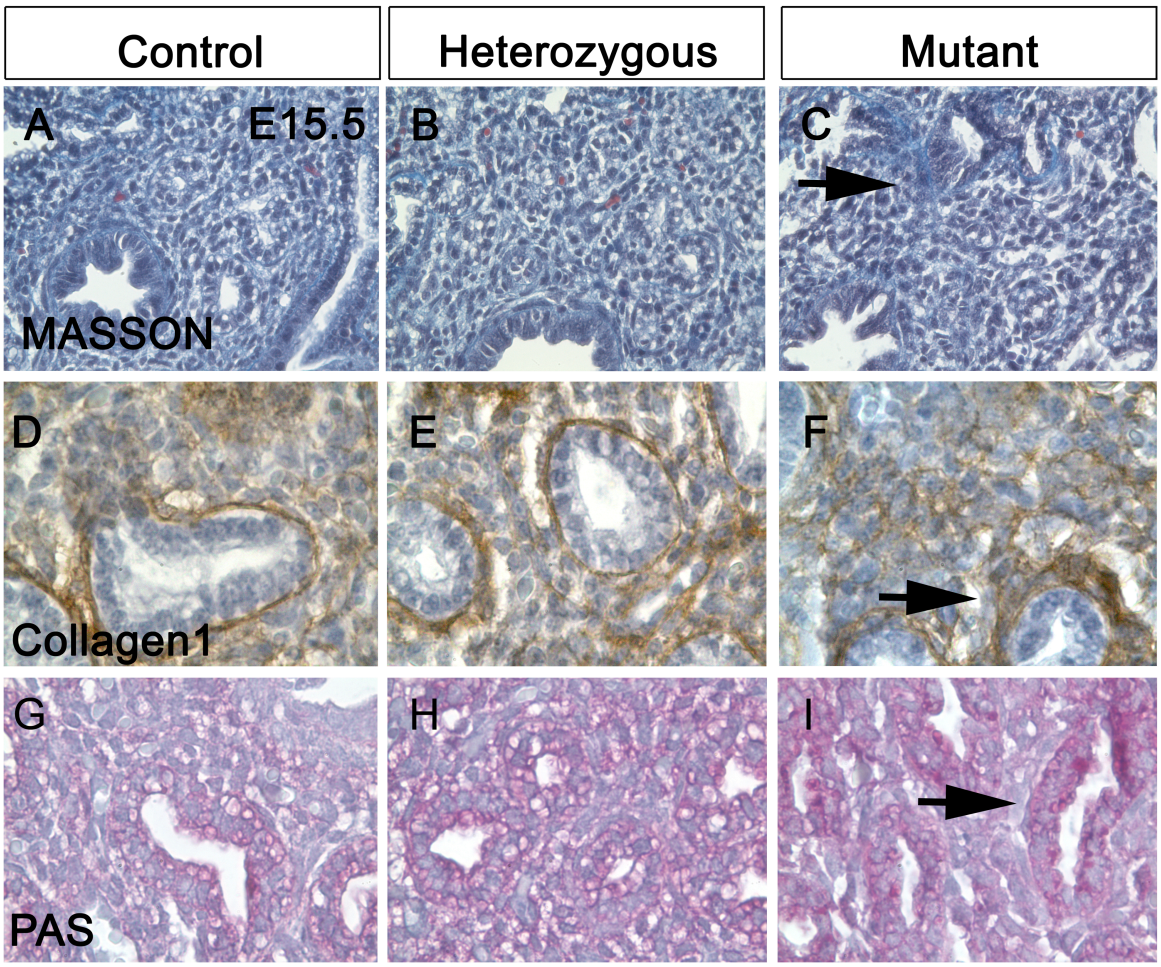


Figure 14: Mesenchymal deletion of *Pten* does not affect mesenchymal differentiation.

(**A-C**): Immunofluorescence for α-SMA at E15.5: the mutant and the heterozygous do not show an increase on the staining compare to the control (n=4 for each group). Magnification 20X.
(**C-E**): PECAM staining at E15.5 shows no differences in the mutant (F) or in the heterozygous (E) compared to the control (D). (n=4 for each group). Magnification 40X.

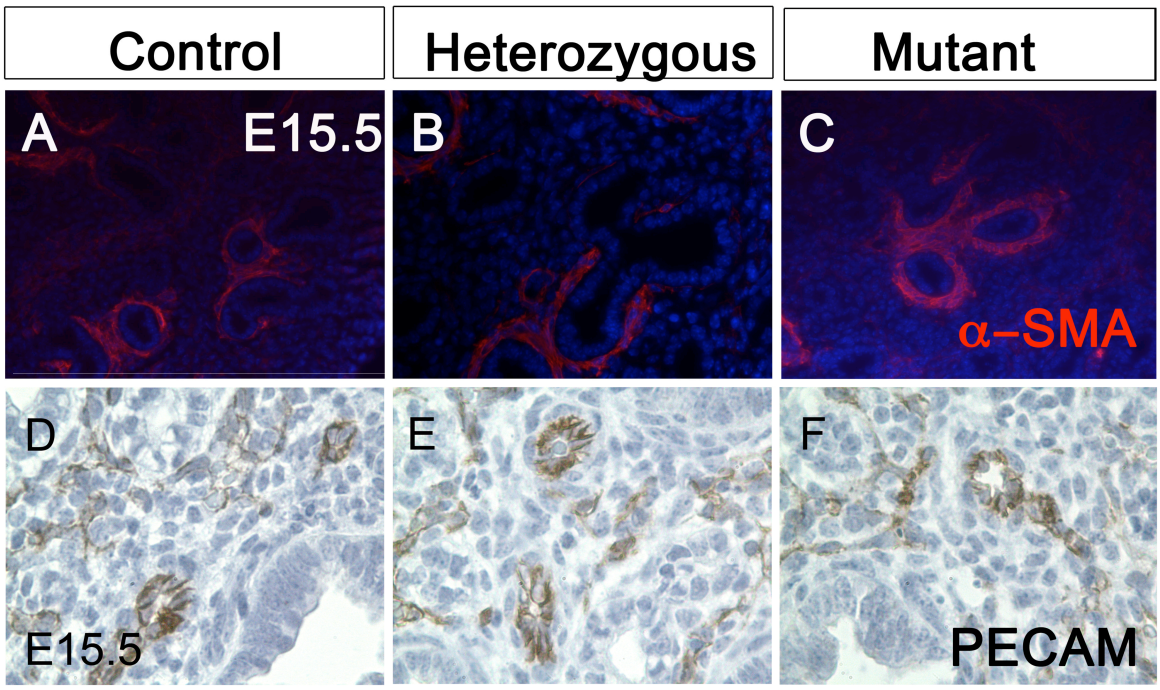


Figure 15: Absence of *Pten* increases collagen1 and extracellular matrix deposition.

(**A-F**): In the mutant and in heterozygous, an increase of collagen1 is detected compared with the control with immunohistochemistry for collagen 1 (C and B compare to A) and MASSON staining (compare F and E to D).
(**G-I**): Increase extracellular matrix deposition was detected by PAS staining in the mutant and heterozygous (I and H) compare to the control (G).