Signaling via Alk5 Controls Ontogeny of Lung Clara Cells

Yiming Xing\textsuperscript{1}, Changgong Li\textsuperscript{1}, Aimin Li\textsuperscript{1}, Somyoth Sridurongrit\textsuperscript{2}, Caterina Tiozzo\textsuperscript{1}, Saverio Bellusci\textsuperscript{3}, Zea Borok\textsuperscript{4}, Vesa Kaartinen\textsuperscript{2}, & Parviz Minoo\textsuperscript{1}

\textsuperscript{1}Department of Pediatrics, Division of Neonatology, University of Southern California, Keck School of Medicine, Los Angeles, CA. \textsuperscript{2}University of Michigan, Department of Biologic and Materials Science, Ann Arbor, MI. \textsuperscript{3}Saban Research Institute of Children\textquotesingle s Hospital Los Angeles, CA, & \textsuperscript{4}Will Rogers Institute Pulmonary Research Center, Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Southern California, Keck School of Medicine, Los Angeles, CA.

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Key Words: Alk5, TGF-beta, receptor, Lung, Morphogenesis, Clara, Ciliated, stem, progenitor, differentiation, cell fate.

Address correspondence to: Parviz Minoo, Ph.D.
General Laboratories Building
1801 E. Marengo Street, Room 1G1
Los Angeles, CA 90033
Tel. (323) 226-4340
Fax. (323) 226-5049
e-mail: minoo@usc.edu
Abstract

Clara cells, together with ciliated and pulmonary neuroendocrine cells, make up the epithelium of the bronchioles along the conducting airways. Clara cells are also known as Progenitor/Stem cells during lung regeneration after injury. The mechanisms of Clara cell differentiation are largely unknown. Transforming growth factor-beta is a multifunctional molecule with roles in normal development and disease pathogenesis. In this study, we deleted the TGF-β type I receptor, Alk5 in the embryonic lung epithelium using Gata5-Cre mice. Absence of Alk5 blocked Clara cell differentiation but had no effect on ciliated or pulmonary neuroendocrine cells. Hairy/Enhancer of Split-1, which is expressed in Clara cell putative “progenitors” was found to be a downstream target of Alk5 in vivo and in vitro. Loss of Alk5-mediated signaling also stimulated Pten gene expression and inhibited ERK phosphorylation in vivo. Using lung epithelial cells, we show that Alk5-regulated Hes1 expression is stimulated through Pten, MEK/ERK and PI3K/AKT pathways. Thus, the signaling pathway by which TGF-β/ALK5 regulates Clara cell differentiation may entail inhibition of Pten expression, which in turn activates ERK and AKT phosphorylation.

Key words: Lung Morphogenesis, TGF-beta, receptor, Alk5, Clara cell differentiation, Progenitor, Stem cells, Hes1, Pten, ERK, AKT, Gata5.
Introduction

During embryogenesis, developmentally committed tissue progenitor cells make cell fate decisions and differentiate along specific cell lineages to form specialized cell types. Some are thought to remain uncommitted or partially differentiated and these may be important in normal maintenance and regeneration or repair after injury. Both diffusible, as well as cell-cell contact-mediated signaling and communication likely drive cell fate decisions. Ultimately, signaling in most cases is transduced by activation or inhibition of downstream transcription factors that define the differentiation status of the particular cell types. The present knowledge of the mechanisms involved in cell-fate determination or differentiation remains limited.

The cellular composition of the mammalian lung is said to exceed 40 distinct specialized varieties. Thus the lung represents an attractive model in which to study mechanisms of cell fate determination and differentiation during embryonic development. The specialized cell types in the lung are derived from at least two embryonic sources, endoderm and the mesoderm. The decision-making pathways that control the emergence of these highly specialized cell types are dauntingly complex. Within the endodermal derivatives, three types of progenitor/stem cells have been identified that play key roles in differentiation of the lung epithelium. These are the “basal cells” which are thought to generate “Clara” and “ciliated” cells, the variant Clara cells which generate Clara cells and perhaps “neuroendocrine cells”, and the “bronchialveolar stem cells” which generate Clara cells, alveolar type I and Type II cells (Giangreco et al., 2007).

Clara cells, which are nonciliated bronchiolar epithelial cells, are characterized by abundant, discrete electron-dense granules (Plopper et al., 1980). In the normal lung, they are positive for Clara cell specific 10-kDa protein designated CC10 or CCSP. Clara cells are most prominent in the terminal and respiratory bronchioli, but to varying degrees can also be found in larger airways, including bronchi (Broers et al. 1992). As they exhibit the capacity to rapidly repopulate damaged or denuded airway epithelium, Clara cells are
presumed to be progenitors for themselves and ciliated cells (Reynolds et al., 2007). Clara
cells are thought to be derived from an unknown progenitor in the proximal airways late in
lung development (in mouse embryonic day 16, E16) but the mechanism remains
unsubstantiated (Cardoso, 2001). However, the gene, *hairy and enhancer of split 1 (Hes1)* is
expressed in Clara cell precursors and has been suggested to play a role in Clara cell lineage
determination (Ito et al., 2000).

Several classes of diffusible signaling molecules act as mediators of cell fate determination
and differentiation during development. The role of transforming growth factor (TGF)-β
family members is critical to both lineage selection and progression of differentiation.
These molecules also have the ability to redirect cell lineage determination (for review,
Derynck et al., 2007). TGF-β ligands bind to receptor complexes that consist of type I and
type II serine/threonine kinases. Blocking the type I, Alk5 (activin receptor-like kinase 5)
receptor function abrogates TGF-β signaling and inhibits epithelial cell morphology, but
stimulates smooth muscle cell differentiation in epicardial cells (Compton et al., 2006).
Blocking Alk5 also inhibits keratinocyte differentiation (Shukla et al., 2008) and epithelial-
mesenchymal transformation (Tojo et al., 2005). Upon activation of the receptors, both
Smad and non-Smad intracellular signaling can be engaged. Mitogen-activated protein
kinase/extracellular signal-regulated kinase, abbreviated as MEK/ERK is central to non-
Smad signaling pathways. The ERK cascade is activated and phosphorylated by
extracellular signal, MEK (Crews et al., 1992). Upon its activation, the cascade controls cell
cycle progression, proliferation and differentiation (Murphy et al., 2002). Erk knockout
mice showed defects in T cell differentiation (Pages et al., 1999) and mesoderm
differentiation (Yao et al., 2003). Smad and non-Smad pathways can direct and redirect
differentiation in many vertebrate tissues, including the immune and hematopoietic
compartments and during neuronal differentiation (Larsson et al., 2005).

Pten, a multifunctional tumor suppressor was initially identified as a TGF-β-regulated and
epithelial cell-enriched phosphatase (Li et al., 1997). Deletions and mutations in the *Pten*
gene have been associated with multiple forms of human cancers (Steck et al., 1997). Both
in vivo and in vitro results showed that *Pten* is negatively regulated by TGF-β (Kattla et al., 2008; Chow et al., 2008). In glioblastomas, MEK/ERK activity is downregulated by Pten through inhibition of SHC phosphorylation (Gu et al., 1998). Consistent with the latter results, increased phosphorylation of ERK is found in *Pten*-null hepatocytes (Xu et al., 2006). We showed that TGF-β-induced inhibition of lung endodermal cell proliferation and morphogenesis is mediated via *Pten* (Xing et al., 2008). Although its precise role in lung development remains unknown, *Pten* appears to control lung epithelial progenitor/stem cell pool sizes (Yanagi et al., 2007).

In the present study, we examined the role of *Alk5* in lung development by generating mice deficient for *Alk5* specifically in endodermally-derived epithelial cell lineages via a *Gata5-Cre* driver mouse line. Our results provide the first comprehensive evidence that signaling via *Alk5* plays a necessary role for emergence of Clara, but not ciliated cells through *Pten*-regulated ERK phosphorylation.
Materials & Methods

Mouse Genotypes and Cell Lines
Alk5$^{+/0}$ mice were generated as described (Sridurongrit et al., 2008). The generation of Gata5-Cre mice has been previously reported (Merki et al., 2005). Pten$^{+/4}$ mice were generated by crossing Pten$^{floxed/floxed}$ mice (ATCC, Manassas, VA) with Nkx2.1-cre mice (Xu et al., 2008; Xing et al., 2008). Human pulmonary carcinoma H441, A549 cell lines and human fibroblast MRC5 cell lines were purchased from ATCC. SV40 transformed mouse lung epithelial cell line MLE15 was generated as described (Wikenheiser et al., 1993) and was a gift from Dr. Whitsett (Childrens Hospital, Cincinnati, OH). Mouse Smad3 knockout lung alveolar type II cell line was generated from Smad3$^{-/-}$; SPC-SV40 double transgenic lungs in our laboratory.

Histology and Immunohistochemistry (IHC)
Lungs were fixed in 4% paraformaldehyde in PBS and processed into serial paraffin sections using standard procedures. Immunostaining were performed as previously described (Li et al., 2005). IHC analysis was performed using an indirect method with antibodies recognizing the following proteins: β-Galactosidase, ALK5(TβRI), CGRP and PAI-1 define CGRP and PAI-1 (Abcam, Cambridge, MA), SP-C, SP-B and CCSP (Seven Hill Bioreagents, Cincinnati, OH), NKX2.1 (LAB Vision, Fremont, CA), FOXJ1 and HES1 (Santa Cruz Biotechnology, INC. Santa Cruz, CA), β-tubulin (BioGenex, San Ramon, CA), phosphor-MEK1/2 and PTEN (Cell Signaling Technology, Danvers, MA), PGP9.5 (AnaSpec, San Jose, CA), SSEA1 (Millipore, Billerica, MA).

Cell Culture, Transient Transfection Assay and Pathway Inhibitors
Mouse lung epithelial MLE15 and Smad3 knockout cell lines were maintained in RPMI 1640 (GIBCO, Carlsbad, CA), supplemented with 10nM hydrocortisone, 5µg/ml insulin, 5µg/ml human transferring, 10nM β-estradiol, 5µg/ml selenium, 2mM L-glutamine, 10nM HEPES, 100u/ml penicillin, 100µg/ml streptomycin, and 2% FBS (Sigma, St. Louis, MO). Cells were grown to 80-90% confluence in 12-well plates for transient transfection or
conditional media treatment. For transient transfection, cells were transfected using SuperFect (Qiagen, Valencia, CA) with 1 μg Hes1 promoter construct (gift from Dr. Slack, University of Ottawa, Canada) (Vanderluit et al., 2007), or CMV-Pten (gift from Dr. Yamada, National Institutes of Health, Bethesda, MD)(Gu et al., 1998). The plasmids pGL3 (Promega, Madison, WI) or CMV-vector were used as negative controls. The PAI-1 promoter-luciferase construct (van Zonneweld et al., 1988) was used as a positive control for transfection and TGFβ1 treatment efficiency. Cells were treated with MEK inhibitor U0126 (20μM), p38 MAPK inhibitor SB203580 (10μM) (Promega, Madison, WI), PI3K inhibitor LY294002 (15μM) or ALK5 inhibitor SB525334 (50μM) (Sigma, St. Louis, MO) with or without TGF-β1 (20ng/ml) for 48 hr. Cells were lysed for assay of luciferase and β-Galactosidase activities or purification of RNA or protein for real-time PCR, or western blot analyses, respectively.

**RNA extraction and Real-Time PCR**

Total RNA was isolated from lungs using Trizol ( Gibco, Carlsbad, CA ). The cDNA was synthesized from 1μg total RNA by following the protocol of the SuperScript™ First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA). Quantification of the selected genes by real-time PCR was performed using a LightCycler (Roche Diagnostics, Mannheim, Germany) as previously described (Li et al., 2005). Sequence of the primers were as follows: Pten: 5’-CCACAAACAGAACAACAGATGCTC-3’ and 5’-TTCCATTTTCCACTTTCTC TGAG-3’; Hes1: 5’-CAGCCAGTGTCAACACGACAC-3’ and 5’-TGGTTCTACCTCGTCTGCT-3’. CC10: Nkx2.1: 5’-AAAACGTGGGAGATCTGAG-3’ and 5’-CTGTTGGAGACTCATCGACAT-3’; SpC: 5’-CAAAACACATACTGAGATGGTGAGT-3’ and 5’-TCTCTTCTCCCGAACAGC-3’; SpB: 5’-TGGTGCATGCTTTTATCCA-3’ and 5’-CCTGGAACTTTGCTCTGAGAC-3’. Real-time PCR was performed by a LightCycler System (Roche). Primer sets for the following genes were used for RT-PCR: Gata5: 5’-GGCAACACAGCCCTAT TTGT-3’ and 5’-CTGACTCCCTTGGTTCTTG-3’; Alk5: 5’-TGTTTGAGCTGTTTTAC-3’ and 5’-TAAACAGAGCCCAGCTGTT-3’.

**Western Blot Analysis**

Cells or lung tissue were harvested and frozen in liquid nitrogen and stored at -80°C. Protein extracts were prepared in RIPA buffer (Sigma, St. Louis, MO) by homogenization,
and equal amounts of protein were separated on 4-12% NuPAGE gels (Invitrogen, Carlsbad, CA). Proteins were transferred onto Immobilon-P transfer membranes (Millipore Corp. Billerica, MA) and analyzed by western blotting using antibodies recognizing the following proteins: phosphor-MEK1/2, PTEN and FOXJ1.
RESULTS

Epithelial Specific Deletion of Alk5 in the Murine Lung.
To determine the potential role of epithelial Alk5-mediated TGF-β signaling in lung morphogenesis, we used a Gata5-cre mouse line to generate Alk5 deletions in the lung epithelium. As the expression of Gata5 has not been described in the lung, we examined it, along with expression of Alk5 using RT-PCR (Figure 1, Panel A). During mouse development, both Alk5 and Gata5 are expressed as early as E12.5 and continue to be expressed to adulthood. Alk5 is also expressed in H441 and A549 human lung carcinoma cell lines, SV40 transformed mouse lung epithelial cell line MLE15, and human fetal mesenchymal cell line MRC5. We found Gata5 expression only in A549 and MLE15 cell lines but not in H441 or MRC5 cells. The pattern, efficiency and cell type-specificity of the Gata5-cre mouse line in mediating LoxP-dependent DNA excision in the lung was determined using Rosa26-LacZ reporter mice. In E12.5 Gata5-cre; ROSA26-LacZ double transgenic embryos, LacZ activity was found to be uniform and limited to the endodermally derived lung epithelium (Figure 1, Panel B, a). Immunohistochemistry with an anti-LacZ antibody showed similar epithelial cell type specificity in E15.5 lungs (Figure 1, Panel B, b).

Alk5-deficient mice, referred to simply as Alk5Δ/KO were generated by crossing Alk5<sup>flox/KO</sup> mice with Gata5-Cre driver mice. Alk5<sup>flox/KO</sup> mice carry a heterozygous deletion of exon 3 plus a floxed allele of this exon. Their generation was described previously (Sridurongrit et al., 2008)(Figure 2, Panel A). Alk5Δ/KO mice failed to develop ventral skin and died after birth. Consequently, our analysis of the lung phenotype is limited to embryonic day E18.5. Using a commercially available antibody, we found ALK5 to be localized primarily to the apical surface of epithelial cells forming the bronchiolar epithelium, but also sporadic cells localized in the intraparenchymal regions of wild type E18.5 lungs (Figure 2, Panel B, a). Immunostaining for ALK5 showed high efficiency of Gata5-Cre mediated recombination of the LoxP sites as ALK5 was not detected in the airway epithelium in Alk5Δ/KO lungs (Figure 2, Panel B, b). Immunostaining for plasminogen activator inhibitor, PAI, a well-established
downstream target of TGF-β signaling confirmed that the TGF-β pathway was blocked, or significantly reduced in Alk5Δ/KO lungs (Figure 2, Panel B, c & d).

Gross & Histological Morphology of Alk5Δ/KO Lungs
To characterize the phenotype of the Alk5Δ/KO lungs, we isolated embryonic lungs from E15.5 and E18.5 control and mutant embryos. In E15.5 embryos, the Alk5Δ/KO lungs were consistently smaller in overall size compared to the controls (Figure 3, Panel 3.1, A & H). The Alk5Δ/KO lungs contain the correct number of lobes as the control lungs, but the individual lobes are smaller in size (Figure 3, Panel 3.1, A-F & H-M). This differential size characteristic persists to embryonic day 18.5 as shown in Figure 3, Panel 3.2. In addition, compared to the 5 lobes found in the E18.5 control lungs, the Alk5Δ/KO lungs contain what appears to be only 4 lobes, most likely due to fusion between the right median and the right caudal lobes (Figure 3, Panel 3.2, l & m). Remarkable abnormalities in the trachea, if any, were not discernible in Alk5Δ/KO lungs (Figure 3, Panel 3.1 & 3.2, G & N; g & n).

Histologically, the hematoxylin-eosin (H&E) stained sections of lungs from E15.5 Alk5Δ/KO embryos showed enlarged airways compared to the control lungs (Figure 4, compare A & D). Closer inspection of the lung sections revealed abnormalities in the epithelial layers in both proximal (Figure 4, compare B & E) as well as in distal (Figure 4, C & F) airways. Compared to the highly organized arrangement of the epithelial cells forming the distal airways in the control lungs (Figure 4, C), the mutant airways appeared to be composed of highly disorganized cells (Figure 4, E & F, arrows). In wild type E18.5 embryos, maturation of the lung is associated with the thinning of the saccular walls as seen in Figure 4, a. Absence of epithelial ALK5 activity blocked or impeded late lung development as the mutant lungs appear to be more cellular, contain immature alveoli and reduced airspace in distal airways (Figure 4, d – f; Figure 9). As in the earlier time point, the E18.5 mutant lungs were also characterized by airways consisting of a disorganized and multi-layered epithelium as seen in Figure 4, e & f, arrows.
Reduced Clara Cell Numbers in Alk5\textsuperscript{Δ/KO} Lungs

In the mouse, the endodermally-derived lung epithelium is organized into functional compartments along its proximo-distal axis. The major differentiated cellular constituents of the proximal lung epithelium include Clara cells, ciliated cells and neuroendocrine cells. To determine whether epithelial deletion of Alk5 causes abnormalities in the composition of the bronchial and bronchiolar epithelium of the lung, the expression of cell-specific markers for Clara, ciliated and neuroendocrine cells was examined by immunohistochemistry. Antibodies to CC10 and β-tubulin were used as Clara and ciliated cell markers respectively. In E18.5 Alk5\textsuperscript{Δ/KO} lungs, Clara cell numbers were significantly reduced compared with the numbers in control airways (Figure 5, Panel A, a & b). During fetal lung development, hairy and enhancer of split 1 (Hes1) has been found to exhibit a regulation pattern that indicates a role in determination of Clara cell differentiation (Ito et al., 2000). Hes1 is expressed in the Clara cell putative progenitor cells (Santos et al., 2007; Ito et al., 2000). Consistent with the reduced number of Clara cells, the expression of Hes1 was also significantly reduced in E18.5 Alk5\textsuperscript{Δ/KO} lungs (Figure 5, Panel A, c & d). Real-time PCR analysis of CC10 and Hes1 mRNA validated the latter findings (Figure 5, Panel B). In contrast to Clara cells, the number of ciliated cells in the mutant lungs appeared unchanged (Figure 5, Panel C, e & f). The transcription factor FoxJ1 otherwise known as hepatocyte nuclear factor-3/forkhead homologue 4 (Hfh-4) is expressed in ciliated cells and is required for ciliogenesis (Blatt et al., 1999). Immunohistochemistry and western blot analysis for FOXJ1 showed a similar pattern and level of expression in E18.5 Alk5\textsuperscript{Δ/KO} and control airways, indicating no changes in the distribution or number of ciliated cells (Figure 5, Panel C, g & h; Panel D). Actual counting of Clara cells and ciliated cells in multiple histological preparations, subsequent to immunohistochemical identification of the cell types by specific antibodies confirmed the paucity of Clara cells in Alk5\textsuperscript{Δ/KO} airways (Figure 6). In Alk5\textsuperscript{Δ/KO} airways, a mere 9.4% of the total epithelial cells were positive for CC10, compared to 85% in the control lungs. Ciliated cells accounted for 14% of the total number of epithelial cells in both Alk5\textsuperscript{Δ/KO} and control airways (Figure 6).

Another highly specialized cell population in the mouse airway epithelium is the
neuroendocrine cells. These cells are few and occur both as solitary cells and clusters known as neuroendocrine bodies. Calcitonin-gene-related peptide (CGRP) and Protein gene product 9.5 (PGP9.5) have been used to identify neuroendocrine cells (Ito et al., 2000; Poulson et al., 2008). Using antibodies against the latter two markers, no significant differences between E18.5 mutant and control lungs could be found (Figure 7). Therefore, epithelial-specific deletion of Alk5 in the lung appears to not affect neuroendocrine cell differentiation.

Absence of ALK5 in Epithelial Cells Affects Endodermal Progenitor Cell Differentiation

Initial histological assessment of the Alk5A/KO lungs showed that they are immature in comparison to age-matched controls (Figure 4). To identify whether the mutant airway epithelial cells are arrested in a progenitor state, we used a screening panel of antibodies against mouse progenitor cell markers (CHEMICON, ES Cell Marker Kit). Stage-specific embryonic antigen-1, SSEA1 is a marker of mouse embryonic stem (ES) cells. SSEA1 expression is lost upon differentiation of murine ES cells (Henderson et al., 2002). Immunoreactivity for SSEA1 was detectable by embryonic day E12.5 in the proximal airways of wild-type mouse lungs (Figure 8. Panel A). SSEA1 positive cells were hardly found in adult lungs (Figure 8. Panel B). In E15.5 embryos and preceding the onset of differentiation for many epithelial cell types, SSEA1 is expressed in the proximal airway epithelium in both control and Alk5A/KO lungs (Figure 8. Panel C, e & f). Interestingly, there was a significant pool of positive cells for SSEA1 in E18.5 Alk5A/KO lungs compared to age-matched controls (Figure 8, Panel C, compare h to g). Using the anti-SSEA1 antibody three groups of cells could be distinguished in E18.5 control lungs; SSEA1positive, progenitor cells (Figure8, Panel C, g, thin arrow); SSEA1positive; CC10positive, differentiating Clara cells (Figure 8, Panel C, g, arrowhead); and CC10positive terminally differentiated Clara cells (Figure 8, Panel C, g, thick arrow). A gradient of less SSEA1positive cells and more CC10positive cells are localized along the proximal to distal axis of the airways in the wild type lung (Figure 8. Panel C, g & i). In contrast, CC10positive cells (terminally differentiated Clara cells) are greatly reduced and replaced by SSEA1positive cells (progenitor cells) in E18.5 Alk5A/KO lungs (Figure 8. Panel C, h & j).
The specialized epithelial cell types populating the distal airways and the alveoli begin to emerge during the terminal sac stage of lung development (Cardoso & Whitsett 2008). To determine whether epithelial-specific deletion of Alk5 perturbs the differentiation or distribution of alveolar epithelial cells, we examined the expression of their distinctive markers by immunohistochemistry. The expression levels and pattern of the alveolar type II cell marker, pulmonary surfactant proteins-B (SP-B) and C (SP-C) showed no significant differences between Alk5Δ/KO and the control lungs in E18.5 embryos (Figure 9. Panel A, a-d). NKX2.1, a homeodomain transcription factor is widely acknowledged as an early marker of lung endodermal cell specification. Distribution of NKX2.1 was also examined by immunohistochemistry which showed NKX2.1 to be expressed in both airway and alveolar epithelial cells in Alk5Δ/KO and control lungs (Figure 9, Panel A, e & f). Also, analysis of steady state levels of mRNA for Sp-B, Sp-C and Nkx2.1 by real-time PCR revealed no significant differences between Alk5Δ/KO and control lungs (Figure 9. Panel B). Therefore, epithelial deletion of ALK5 affects bronchiolar epithelial progenitor cell differentiation, but spares alveolar epithelial cell differentiation.

**TGF-β Activation of Hes1; The role of Alk5**

The *in vivo* observations in Alk5Δ/KO lungs suggest that TGF-β signaling via ALK5 is necessary for emergence of Clara cells during lung development. Two possibilities were considered. First, TGF-β signaling via ALK5 may directly stimulate CC10 gene expression in Clara cell progenitor cells. Alternatively, the pathway that leads to Clara cell differentiation may be the target of Alk5-mediated TGF-β signaling. To examine these possibilities we used *in vitro* experiments employing the MLE15 mouse epithelial cell line. Treatment of MLE15 cells with various doses of TGF-β1 had no discernible impact on endogenous CC10 mRNA levels, suggesting that TGF-β1 does not directly stimulate CC10 gene expression (data not shown). To examine the alternative possibility, we examined the effect of TGF-β on *Hes1*, a gene that encodes a transcription factor associated with Clara cell lineage determination (Ito et al., 2000). MLE15 cells were transfected with either pGL3-luciferase (control vector) or the *Hes1* promoter-luciferase construct and treated with TGF-β1. To verify TGF-β1 bioactivity (positive control), we used a PAI-1-promoter-luciferase construct (pRK5-PAI).
Promoter activity increased more than 7 fold in response to TGF-β1 (Figure 10, Panel A). Similarly, TGF-β1 treatment stimulated luciferase activity by 2 fold in MLE15 cells transfected with Hes1 promoter-luciferase construct (Figure 10, Panel A). The latter findings were validated by examining the response of the MLE15 endogenous Hes1 gene to TGF-β1 treatment. MLE15 cells were treated either with TGF-β1 or its carrier, DMSO and steady state levels of Hes1 mRNA were determined by real-time PCR. When compared to DMSO control, TGF-β1 increased Hes1 mRNA by 2.5 fold (Figure 10, Panel B). In addition, TGF-β1-mediated increase in Hes1 was almost entirely blocked by SB-52334, a specific inhibitor of Alk5 function (Grygielko et al., 2005). SB-52334 alone had no effect on TGF-β1-induction of Hes1 mRNA (Figure 10, Panel B). These finding demonstrate that the endogenous Hes1 gene in lung epithelial cells can be stimulated by TGF-β1 signaling via the type I receptor, ALK5.

**TGF-β Activation of Hes1; Downstream of receptor signaling**

Signaling through the TGF-β receptor complex can be transduced via Smad or non-Smad pathways. Using a recently developed alveolar type II cell line from Smad3−/−; SPC-SV40 double transgenic lungs we examined the potential involvement of Smad3 in TGF-β1 regulation of Hes1 mRNA. Smad3−/− and MLE15 (control) cells were treated either with TGF-β1 or DMSO and steady state levels of Hes1 mRNA were determined by real-time PCR (Figure 10, Panel C). Absence of Smad3 had little to no impact on TGF-β1-induced expression of Hes1 (Figure 10, Panel C).

Intracellular TGF-β signal transduction can be mediated by a number of so called non-Smad dependent pathways (For a review see Zhang, 2009). Blocking the MEK/ERK pathway by a specific inhibitor, U0126 blocked TGF-β induction of Hes1 mRNA (Figure 11, Panel A). Another inhibitor, LY-294002 which blocks Akt activity, caused only partial interference with the impact of TGF-β1 on Hes1 mRNA, whereas SB 203580, a p38 inhibitor had no effect (Figures 11, Panel B & C). Further support for the involvement of MEK/ERK pathway was provided by western blot analysis with anti-phospho-ERK antibody. In MLE15 cells, ERK phosphorylation was increased by TGF-β1 treatment (Figure 12, Panel A, Lane b) and
this response was blocked by the ALK5 inhibitor, SB-52334 (Figure 12, Panel A, Lane c). Consistent with these *in vitro* results, western blot analysis showed decreased phospho-ERK in E18.5 *Alk5Δ/KO* lungs (Figure 12, Panel B). These data clearly demonstrate that the MEK/ERK pathway, and to a lesser extent, the AKT pathway serve as the intracellular transducers of TGF-β signaling-induced *Hes1* gene regulation.

**TGF-β Activation of Hes1: The role of Pten**

Pten is a negative regulator of MEK/ERK cascade (Gu et al., 1999). *Pten* is also negatively regulated by TGF-β1 via Smad-independent signaling (Chow et al., 2008). In similar experiments to those described above, we used MLE15 cells and assayed for ERK phosphorylation and expression of a number of genes in the presence and absence of TGF-β1 and SB-52334, an ALK5-specific inhibitor. Treatment of MLE15 cells with TGF-β1 reduced steady state level of Pten protein (Figure 12, Panel A, Lane b). This effect of TGF-β1 was mediated via ALK5 as it was blocked by SB-52334 (Figure 12, Panel A, Lane c). These observations raised the possibility of involvement of *Pten* in TGF-β-induced *Hes1* gene regulation. Consistent with this hypothesis, real-time PCR analysis showed significantly increased *Pten* mRNA in E18.5 *Alk5Δ/KO* lungs compared to controls (Figure 12, Panel C). We reasoned that if the impact of TGF-β on Clara cell lineage determination is mediated through *Pten*, it can be predicted that *Pten* deletion in lung epithelial cells may increase the number of Clara cells or its progenitors during lung morphogenesis *in vivo*. To investigate this possibility, we generated mice carrying conditional homozygous lung epithelial deletion of *Pten* by crossing *Pten*^flox/flox^ mice with the recently generated *Nkx2.1-Cre* mice (Xing et al., 2008). *Nkx2.1* encodes a key endodermally-specific transcriptional regulator of lung morphogenesis. Previous studies have shown that in the lung *Nkx2.1-Cre* activity is strictly epithelial-specific (Xing et al., 2008). Analysis of cell differentiation in *PtenΔ/Δ* and control embryonic lungs revealed a significantly increased number of Clara cells in *PtenΔ/Δ* lungs (Figure 13, Panel A, compare a to b or e to f). Significantly, and consistent with the findings in *Alk5Δ/KO* lungs, differentiation of ciliated cells remained intact (Figure 13, Panel A, compare c and d). Real-time PCR analysis showed increased *CC10* and *Hes1* mRNA in *PtenΔ/Δ* lungs (Figure 13, Panel B). Interestingly, ERK
phosphorylation was significantly increased due to absence of Pten activity in Pten⁻/⁻ lungs (Figure 13, Panel C). The above findings were validated by examining the response of Hes1 expression and phospho-ERK to PTEN in vitro. MLE15 cells were transfected with either CMV-vector (control plasmid) or CMV-Pten expression constructs (Gu et al., 1998). PTEN protein levels were measured to verify the activity of CMV-Pten plasmid and efficiency of transfection (Figure 14, Panel B). Endogenous Hes1 mRNA and phospho-ERK levels were examined by real-time PCR and western blot respectively. Compared to the CMV-vector transfection (control), CMV-Pten reduced Hes1 mRNA levels (Figure 14, Panel A) concomitant with blocking ERK phosphorylation (Figure 14, Panel B). Thus, both in vitro and in vivo data are consistent with a role for Pten in regulating Clara cell lineage determination mediated by the TGF-β/Alk5 pathway.
Discussion

The purpose of the current study was to determine the precise role of endodermal-specific TGF-β signaling, via its type I receptor, Alk5 in cell fate determination and differentiation in a model of embryonic lung morphogenesis. The mammalian lung represents an attractive model as its cellular composition is said to exceed 40 distinct specialized varieties. Endodermal deletion of Alk5 resulted in abnormal lung morphogenesis, characterized by enlarged airways in E15.5 lungs. As development progressed, this phenotype evolved into immature alveoli and formation of disorganized and multi-layered epithelium in the proximal airways of E18.5 lungs. Examination of cell differentiation in Alk5 mutant lungs revealed a marked reduction in the number of Clara cells, a highly specialized airway epithelial cell type. In contrast, abundance and distribution of ciliated and neuroendocrine cells remained unchanged. Thus, Alk5 appears to regulate either the determination or expansion of the Clara cell lineage, without affecting those of ciliated or neuroendocrine cells. These results suggest that contrary to the prevailing concept, the three epithelial cell types may originate from independent progenitor/precursor cells, or that distinct pathways are involved in their differentiation or expansion.

It is currently thought that peripheral pulmonary epithelial cells including those in the conducting airways and the respiratory alveoli share a common lineage, distinguished by expression of Nkx2.1 (for a review see Cardoso and Whitsett, 2008). In the proximal lung, an unknown progenitor cell population is thought to undergo differentiation and generate multiple, highly specialized cell types, including Clara cells, ciliated cells and pulmonary neuroendocrine cells. Together, these cells compose the bronchiolar and conducting airway epithelium (Nettesheim et al., 1990).

There are presently no definitive data linking the origin of the latter cell types to a common progenitor. Much of what is available comes from studies of induced lung injury combined with observations of cell population dynamics during recovery (Rawlins et al., 2007). For example, acute airway injury that targets ciliated cells is rapidly resolved through what
appears be proliferation and differentiation of Clara cells, thus linking Clara and ciliated cells (Raynolds et al., 2007). Also, in a naphthalene injury model, Clara cells die within the first 24 hours after exposure to this toxicant. However, a variant subpopulation of Clara cells that does not express Cyp2f2, is thought to survive, undergo expansion and differentiation in 2 to 4 weeks to re-establish the normal airway epithelial cell composition. Thus, Clara cells are thought to serve as progenitor/stem cells for the bronchial epithelium. Little in the way of experimental information is available on the precise pathway(s) that lead to Clara cell differentiation.

Clara cells are thought to derive from an unknown progenitor, perhaps basal cells in the proximal airways late in lung development, but this remains unsubstantiated (Cardoso, 2001). The transcription factor Hes1 is expressed in Clara cell putative “progenitors” (Ito et al., 2000). In the present study, we found that loss of Alk5 in the lung epithelium reduced the number of Clara cells in association with reduced Hes1. This suggested that TGF-β signaling may be an important determinant of Clara cell differentiation. The observation that Alk5−/−/− lungs are not entirely devoid of Clara cells leads to two alternative interpretations. First, it is possible that the presence of the few Clara cells seen in Alk5 mutant lungs is merely related to incomplete Cre recombination activity. This is not supported by the data in Figure 1 where LacZ was uniformly expressed in what appears to be the entire epithelium. Alternatively, TGF-β signaling via ALK5 may be necessary for expansion rather than determination of Clara cell lineage. The finding that Hes1 is directly controlled by TGF-β via Alk5 argues against the latter, but supports the former possibility, although this requires further validation.

Alk5 deficiency in epithelial cells only affected Clara cell differentiation, but not that of ciliated nor pulmonary neuroendocrine cells. This finding raises the possibility that the three cell types, previously thought to have a common progenitor may either arise from different progenitor cells, or alternatively, differentiate via independent pathway(s). To investigate these possibilities, we examined the temporal and spatial pattern of SSEA1 expression, which had hitherto remained unknown, during lung development (Figure 8).
The results are consistent with a model whereby airway epithelial cells emerge from a common progenitor cell population. However, each specialized cell type may require an independent mechanism for initiation of cell differentiation. The latter is supported by the *in vitro* observation that the ciliated cell marker, *Foxj1*, is unresponsive to, whereas *Hes1* is increased by TGF-β1 signaling (Figure 12, Panel I). Thus, TGF-β/ALK5 signaling may not be involved in regulation of ciliated cell differentiation.

TGF-β/ALK5-mediated signaling controls many processes during cell and tissue differentiation. Ligand-binding activates the TGF-β/ALK5 downstream signal transduction cascade that includes Smad and non-Smad pathways (Rahimi et al., 2007). The abilities of both Smad and non-Smad pathways to direct and redirect differentiation have been demonstrated in many vertebrate tissues. For example, Smad3 mediates TGF-β inhibition of cell differentiation and progenitor cell determination (Alliston et al., 2001). However, absence of Smad3 did not block *Hes1* response to TGF-β1 treatment, indicating that the role of Smad3 in TGF-β1/ALK5-induced *Hes1* expression and hence Clara cell differentiation is at best limited.

TGF-β signaling is also mediated by the so called Smad-independent pathways, including MEK/ERK, PI3K/AKT and p38 MAP kinase (Niculescu-Duvaz et al., 2007; Giehl et al., 2000, Lee et al., 2008). We found that the MEK/ERK pathway accounts for the observed TGF-β/ALK5-induced *Hes1* gene regulation *in vitro*. Blockade of ERK phosphorylation led to loss of *Hes1* response to TGF-β1 (Figure 11, Panel I). *Hes1* induction by TGF-β1 was partially blocked also by treatment with AKT inhibitor suggesting a minor requirement for PI3/AKT activity (Figure 11, Panel II). Recent findings in human neuroblastoma cells also contend that *Hes1* response is dependent on activation of ERK and AKT phosphorylation (Slockhausen et al., 2005). Consistent with our *in vitro* data, we found that deletion of ALK5 caused reduction of phosphorylated ERK *in vivo*, linking ALK5-regulated Clara cell differentiation to the MEK/ERK pathway.
Several studies have emphasized a role for Pten in progenitor/stem cell homeostasis (Yanagi et al., 2007; Zheng et al., 2008). Pten was initially identified as a negatively regulated TGF-β target (Li et al., 1997; Kattla et al., 2008). In the current study, we found that Alk5 deficiency led to increased Pten expression in vivo and that Pten was inhibited by TGF-β1 in vitro (Figure 12, Panel I & III). Moreover, Pten negatively regulates the phosphorylation of ERK in vivo (Figure 13, Panel III) and in vitro (Figure 14, Panel II). Reciprocal and balanced Pten/ERK regulation has also been reported and proposed as a mechanism for maintaining the cellular steady state condition (Chow et al., 2007). Based on these findings, we propose a model (Figure 15) whereby the impact of TGF-β via ALK5 on Clara cell ontogeny in the lung is mediated through activation of Pten and phospo-ERK. A number of observations support the validity of this hypothetical model. First, TGF-β modulation of Pten, as the former’s effect on Hes1, occurs independently of Smad signaling (Chow et al., 2008). Second, and as we found in this study (Figure 13 & 14), Pten is known to inhibit activation of the MEK/ERK pathway (Gu et al., 1998). TGF-β/Alk5 regulation of the Clara cell progenitor marker Hes1 also involves MEK/ERK activation (Figure 12). In addition, Pten is a negative regulator of AKT phosphorylation (for review see Yamada et al., 2001), a pathway that is partly involved in Hes1 regulation. Finally, the model is directly supported by the observation that conditional ablation of Pten in lung epithelial cells caused expansion of the Clara cell population and increased ERK phosphorylation without affecting ciliated cell differentiation (Figure 13). In summation, the results of this study suggest that TGF-β signaling via Alk5 through a pathway that involves Pten-regulated ERK and AKT activation may be required for Clara cell ontogeny but not those of ciliated or pulmonary neuroendocrine cells during lung development.

**Acknowledgments:**
We thank Dr. Pilar Ruiz-Lozano for the Gata5-Cre mice; Dr. Jonas Larsson for the Alk5^{+/−} mice; Dr. Ruth S. Slack for pGL3-Hes1-promoter construct and Dr. Kenneth M Yamada for CMV-Pten construct. This work was supported by NIH/NHLBI and generous funds from the Hastings Foundation.
FIGURE LEGENDS

Figure 1. Alk5, Gata5 expression & Gata5-cre activity.
Panel A: Alk5 and Gata5 expression during lung development and in select cell lines as analyzed by real time PCR. Beta-actin was used as control. Panel B. LacZ activity (a) and β-gal (b) immunostaining in Gata5-cre; R26R lungs. Arrows show epithelial-specific activity of Gata5-cre activity in embryonic lungs.

Figure 2. Schematic map of Alk5 alleles.
Panel A. Map of the floxed and deleted Alk5 alleles in heterozygous Alk5Δ/flox mutant mice as reported by Sridurongrit et al. (2008). Mating with Gata5-cre results in generation of homozygous Alk5 deleted alleles, Alk5Δ/KO. Panel B. Immunolocalization of TβRI (ALK5) and PAI-1 in control (a & c) and Alk5Δ/KO (b & d) E18.5 lungs. Arrows show spatial distribution of ALK5 and PAI-1 in control airways (a & c), and their reduction or absence in mutant airways (b & d).

Figure 3. Gross morphology of the Alk5Δ/KO lungs.
Panel 3.1. Individual lobes and trachea of mutant and control lungs are shown. Panels A to G, E15.5, control lungs. Panels H to N, E15.5, Alk5Δ/KO lungs. Panel 3.2. Panels a to g, E18.5, control lungs. Panels h to n, E18.5, Alk5Δ/KO lungs. The right median and the right caudal lobes appear to be fused in Panel I.

Figure 4. Histology of the Alk5Δ/KO lungs.
H&E sections of control and Alk5Δ/KO lungs at two embryonic stages. Arrows indicate enlarged airways in Panels D and E. Disorganized epithelium in the Alk5Δ/KO lungs is shown in Panels e and f by arrows.

Figure 5. Reduced Clara cells and their putative progenitors in Alk5Δ/KO lungs.
Panel A. Immunolocalization of Clara cells and their putative progenitors by anti-CC10 (a & b) and anti-HES1 antibodies (c & d) in Alk5Δ/KO and control lungs. Arrows indicate presence (a & b) or absence (c & d) of immunoreactivity with each antibody. Panel B. Realtime PCR
analysis of CC10 and Hes1 mRNAs in E18.5 lungs. Control values are normalized to unity.

**Panel C.** Immunolocalization of ciliated cells using anti-β-tubulin (e & f) or anti-FOXJ1 (g & h) antibodies. Arrows indicate β-tubulin or FOXJ1 positive cells in control or mutant airways. **Panel D.** Western blot analysis of FOXJ1. α-tubulin was used as control.

**Figure 6. Relative ratio of Clara and ciliated cell numbers in airway epithelium.**
The numbers of Clara, ciliated, and total epithelial cells were determined and plotted as percent of total epithelial cell numbers.

**Figure 7. Neuroendocrine cell differentiation in Alk5Δ/KO lungs.**
Immunolocalization of pulmonary neuroendocrine cells using anti-CGRP (a & b) and anti-PGP9.5 (c & d) antibodies in E18.5 Alk5Δ/KO and control lungs (arrows).

**Figure 8. Expression pattern of SSEA1 in Alk5Δ/KO and control lungs.**
**Panel A.** Immunolocalization of SSEA1 in E12.5 wild type lungs. Image in b is higher magnification of image in a. Arrows point to SSEA1 positive cells. **Panel B.** SSEA1/CC10 double immunofluorescence in 3 month old wild type lungs. Note few double positive cells in c (arrow). **Panel C.** SSEA1 single (e & f) and SSEA1/CC10 double (g to j) immunofluorescence in E15.5 (e & f) and E18.5 (g to j) control (e, g & i) and Alk5Δ/KO (f, h & j) lungs. SSEA1positive cells (red, thin arrow), CC10positive cells (green, thick arrow) and SSEA1positiveCC10positive cells (yellow, arrowhead) are shown. The interrupted line indicates the basement membrane of distal airways.

**Figure 9. Cell differentiation in Alk5Δ/KO Lungs.**
**Panel A.** Immunolocalization of SP-C (a & b), SP-B (c & d) and NKX2.1 (e & f) in E18.5 control and Alk5Δ/KO lungs. **Panel B.** Real-time PCR quantification of mRNA for Sp-C, Sp-B and Nkx2.1 in mutant and control lungs.

**Figure 10. Regulation of Hes1 by TGF-β1.**

22
Panel A. The mouse *Hes1*-promoter-luciferase construct, *pGL3-Hes1*, was transfected into MLE15 cells which were then treated with DMSO or TGF-β1 for 48hr. Normalized luciferase values are shown. *PAI-1*-promoter-luciferase construct, *pRK5-PAI*, was used as positive control. Panel B. MLE15 cells were treated with DMSO (control), TGF-β1, ALK5-inhibitor (SB525334) or TG-β1 plus ALK5-inhibitor. After 48 hours, *Hes1* mRNA was analyzed by real-time PCR. Panel C. MLE15 and *Smad3(-/-)* cells were treated with TGF-β1. *Hes1* mRNA was analyzed by real-time PCR.

Figure 11. *Regulation of Hes1 by TGF-β1 downstream signaling.*
Real-time PCR was used to analyze *Hes1* induction by TGF-β1 in MLE15 cells in presence or absence of one of the following: U0126, a MEK inhibitor (Panel A); LY294002, a PI3K inhibitor (Panel B); SB203580, a p38 MAPK inhibitor (Panel C). DMSO was used as a TGFβ1 control and set to unity for comparison. Show significance *

Figure 12. *Alk5 Signaling Controls ERK Phosphorylation and Pten expression.*
Panel A. MLE15 cells were treated with DMSO (control), TGF-β1, ALK5-inhibitor or ALK5-inhibitor plus TGF-β1. Western blot analysis was used to assess the steady state levels of phosphorylated ERK, Pten, FOXJ1, PGP9.5 and β-tubulin (loading control). Panel B. Western blot analysis of phosphorylated ERK in lung tissue from E18.5 control and *Alk5*/*Ko* embryos. β-tubulin was used as loading control. Panel C. Real-time PCR measurement of *Pten* mRNA in lung tissue from E18.5 control and *Alk5*/*Ko* embryos.

Figure 13. *Conditional deletion of Pten increases Clara cell population.*
Lung epithelial deletion of *Pten* was accomplished by using an *Nkx2.1-cre* driver line as described in Materials & Methods. Panel A. Immunolocalization for CC10 in control and *Pten*/*A* lungs (a & b respectively, arrows). Immunolocalization of β-tubulin in control and *Pten*/*A* lungs (c & d respectively, arrows). CC10/ β-tubulin merge (e & f). Panel B. Real-time PCR analysis of *Hes1* and CC10 mRNA in *Pten*/*A* and control lungs. Panel C. ERK phosphorylation in *Pten*/*A* and control lungs was determined by western blot.
Figure 14. *Pten negatively regulates Hes1 expression and ERK phosphorylation in MLE15.*
MLE15 cells were transfected with either *CMV-Pten* or *CMV-vector* (control) plasmid. **Panel A.** *Hes1* mRNA was measured by real-time PCR. **Panel B.** PTEN protein and ERK phosphorylation were examined by western blot analysis. α-tubulin was used as control.

Figure 15. *A simplified model illustrating TGFβ/ALK5 signaling in lung airway cell differentiation.* TGFβ signaling via ALK5 negatively regulates *Pten* expression, which in turn negatively regulates phosphorylated ERK and phosphorylated AKT. Phosphorylated ERK and AKT activate *Hes1* expression. TGFβ/Alk5 signaling does not affect ciliated cell nor pulmonary neuroendocrine cell differentiation.
REFERENCES


Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J. 2002. Molecular interpretation of


Fig. 1

A. Wild-type mouse lung

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Cell line

B.

E12.5 Gata5/R26-LacZ

E15.5 β-Gal

Scale bar
Fig. 2

A.

LoxP neo LoxP Exon III Exon IV

Alk5\textsuperscript{flox/+} recombinant allele

Alk5\textsuperscript{ko/+} allele

↓ \( \times \) Gata5-Cre

LoxP

Alk5\textsuperscript{Δ/+} Cre excised allele

Alk5\textsuperscript{ko/+} allele
Fig. 2

B.

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PAI-1

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Fig. 4

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Arrow indicates the region of interest.
Fig. 5

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Fig. 6

- Control
- Alk5Δ/KO

*P = 0.0001

Percentage of Clara or ciliated cells in airway epithelium

Clara Cell

Ciliated Cell
Fig. 7

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Fig. 8

A. E12.5 Wild type

B. 3 month Wild type

C. Control Alk5Δ/Δ}
Fig. 9

A. | Control | Alk5Δ/KO |
---|---------|---------|
SP-C | ![Image](image1) | ![Image](image2) |
SP-B | ![Image](image3) | ![Image](image4) |
NKX2.1 | ![Image](image5) | ![Image](image6) |

B. 

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Fold Difference
Fig. 10

A. 

![Graph showing luciferase activity fold difference](image)

B. 

![Bar graph showing Hes1 fold difference](image)

C. 

![Bar graph showing Hes1 fold difference](image)
Fig. 11

A.

![Bar graph showing Hes1 Fold Difference for DMSO, +TGFβ1, MEK-Inhibitor, and MEK-Inhibitor + TGFβ1 conditions.]

B.

![Bar graph showing Hes1 Fold Difference for DMSO, +TGFβ1, Akt-Inhibitor, and Akt-Inhibitor + TGFβ1 conditions.]

C.

![Bar graph showing Hes1 Fold Difference for DMSO, +TGFβ1, p38-Inhibitor, and p38-Inhibitor + TGFβ1 conditions.]

Fig. 12

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B. 

Control vs Alk5Δ/KO

C. 

Fold Difference

Control vs Alk5Δ/KO
Fig. 13

A.  

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C.  

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Fig. 14

A. 

Hes1 Fold Difference

- CMV-vector
- CMV-Pten

B. 

- CMV-vector
- CMV-Pten

- ERK-P
- PTEN
- α-tubulin
Summary

TGFβ

Type II
ALK5

Pten

ERK$^\text{-P}$
AKT$^\text{-P}$

Hes1