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PTEN regulates lung endodermal morphogenesis through MEK/ERK pathway

Yiming Xing [a,*], Runming Wang [a], Changgong Li [b], Parviz Minoo [b]

[a] The State Key Laboratory for Agro-biotechnology, China Agricultural University, Beijing 100191, PR China
[b] Department of Pediatrics, Division of Neonatology, University of Southern California, Keck School of Medicine, Los Angeles, CA 90033, United States

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ABSTRACT

Pten is a multifunctional tumor suppressor. Deletions and mutations in the Pten gene have been associated with multiple forms of human cancers. Pten is a central regulator of several signaling pathways that influences multiple cellular functions. One such function is in cell motility and migration, although the precise mechanism remains unknown. In this study, we deleted *Pten* in the embryonic lung epithelium using *Gata5-cre* mice. Absence of *Pten* blocked branching morphogenesis and ERK and AKT phosphorylation at E12.5. In an explant model, $Pten^{\Delta/\Delta}$ mesenchyme-free embryonic lung endoderm failed to branch. Inhibition of budding in $Pten^{\Delta/\Delta}$ explants was associated with major changes in cell migration, while cell proliferation was not affected. We further examined the role of ERK and AKT in branching morphogenesis by conditional, endodermal-specific mutants which blocked ERK or AKT phosphorylation. $MEK^{DM/+}$; *Gata5-cre* (blocking of ERK phosphorylation) lung showed more severe phenotype in branching morphogenesis. The inhibition of budding was also associated with disruption of cell migration. Thus, the mechanisms by which *Pten* is required for early endodermal morphogenesis may involve ERK, but not AKT, mediated cell migration.

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1. Introduction

The mammalian lung is a highly precise branched structure consisting of epithelial airways and alveoli as well as mesenchymal stroma and vascular networks. Each of the latter components is essential to proper respiratory functions. The formation of the lungs involves the structuring of epithelial tissues into complex but highly organized tubular networks that transport gases. This process is generally referred to as “branching morphogenesis”. In mammalian lung, branching morphogenesis starts from formation of an anlagen and invagination of the epithelium to branch initiation and outgrowth. The branching pattern is controlled genetically and gives rise to successive rounds of branched structures in a predictable manner.

The formation of lung via branching morphogenesis can generally be subdivided into a series of steps: formation of anlagen; formation of primary bud; branch initiation; branch outgrowth; organization of successive branching events and differentiation of proximal–distal structures. To generate this arborized epithelial network, each individual step depends on proper execution of cell proliferation, migration, apoptosis and changes in cell shape. The pattern of arborization depends on positive regulation by growth factors and receptors to promote proliferation and migration and negative regulation to inhibit excessive budding and bifurcation. How potential positive and negative signals are integrated to control cell movement, while retaining the essential character of an invasive epithelium, remains unclear.

A sequence of induction of factors was found. The implication of these factors in lung development was based on their association with specific lung defects in knockout mouse models. The major factors found regulating the branching are Fibroblast Growth Factors (FGFs), Transforming Growth Factor-β (TGF-β) superfamily, Wnt and Sonic Hedgehog. Recently Pten has been identified to control lung morphogenesis (Yanagi et al., 2007). PTEN has a dual specificity protein phosphatase and lipid phosphatase activity that can dephosphorylate serine, threonine and tyrosine residues. This positions PTEN as a critical negative regulator of PI3K/AKT signaling pathway (Vivanco and Sawyers, 2002). 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). Thus, much of our current knowledge regarding the roles of PTEN in development is acquired from animals with tissue-specific Pten deletion using the Cre–LoxP system. The functions of PTEN in individual tissues include stem cell self-renewal and proliferation, cell differentiation

* Correspondence to: China Agricultural University, Life Science Building, Room 2025, #2 W, Yuan-Ming-Yuan Road, Beijing 100191, PR China.
E-mail address: ymxing@cau.edu.cn (Y. Xing).

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and migration, organ size control, and the hormone-regulated organogenesis (Stiles et al., 2004).

The mitrogen-activated protein kinase (MAPK) /extracellular signal-regulated kinase (ERK) cascade is conserved in mammals and controls early developmental processes, including determination of morphology, organogenesis, synaptic plasticity and growth (McKay and Morrison, 2007). Signal transmission via this cascade is initiated by the activation of cell surface receptors by extracellular ligands, which results in the phosphorylation and activation of the MAPK/ERK kinases. Activation of the MAPK/ERK pathway leads to transcriptional control of genes important for cell proliferation and differentiation (Sanges and Cosma, 2010). Evidence is provided that MAPK/ERK kinases influence the cells' motility machinery. Inhibition of ERK activity causes decreased cell migration on extracellular matrix protein (Klemke et al., 1997).

In the current study, we assessed the direct impact of epithelial Pten inactivation on lung morphogenesis. The results demonstrate that Pten controls early stage branching morphogenesis by regulating epithelial cell migration, without major or measurable changes in cell proliferation. This function is mediated via phosphorylation of ERK, which is also essential for embryonic lung epithelial cell migration.

2. Materials and methods

2.1. Mouse lines

Pten$^{\Delta/\Delta}$mice were generated by crossing *Pten*$^{fl/fl}$ (Xing et al., 2008) with *Gata5-Cre* (Xing et al., 2010) mice. *MEK*$^{DN/+}$, *R26StopFL-Pik3ca* and *mT/mG* mice were purchased from The Jackson Laboratory. *MEK*$^{DN/+}$; *Gata5-Cre* and *R26StopFL-Pik3ca; Gata5-Cre* mice were generated by crossing *MEK*$^{DN/+}$ and *R26StopFL-Pik3ca* with *Gata5-Cre* mice.

2.2. 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, Carlsbad, CA) growth medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), with 20 μM MEK inhibitor U0126 (Promega, Madison, WI) or same amount of DMSO as control. Heparin beads (Sigma) were incubated with FGF10 (10 μl of a 50 ng/μl solution; R&D Systems, MN) at 37 °C for 2hr, grafted onto lung explants and cultured for 48 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 E13.5 and treated with Dispase (50 U/ml, BD Biosciences, Franklin Lakes, NJ) 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, Fremont, CA), 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 FGF10 and cultured at 37 °C at 5% CO_2 for various lengths of time as indicated. FGF10-saturated beads were prepared with acrylic beads with immobilized heparin (Sigma). Beads were rinsed with PBS three times, manually selected under dissection microscope and then soaked in 50ng/μl recombinant human FGF10 at 37 °C for 1hr. The beads were then washed in DMEM medium for 1hr at 37 °C before use (Li et al., 2005). MEK inhibitor U0126 was purchased from Promega.

2.3. Migration assay

Mesenchyme-free explants were treated with 0.25% trypsin/EDTA and DNase I(1:1) at 37 °C for 15 min. E18.5 lung epithelial cells were isolated according to Corti et al. (1996). The lungs were carefully chopped into pieces and treated with dispase at 37 °C for 15 min. DMEM was added to terminate the reaction. The cell suspension was filtered through 100 μm, 40 μm and 20 μm filters. Then the epithelial cells were plated on culture dish (BD Bioscience) and culture in DMEM and 10% FBS at 37 °C. Cell monolayers were wounded with a plastic tip. Cell migration was followed for 6hr at 37 °C and photographed.

2.4. Proliferation assay

Subsequent to culturing, endodermal explants were treated with bromodeoxyuridine (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 (Invitrogen, Carlsdad, CA) 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.

2.5. Cell death analysis

The cultured endodermal explants were fixed, dehydrated and embedded in paraffin and 5 μm sections were prepared. The "In Situ Cell Death Detection Kit, Fluoresein" kit (Roche Allied Science, Indianapolis, IN) was used for detection and quantification of cell apoptosis at single cell level, based on labeling of DNA strand breaks (TUNEL technology). The sizes of labeled areas and whole sections were calculated for the percentage of cell death. 5 sections from each treatment were analyzed.

2.6. Immunohistochemistry

5 μm tissue sections were prepared. Subsequent to deparaffinization, the sections were hydrated, heated in 10 mM citrate buffer (pH6.0) and treated with 10% H_2O_2 in methanol for 20 min and blocked with 10% of nonimmune serum. Sections were then incubated with primary antibodies at 4 °C overnight. Biotinylated secondary antibody and streptavidin-peroxidase conjugate (Vector Laboratories, Inc.) were used to detect the bound antibodies. The sections were developed with diaminobenzidine. GFP and Ki67 primary antibody was purchased from Thermo Scientific.

2.7. RNA extraction and Real-time PCR

Total RNA was isolated from lungs or endodermal explants using Trizol (GIBCO). The cDNA was synthesized from 1 μg total RNA by following the protocol of the SuperScript^{TM} First-Strand Synthesis Kit (Invitrogen). Real-time PCR was performed by a LightCycler System (Roche). Primer sets for following genes were used for Real-time PCR: *Cdc42*: 5′-TGCTCTGCCCTCACACAG-3′ and 5′-GGCTCTTCTTCGGTTCTGG-3′; *Rac1*: 5′-AGATGCAGGCCATCAATGT-3′ and 5′-GAGCAGGCAGGTTTTACCAA-3′; *RhoA*: 5′-GAATGACGAGCACACGAGAC-3′ and 5′-TCCTGTTTGCCATATCTCTGC-3′.

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