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PTEN is required to maintain luminal epithelial homeostasis and integrity in the adult mammary gland

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ABSTRACT

In the mammary gland, PTEN loss in luminal and basal epithelial cells results in differentiation defects and enhanced proliferation, leading to the formation of tumors with basal epithelial characteristics. In breast cancer, PTEN loss is associated with a hormone receptor-negative, basal-like subtype that is thought to originate in a luminal epithelial cell. Here, we show that luminal-specific PTEN loss results in distinct effects on epithelial homeostasis and mammary tumor formation. Luminal PTEN loss increased proliferation of hormone receptor-negative cells, thereby decreasing the percentage of hormone receptor-positive cells. Moreover, luminal PTEN loss led to misoriented cell divisions and mislocalization of cells to the intraluminal space of mammary ducts. Despite their elevated levels of activated AKT, *Pten*-null intraluminal cells showed increased levels of apoptosis. One year after *Pten* deletion, the ducts had cleared and no palpable mammary tumors were detected. These data establish PTEN as a critical regulator of luminal epithelial homeostasis and integrity in the adult mammary gland, and further show that luminal PTEN loss alone is not sufficient to promote the progression of mammary tumorigenesis.
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1. Introduction

In adult epithelial tissues, the balance between cell division and cell death maintains proper cell numbers, and thus, proper tissue architecture and function, in a process referred to as homeostasis (Datta et al., 2011; Macara et al., 2014; Ragkousi and Gibson, 2014). Epithelial integrity also relies on the maintenance of oriented cell divisions, cell–cell adhesion, and apical–basal polarity, which is defined by the distinct localization of lipids and proteins to the apical or basal domains of a cell. In the adult mammary gland, there is a network of hollow epithelial ducts that carry nutrient-rich milk to offspring during lactation (Fu et al., 2014; Inman et al., 2015; Visvader, 2009). The ducts are comprised of a single, outer layer of basal epithelial cells and a single, inner layer of polarized luminal epithelial cells. The luminal compartment contains hormone receptor-positive cells, which regulate the proliferation of neighboring cells via a paracrine mechanism, and hormone receptor-negative progenitor cells. The epithelium of the adult mammary gland is not only subject to hormonally driven changes in proliferation and apoptosis during each pregnancy, but also during each estrous cycle (Fata et al., 2001). Throughout these dynamic phases, preservation of the simple epithelial structure of the mammary ducts is essential for lactation, and more importantly, it is essential for preventing mammary tumorigenesis (McCaffrey and Macara, 2011).

The tumor suppressor phosphatase and tensin homolog (PTEN) plays a central role in regulating organ growth and development (Manning and Cantley, 2007; Song et al., 2012). PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP3), the product of phosphatidylinositol kinase 3 (PI3K), thereby attenuating the nutrient- and growth factor-sensing PI3K pathway. Loss of PTEN leads to an accumulation of PIP3, which recruits AKT to the plasma membrane. At the plasma membrane, AKT is phosphorylated and activated, and via its regulation of many downstream targets, phosphorylated AKT (pAKT) enhances cell growth, proliferation, and survival. These pAKT-mediated effects following PTEN loss drastically disrupt homeostasis in multiple epithelial tissues, including intestine, skin, mammary gland, and several reproductive tissues, with the most commonly reported phenotypes being increased proliferation

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and altered differentiation of specific epithelial populations (Knobbe et al., 2008; Langlois et al., 2009; Miyagawa et al., 2015; Xu et al., 2014). However, recent data indicate that PTEN has additional roles in maintaining epithelial integrity. Several *in vitro* models using mammalian epithelial cells have shown that PTEN regulates mitotic spindle orientation, apical polarity, and lumen formation (Feng et al., 2008; Martin-Belmonte et al., 2007; Toyoshima et al., 2007). Importantly, a recent study provided *in vivo* evidence for each of these functions by showing that PTEN loss in prostate luminal epithelial cells results in randomized mitotic spindle orientation, decreased cell–cell adhesion, and disrupted apical polarity (Wang et al., 2014). Interestingly, loss of PTEN in prostate basal epithelial cells does not affect polarity or mitotic spindle orientation, suggesting that these PTEN functions may be cell-context dependent.

In the mammary epithelium, PTEN loss leads to multiple developmental defects. Deletion of *Pten* in both epithelial compartments of the mouse mammary gland using a mouse mammary tumor virus (MMTV)-driven Cre recombinase during puberty results in increased proliferation, hyperbranching of the mammary ducts, and precocious alveolar differentiation (Li et al., 2002). In adult virgin mice, deletion of *Pten* in luminal and basal epithelial cells also results in a rapid induction of alveolar differentiation, accompanied by milk production (Chen et al., 2012). These studies demonstrate a key role for PTEN in regulating proliferation and alveolar differentiation in mammary epithelium. Interestingly, *in vitro* studies using three-dimensional (3D) mammary epithelial culture models have also shown that PTEN is required for lumen formation and apical polarity (Berglund et al., 2013; Fournier et al., 2009). However, the precise effects of PTEN loss on epithelial architecture, including mitotic spindle orientation, cell–cell adhesion, and apical–basal polarity, have not yet been assessed *in vivo*. Furthermore, the distinct effects of PTEN loss in luminal compared with basal epithelial cells, such as those observed in the prostate, have not yet been investigated in the mammary gland.

Because *PTEN* is one of the most frequently mutated genes in cancer, PTEN is best known for its role in tumor suppression. The hyperactivation of AKT that occurs upon loss of PTEN confers essential properties to cancer cells, such as their enhanced proliferative ability and their ability to evade anoikis, which is a type of cell death that occurs as a result of inappropriate cell or extracellular matrix interactions (Buchheit et al., 2014; Guadamillas et al., 2011). In breast cancer, PTEN loss is correlated with an aggressive, hormone receptor-negative, basal-like tumor phenotype (Cancer Genome Atlas, 2012; Marty et al., 2008; Saal et al., 2005, 2008). Consistent with these findings, loss of PTEN in mammary epithelial 3D culture models results in a reduction in hormone receptor expression and an increase in the expression of basal epithelial markers (Ghosh et al., 2013; Korkaya et al., 2009). Furthermore, mammary tumors that arise in mice with *Pten* deletion in luminal and basal epithelial cells express basal-specific keratins, similar to those expressed in human basal-like breast cancer (Li et al., 2002; Saal et al., 2008). Initially, it was hypothesized that basal-like breast tumors originate from basal epithelial cells; however, accumulating data suggest that, for some tumor-initiating mutations, basal-like tumors originate from hormone-receptor negative, luminal progenitor cells (Lim et al., 2009; Molyneux et al., 2010). Thus, it is important to dissect distinct luminal and basal epithelial-specific effects of cancer-initiating mutations, and furthermore, to elucidate how these epithelial subtype-specific responses can ultimately promote or suppress cancer progression in that particular cell type.

In this study, we deleted *Pten* using an inducible, luminal epithelial-specific Cre recombinase to determine the effects of luminal PTEN loss on epithelial homeostasis and architecture in the adult mammary gland. At 12 weeks post-induction, luminal PTEN loss disrupted the architecture of the luminal compartment and resulted in an accumulation of intraluminal epithelial cell clusters. Not surprisingly, luminal PTEN loss led to increased proliferation, and this was accompanied by a reduced percentage of progesterone receptor (PR)-positive cells. Furthermore, loss of PTEN led to misoriented mitotic spindles in luminal epithelial cells, without affecting cell–cell adhesion or apical polarity. Surprisingly, we also observed an increase in apoptosis of the intraluminal cells, suggesting that, in this model of PTEN loss, activated AKT does not confer anoikis resistance. At 1 year post-induction, the majority of intraluminal cells had cleared and the ducts largely resembled those of control mice, indicating that *Pten*-null luminal cells are lost over time. Moreover, although macroscopic lesions were observed using whole mount analysis, no palpable mammary tumors were detected, suggesting that loss of PTEN in the luminal compartment alone is not sufficient to promote mammary tumorigenesis. Together, these data identify a novel role for the tumor suppressor PTEN in regulating oriented cell divisions in the mammary luminal epithelium, and demonstrate the necessity for PTEN in maintaining luminal epithelial cell homeostasis and epithelial integrity in the adult mammary gland. Furthermore, these results indicate that PTEN loss in luminal epithelial cells is not sufficient to confer anoikis resistance; therefore, *Pten*-null luminal cells are unable to sustain lumen filling and promote palpable mammary tumor formation.

2. Materials and methods

2.1. Animal care

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal research protocol was approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (AN-504). All mice used in this study were maintained and euthanized under the guidelines of the Institutional Animal Care and Use Committee of Baylor College of Medicine. The sources of the mouse lines and the genotyping strategies used for each line were previously described (Choi et al., 2012). Tamoxifen (Sigma-Aldrich, St. Louis, MO) was dissolved into corn oil and was administrated into adult female mice (2 mg/40 g), at 8–12 weeks of age, once a day, for four consecutive days, via intraperitoneal injection.

2.2. MEC isolation and flow cytometric analysis

One week after completion of Tmx treatment (K8mTmG mice), or 12 weeks after Tmx or vehicle treatment (K8-CTR or K8PTEN-KO mice), the third, fourth, and fifth pairs of mammary glands were harvested from virgin female mice. MECs were isolated as previously described (Shore et al., 2012). MECs were resuspended at a concentration of 1×10^8 cells/ml in HBSS supplemented with 10 mM HEPES and 2% FBS (HBSS+). This cell suspension was depleted of lineage-positive cells (CD45, Ter119, CD31, and BP-1) using the EasySep™ Mouse Epithelial Cell Enrichment Kit (Stem Cell Technologies). MECs were subsequently resuspended in HBSS+ at a density of 1×10^7 cells/ml and stained with anti-mouse CD24 conjugated to Allophycocyanin (APC) (Biolegend, 1:100), anti-mouse CD29 conjugated to Pacific Blue (BioLegend, 1:100), and anti-mouse CD49f conjugated to Pacific Blue (Biolegend, 1:100). Cells were analyzed using a BD LSR Fortessa Cell Analyzer. Flow Cytometry data was analyzed using the FloJo software.

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