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Signaling via *Alk5* controls the ontogeny of lung Clara cells

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SUMMARY

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 or stem cells during lung regeneration after injury. The mechanisms of Clara cell differentiation are largely unknown. Transforming growth factor beta (TGFβ) is a multifunctional molecule with roles in normal development and disease pathogenesis. In this study, we deleted the TGFβ 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* and the MEK/ERK and PI3K/AKT pathways. Thus, the signaling pathway by which TGFβ/ALK5 regulates Clara cell differentiation may entail inhibition of *Pten* expression, which in turn activates ERK and AKT phosphorylation.

KEY WORDS: Lung morphogenesis, TGFβ, Receptor, *Alk5*, Clara cell differentiation, Progenitor, Stem cells, *Hes1*, *Pten*, ERK, AKT, *Gata5*, Mouse

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 could be important in normal maintenance and regeneration or repair after injury. Both diffusible and cell-cell contact-mediated signaling and communication probably 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, the 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 airway epithelium. These are the basal cells, which are thought to generate Clara and ciliated cells, the variant Clara cells which generate Clara cells and possibly neuroendocrine cells, and the bronchioalveolar stem cells which generate Clara cells and 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 a 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 [mouse embryonic day 16 (E16)] but the mechanism remains unsubstantiated (Cardoso, 2001). However, the gene hairy/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 beta (TGFβ) family members is crucial to both lineage selection and progression of differentiation. These molecules also have the ability to redirect cell lineage determination (for a review, see Derynck et al., 2007). TGFβ ligands bind to receptor complexes that comprise type I and type II serine/threonine kinases. Blocking the type I kinases, ALK5 (activin receptor-like kinase 5) receptor function abrogates TGFβ 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

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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 TGFβ-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 TGFβ (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 TGFβ-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 the emergence of Clara, but not ciliated, cells through *Pten*-regulated ERK phosphorylation.

MATERIALS AND METHODS

Mouse genotypes and cell lines

Alk5$^{\Delta/KO}$ mice were generated as previously described (Sridurongrit et al., 2008). Generation of *Gata5-Cre* mice was previously reported (Merki et al., 2005). *Pten*$^{\Delta/\Delta}$ mice were generated by crossing *Pten*$^{flox/flox}$ mice (ATCC, Manassas, VA, USA) with *Nkx2.1-Cre* mice (Xu et al., 2008; Xing et al., 2008). Human pulmonary carcinoma H441, A549 cells and human fibroblast MRC5 cells were from ATCC. *SV40*-transformed mouse lung epithelial cell line MLE15 was a gift from Dr Whitsett (Wikenheiser et al., 1993). Mouse *Smad3*$^{-/-}$ 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 was performed as previously described (Li et al., 2005). Antibodies against the following proteins were used: β-galactosidase, ALK5 (TβRI), CGRP and PAI-1 (Abcam, Cambridge, MA, USA), CCSP (Seven Hill Bioreagents, Cincinnati, OH, USA), FOXJ1, HES1 and CYP2F2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), β-tubulin (BioGenex, San Ramon, CA, USA), phosphor-MEK1/2 and PTEN (Cell Signaling Technology, Danvers, MA, USA), PGP9.5 (AnaSpec, San Jose, CA, USA), SSEA1 (Millipore, Billerica, MA, USA) and Ki67 (Thermo Scientific, Fremont, CA, USA).

Cell culture, transient transfection assay and pathway inhibitors

Mouse lung epithelial MLE15 and *Smad3*$^{-/-}$ cell lines were maintained in RPMI 1640 (GIBCO, Carlsbad, CA, USA) as previously described (Wikenheiser et al., 1993). Cells were grown to 80-90% confluence in 12-well plates for transient transfection or other treatments. For transient transfection, SuperFect (Qiagen, Valencia, CA, USA) was used with 1 μg *Hes1* promoter construct (gift from Dr Slack, University of Ottawa, ON, Canada) (Vanderluit et al., 2007) or *CMV-Pten* (gift from Dr Yamada, National Institutes of Health, Bethesda, MD, USA) (Gu et al., 1998). The plasmids *pGL3* (Promega, Madison, WI, USA) or *CMV-vector* were used as negative controls. The *Pai1* promoter-luciferase construct (van Zonneveld et al., 1988) was used as a positive control for transfection and TGFβ1 treatment efficiency. Cells were treated with MEK inhibitor U0126 (20 μM), p38 MAPK inhibitor SB203580 (10 μM) (Promega), PI3K inhibitor LY294002 (15 μM) or ALK5 inhibitor SB525334 (50 μM) (Sigma, St Louis, MO, USA) with or without TGFβ1 (20 ng/ml) for 48 hours. Cells were lysed and assayed for 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). The cDNA was synthesized following the protocol by SuperScript (Invitrogen, Carlsbad, CA, USA). Quantification of selected genes by real-time PCR was performed using LightCycler (Roche Diagnostics, Mannheim, Germany) as previously described (Li et al., 2005). The primer sequences used were as follows: *Pten*: 5′-CCACAAACAGAACAACAAGATGCTC-3′ and 5′-TTCCATTTTCCACTTTTTCTGAG-3′; *Hes1*: 5′-CAGCCAGTGTCA-ACACGACAC-3′ and 5′-TCGTTCATGCACTCGCTG-3′; *CC10*: 5′-GATCGCCATCACAATCACTG-3′ and 5′-TTGAAGAAATCCT-GGGCAGA-3′; *SpA*: 5′-CTGGAGAACATGGAGACAAGG-3′ and 5′-AAGCTCCTCATCCAGGTAAGC-3′. Primer sets for the following genes were used for RT-PCR: *Gata5*: 5′-GGCAACACAGCCCTATTTGT-3′ and 5′-CCTGACTCCCTTGCTTCTTG-3′; *Alk5*: 5′-TGGTTGAGCCTG-GGTTTTAC-3′ and 5′-TTAACAGAGC CCAGCTGCTT-3′.

Western blot analysis

Cells or lung tissues were harvested and frozen in liquid nitrogen. Protein extracts were prepared in RIPA buffer (Sigma) by homogenization, and equal amounts of protein were separated on 4-12% NuPAGE gels (Invitrogen). Proteins were transferred onto Immobilon-P transfer membranes (Millipore Corp., Billerica, MA, USA) and analyzed by western blotting using antibodies recognizing the following proteins: phosphor-MEK1/2, PTEN, FOXJ1 and CYP2F2.

Apoptosis

The apoptotic cells in E18.5 lungs were detected by TUNEL assay using the In Situ Cell Death Detection Kit (Roche Diagnostic Gmb).

RESULTS

Epithelial-specific deletion of *Alk5* in the murine lung

To determine the potential role of epithelial *Alk5*-mediated TGFβ 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 (Fig. 1A). During mouse development, both *Alk5* and *Gata5* are expressed as early as E12.5 and continue to be expressed to adulthood. *Alk5* was also expressed in H441, A549 human lung carcinoma and mouse MLE15 cell lines. We found *Gata5* expression only in A549 and MLE15 cell lines, but not in H441 or MRC5 cell lines (data not shown). 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 (Fig. 1Ba). Immunohistochemistry with an anti-β-galactosidase antibody showed similar epithelial cell type specificity in E15.5 lungs (Fig. 1Bb).

Alk5-deficient mice, referred to simply as *Alk5*$^{\Delta/KO}$ were generated by crossing *Alk5*$^{flox/KO}$ mice with *Gata5-Cre* driver mice. *Alk5*$^{flox/KO}$ mice carry a heterozygous deletion of exon 3 plus a floxed allele of this exon (see Fig. S1 in the supplementary material). Their generation has been previously described (Sridurongrit et al., 2008). *Alk5*$^{\Delta/KO}$ mice failed to develop ventral skin and died after

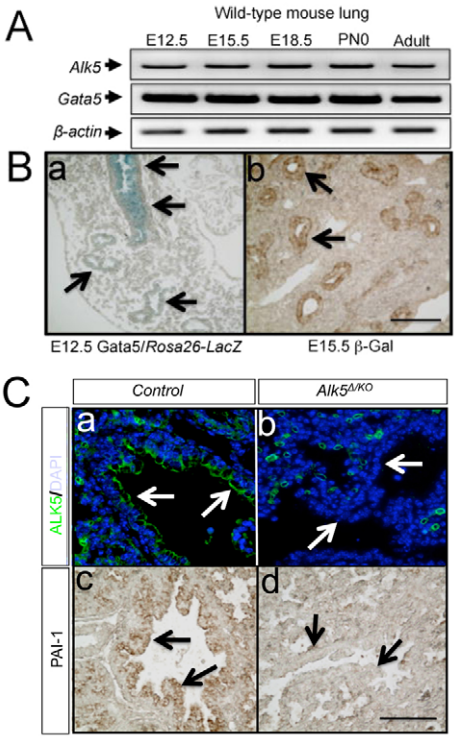

Fig. 1. *Alk5* and *Gata5* expression; *Gata5-Cre* activity and generation of *Alk5* $^{/KO}$ lung. (**A**) *Alk5* and *Gata5* expression during lung development was analyzed by real-time PCR. β-actin was used as control. (**Ba**,**b**) *lacZ* activity (a) and β-gal immunostaining (b) in *Gata5-Cre; ROSA26-lacZ* lungs. Arrows show epithelial-specific activity of *Gata5-Cre* in embryonic lungs. (**Ca-d**) Immunolocalization of TβRI (ALK5) and PAI1 in control (a,c) and *Alk5*$^{\Delta/KO}$ (b,d) E18.5 lungs. Arrows show spatial distribution of ALK5 and PAI1 in control airways (a,c) and their reduction or absence in mutant airways (b,d). Scale bars: 50 μm.

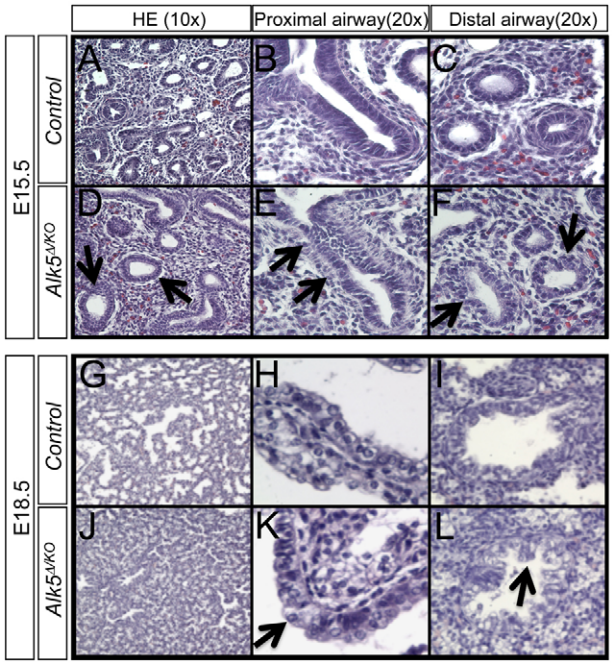

Fig. 2. Histology of the *Alk5* $^{/KO}$ lungs. (**A-L**) Hematoxylin and Eosin sections of control and *Alk5*$^{\Delta/KO}$ lungs at two embryonic stages. Arrows indicate enlarged airways in D-F and disorganized epithelium in the *Alk5*$^{\Delta/KO}$ lungs in K and L.

birth. Consequently, our analysis of the lung phenotype is limited to the embryonic days preceding birth. 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 to sporadic cells localized in the intraparenchymal regions of wild-type E18.5 lungs (Fig. 1Ca). 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 *Alk5*$^{\Delta/KO}$ lungs (Fig. 1Cb). Immunostaining for plasminogen activator inhibitor 1, PAI1, a well-established downstream target of TGFβ signaling, confirmed that the TGFβ pathway was blocked, or significantly reduced, in *Alk5*$^{\Delta/KO}$ lungs starting between E12.5 and E15.5 (Fig. 1Cc,d; see also Fig. S1 in the supplementary material).

Gross and histological morphology of *Alk5* $^{/KO}$ lungs

To characterize the phenotype of the *Alk5*$^{\Delta/KO}$ lungs, we isolated embryonic lungs from E15.5 and E18.5 control and mutant embryos. In E15.5 embryos, the *Alk5*$^{\Delta/KO}$ lungs were consistently smaller in overall size compared with the controls. The *Alk5*$^{\Delta/KO}$ lungs contained the same number of lobes as the control lungs, but the individual lobes were smaller in size (see Fig. S1D in the supplementary material). This differential size characteristic persisted to embryonic day 18.5 (see Fig. S1 in the supplementary material). In addition, compared with the five lobes found in the E18.5 control lungs, the *Alk5*$^{\Delta/KO}$ lungs contained what appeared to be only four lobes, most probably owing to fusion between the right median and the right caudal lobes. Remarkable abnormalities in the trachea, if any, were not discernible in *Alk5*$^{\Delta/KO}$ lungs (see Fig. S1 in the supplementary material).

Histologically, the Hematoxylin and Eosin (H/E) stained sections of lungs from E15.5 *Alk5*$^{\Delta/KO}$ embryos showed enlarged airways compared with the control lungs (Fig. 2A,D). Closer inspection of the lung sections revealed abnormalities in the epithelial layers in both proximal (Fig. 2B,E) and distal (Fig. 2C,F) airways. Compared with the highly organized arrangement of the epithelial cells forming the distal airways in the control lungs (Fig. 2C), the mutant airways appeared to be composed of highly disorganized cells (Fig. 2E,F, arrows). In wild-type E18.5 embryos, maturation of the lung is associated with the thinning of the saccular walls, as seen in Fig. 2G. Absence of epithelial ALK5 activity blocked or impeded late lung development as the mutant lungs appeared to be more cellular and contain immature alveoli and reduced airspace in distal airways (Fig. 2J). As at the earlier time point, the E18.5 mutant lungs were also characterized by airways comprising a disorganized and multi-layered epithelium (Fig. 2K,L, arrows).

Reduced Clara cell numbers in *Alk5* $^{/KO}$ 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

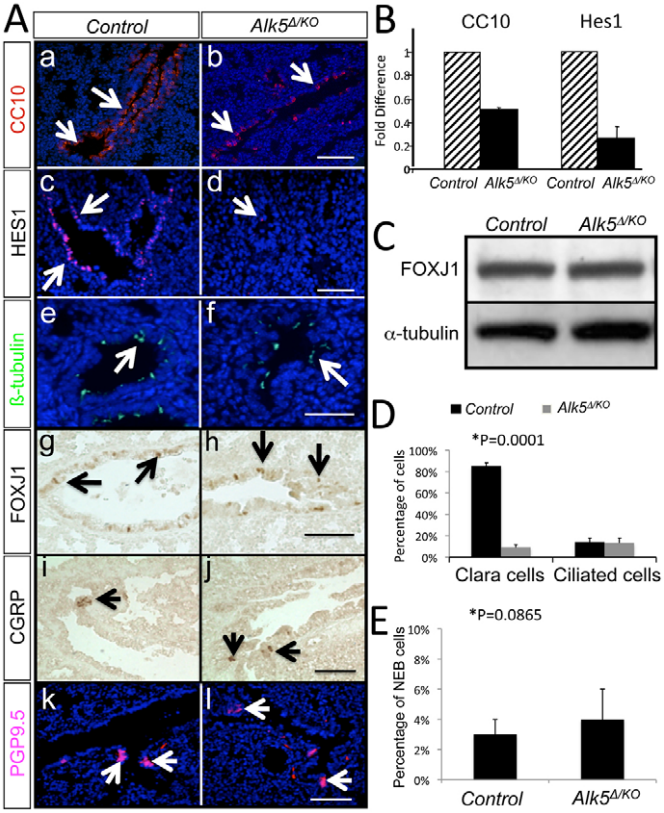

Fig. 3. Reduced Clara cells and their putative progenitors in *Alk5*$^{/KO}$ lungs. (**Aa-l**) Immunolocalization of Clara cells and their putative progenitors by anti-CC10 (a,b) and anti-HES1 antibodies (c,d). Ciliated cells were identified by anti-β-tubulin (e,f) or anti-FOXJ1 (g,h) antibodies. Pulmonary neuroendocrine cells were identified by anti-CGRP (i,j) or anti-PGP9.5 (k,l) antibodies in *Alk5*$^{\Delta/KO}$ and control lungs. Arrows indicate the presence of immunoreactivity with each antibody. (**B**) Real-time PCR analysis of *CC10* and *Hes1* mRNAs in E18.5 lungs. Control values are normalized to unity. (**C**) Western blot analysis of FOXJ1. α-tubulin was used as control. (**D**) Relative ratio of Clara and ciliated cell numbers in the airway epithelium. The numbers of Clara, ciliated and total epithelial cells were determined and plotted as the percent of total epithelial cell numbers. (**E**) The number of neuroendocrine cells was determined and plotted as the percent of total epithelial cell number. Scale bars: 50 μm.

used as Clara and ciliated cell markers, respectively. In E18.5 *Alk5*$^{\Delta/KO}$ lungs, Clara cell numbers were significantly reduced compared with the numbers in control airways (Fig. 3Ab,a, respectively). Reduction in Clara cell numbers was further validated by examining the expression of CYP2F2 and surfactant protein SP-A, both of which were also reduced (see Fig. S2 in the supplementary material).

During fetal lung development, hairy/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*$^{\Delta/KO}$ lungs (Fig. 3Ac,d). Real-time PCR analysis of *CC10* and *Hes1* mRNA validated the latter findings (Fig. 3B). By contrast to Clara cells, the number of ciliated cells in the mutant lungs appeared unchanged (Fig. 3Ae,f). The transcription factor FOXJ1, otherwise known as hepatocyte nuclear factor 3/forkhead homologue 4 (*Hfh4*), 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*$^{\Delta/KO}$ and control airways, indicating no changes in the distribution or number of ciliated cells (Fig. 3Ag,h,C). 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*$^{\Delta/KO}$ airways (Fig. 3D). In *Alk5*$^{\Delta/KO}$ airways, a mere 9.4% of the total epithelial cells were positive for CC10, compared with 85% in the control lungs. Ciliated cells accounted for 14% of the total number of epithelial cells in both *Alk5*$^{\Delta/KO}$ and control airways.

Another highly specialized cell population in the mouse airway epithelium is that of 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 (Fig. 3Ai-l,E). 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 *Alk5*$^{\Delta/KO}$ lungs showed that they are immature in comparison to age-matched controls (Fig. 2). 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 (Fig. 4A). SSEA1-positive cells were hardly found in adult lungs (Fig. 4B). 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 *Alk5*$^{\Delta/KO}$ lungs (Fig. 4Ca,b). Interestingly, there was a significant pool of positive cells for SSEA1 in E18.5 *Alk5*$^{\Delta/KO}$ lungs compared with age-matched controls (Fig. 4Cc-f). Using the anti-SSEA1 antibody, three groups of cells could be distinguished in E18.5 control lungs; SSEA1-positive progenitor cells (Fig. 4Cc, thin arrow); SSEA1- and CC10-positive differentiating Clara cells (Fig. 4Cc, arrowhead), and CC10-positive terminally differentiated Clara cells (Fig. 4Cc, thick arrow). A gradient of fewer SSEA1-positive cells and more CC10-positive cells was localized along the proximal-to-distal axis of the airways in the wild-type lung (Fig. 4Cc,e). The latter cells probably represent quiescent undifferentiated progenitors of airway epithelial cells as double-immunofluorescent histochemistry showed an absence of apoptosis and either ciliated or neuroendocrine cell markers (see Fig. S3 in the supplementary material).

The specialized epithelial cell types populating the distal airways and the alveoli begin to emerge during the terminal sac stage of lung development (Cardoso and 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

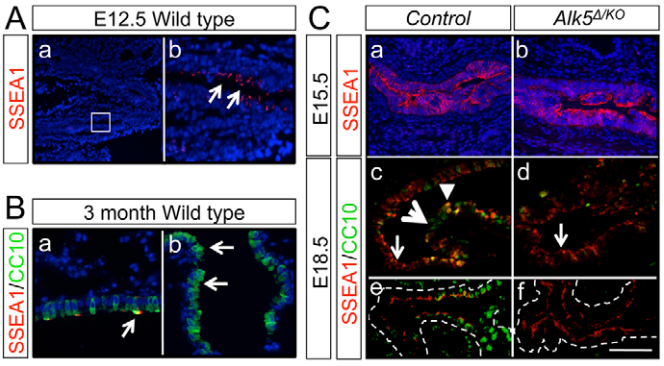

Fig. 4. Expression pattern of SSEA1 in *Alk5* $^{/KO}$ and control lungs. (**Aa,b**) Immunolocalization of SSEA1 in E12.5 wild-type lungs. The image in b is a higher magnification of the image in a. Arrows point to SSEA1-positive cells. (**Ba,b**) SSEA1/CC10 double immunofluorescence in 3-month-old wild-type lungs. Note that there are few double-positive cells in a and b (arrow). (**Ca-f**) SSEA1 single (a,b) and SSEA1/CC10 double (c-f) immunofluorescence in E15.5 (a,b) and E18.5 (c-f) control (a,c,e) and *Alk5*$^{\Delta/KO}$ (b,d,f) lungs. SSEA1-positive cells (red, thin arrow), CC10-positive cells (green, thick arrow) and SSEA1-positive, CC10-positive cells (yellow, arrowhead) are shown. Proximal airways are shown in c and d. The interrupted line indicates the basement membrane of distal airways (e,f). Scale bar: 50 μm.

surfactant proteins-B (SP-B) and -C (SP-C), showed no significant differences between *Alk5*$^{\Delta/KO}$ and the control lungs in E18.5 embryos (see Fig. S4 in the supplementary material). 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 *Alk5*$^{\Delta/KO}$ and control lungs. 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 *Alk5*$^{\Delta/KO}$ and control lungs. Therefore, epithelial deletion of ALK5 affects bronchiolar epithelial progenitor cell differentiation but spares alveolar epithelial cell differentiation. There were no significant differences observed in cell proliferation and survival between mutant and control lungs (see Fig. S4 in the supplementary material).

TGFβ activation of *Hes1*: the role of *Alk5*

The in vivo observations in *Alk5*$^{\Delta/KO}$ lungs suggest that TGFβ signaling via ALK5 is necessary for the emergence of Clara cells during lung development. Two possibilities were considered. First, TGFβ signaling via ALK5 might directly stimulate *CC10* gene expression in Clara cell progenitor cells. Alternatively, the pathway that leads to Clara cell differentiation could be the target of *Alk5*-mediated TGFβ signaling. To examine these possibilities, we used in vitro experiments employing the MLE15 mouse epithelial cell line, which exhibits a baseline low level *CC10* expression. Treatment of MLE15 cells with or without transfected *CC10* promoter-luciferase plasmids, with various doses of TGFβ1, had no discernible impact on either endogenous *CC10* mRNA levels or luciferase activity, suggesting that TGFβ1 alone does not directly stimulate *CC10* gene expression (see Fig. S5 in the supplementary material).

To examine the alternative possibility, we examined the effect of TGFβ 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 TGFβ1. To verify TGFβ1 bioactivity (positive control), we used a PAI1 promoter-luciferase construct (*pRK5-PAI*). PAI promoter activity increased more than 7-fold in response to TGFβ1 (Fig. 5A). Similarly, TGFβ1 treatment stimulated luciferase activity 2-fold in MLE15 cells transfected with the *Hes1* promoter-luciferase construct (Fig. 5A). The latter findings were validated by examining the response of the MLE15 endogenous *Hes1* gene to TGFβ1 treatment. MLE15 cells were treated either with TGFβ1 or its carrier, DMSO, and steady-state levels of *Hes1* mRNA were determined by real-time PCR. When compared with DMSO control, TGFβ1 increased *Hes1* mRNA 2.5-

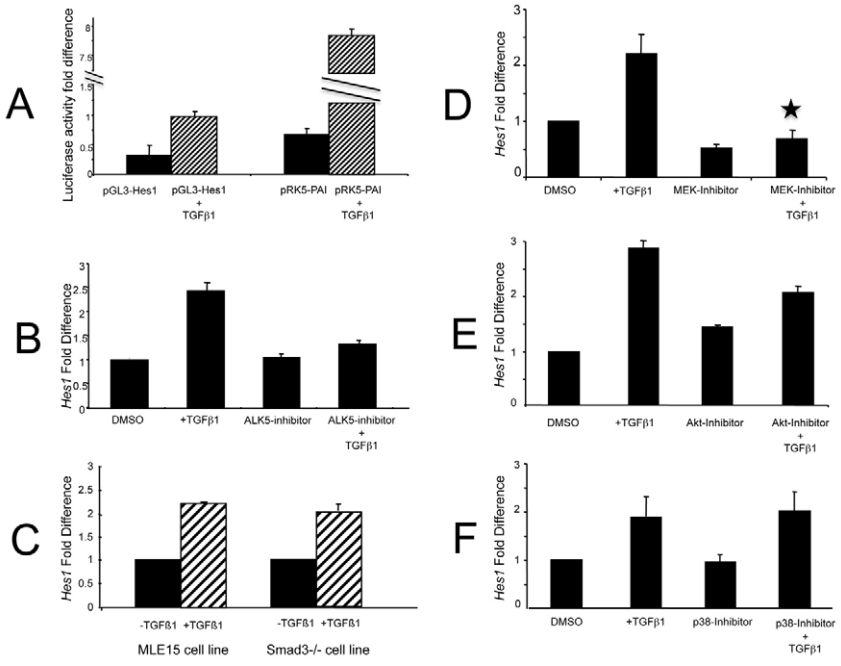

Fig. 5. Regulation of *Hes1* by TGFβ1 and its downstream signaling. (**A**) The mouse *Hes1*-promoter-luciferase construct, *pGL3-Hes1*, was transfected into MLE15 cells, which were then treated with DMSO or TGFβ1 for 48 hours. Normalized luciferase values are shown. The PAI1-promoter-luciferase construct, *pRK5-PAI*, was used as a positive control. (**B**) MLE15 cells were treated with DMSO (control), TGFβ1, ALK5-inhibior (SB525334) or TGFβ1 plus ALK5-inhibitor. After 48 hours, *Hes1* mRNA was analyzed by real-time PCR. (**C**) MLE15 and *Smad3*$^{-/-}$ cells were treated with TGFβ1. *Hes1* mRNA was analyzed by real-time PCR. (**D-F**) Real-time PCR was used to analyze *Hes1* induction by TGFβ1 in MLE15 cells in the presence or absence of one of the following: U0126, a MEK inhibitor (D); LY294002, a PI3K inhibitor (E); or SB203580, a p38 MAPK inhibitor (F). DMSO was used as TGFβ1 control and set to unity for comparison. The star denotes statistical significance.

fold (Fig. 5B). In addition, the TGFβ1-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 TGFβ1 induction of *Hes1* mRNA (Fig. 5B). These findings demonstrate that the endogenous *Hes1* gene in lung epithelial cells can be stimulated by TGFβ1 signaling via ALK5. It should be noted that, although *Hes1* was induced by TGFβ/*Alk5*, we observed no changes in *CC10* gene or promoter activity (please see above). Thus, at least in MLE15 cells, induction of *Hes1* alone is insufficient to stimulate *CC10* gene expression.

TGFβ activation of *Hes1*: downstream of receptor signaling

Signaling through the TGFβ 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 TGFβ1 regulation of *Hes1* mRNA. *Smad3*$^{-/-}$ and MLE15 (control) cells were treated either with TGFβ1 or DMSO and steady-state levels of *Hes1* mRNA were determined by real-time PCR (Fig. 5C). An absence of *Smad3* had little to no impact on TGFβ1-induced expression of *Hes1* (Fig. 5C).

Intracellular TGFβ signal transduction can be mediated by a number of so-called non-Smad-dependent pathways (Zhang, 2009). Blocking the MEK/ERK pathway by a specific inhibitor, U0126 blocked TGFβ1 induction of *Hes1* mRNA (Fig. 5D). Another inhibitor, LY-294002, which blocks *Akt* activity, caused only partial interference with the impact of TGFβ1 on *Hes1* mRNA, whereas SB 203580, a p38 inhibitor, had no effect (Fig. 5E,F). Further support for the involvement of the MEK/ERK pathway was provided by western blot analysis with an anti-phospho-ERK antibody. In MLE15 cells, ERK phosphorylation was increased by TGFβ1 treatment (Fig. 6Ab) and this response was blocked by the ALK5 inhibitor SB-52334 (Fig. 6Ac). Consistent with these in vitro results, western blot analysis and quantification showed decreased phospho-ERK in E18.5 *Alk5*$^{\Delta/KO}$ lungs (Fig. 6B). These data clearly demonstrate that the MEK/ERK pathway, and to a lesser extent, the AKT pathway, serve as the intracellular transducers of TGFβ signaling-induced *Hes1* gene regulation.

TGFβ activation of *Hes1*: the role of *Pten*

Pten is a negative regulator of the MEK/ERK cascade (Gu et al., 1999). *Pten* is also negatively regulated by TGFβ1 via Smad-independent signaling (Chow et al., 2008). In similar experiments to those described above, we used MLE15 cells and assayed for expression of a number of genes in the presence and absence of TGFβ1 and SB-52334, an ALK5-specific inhibitor. Treatment of MLE15 cells with TGFβ1 reduced the steady-state level of *Pten* protein (Fig. 6Ab). This effect of TGFβ1 was mediated via ALK5 as it was blocked by SB-52334 (Fig. 6Ac). No changes were observed in FOXJ1 and PGP9.5 (Fig. 6A). These observations raised the possibility of involvement of *Pten* in TGFβ-induced *Hes1* gene regulation. Consistent with this hypothesis, real-time PCR analysis showed significantly increased *Pten* mRNA in E18.5 *Alk5*$^{\Delta/KO}$ lungs compared with controls (Fig. 6C). We reasoned that if the impact of TGFβ on Clara cell lineage determination is mediated through *Pten*, it can be predicted that *Pten* deletion in lung epithelial cells might increase the number of Clara cells or their progenitors during lung morphogenesis in vivo. To investigate this possibility, we generated mice carrying a 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

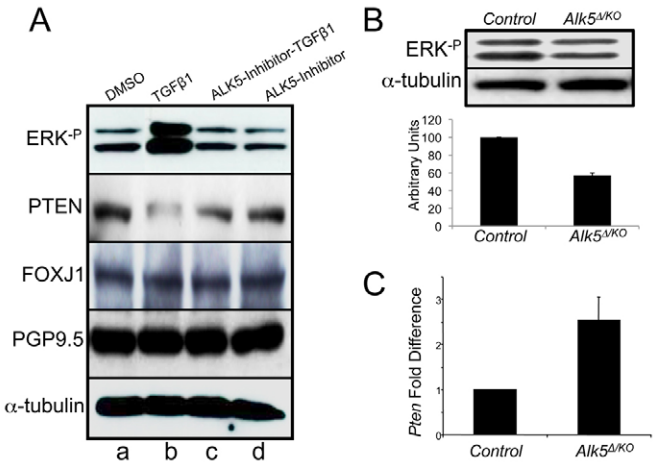

Fig. 6. *Alk5* signaling controls ERK phosphorylation and *Pten* expression. (**A**) MLE15 cells were treated with DMSO (control), TGFβ1, ALK5 inhibitor or ALK5 inhibitor plus TGFβ1. Western blot analysis was used to assess the steady-state levels of phosphorylated ERK, PTEN, FOXJ1, PGP9.5 and α-tubulin (loading control). (**B**) Western blot analysis and quantification of phosphorylated ERK in lung tissue from E18.5 control and *Alk5*$^{\Delta/KO}$ embryos. α-tubulin was used as a loading control. (**C**) Real-time PCR measurement of *Pten* mRNA in lung tissue from E18.5 control and *Alk5*$^{\Delta/KO}$ embryos.

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 *Pten*$^{\Delta/\Delta}$ and control embryonic lungs revealed a significantly increased number of Clara cells in *Pten*$^{\Delta/\Delta}$ lungs (Fig. 7Aa,b,e,f). Significantly, and consistent with the findings in *Alk5*$^{\Delta/KO}$ lungs, differentiation of ciliated cells remained intact (Fig. 7Ac,d; see Fig. S6 in the supplementary material). Real-time PCR analysis showed increased *CC10* and *Hes1* mRNA in *Pten*$^{\Delta/\Delta}$ lungs (Fig. 7B). Interestingly, ERK phosphorylation was significantly increased owing to an absence of *Pten* activity in *Pten*$^{\Delta/\Delta}$ lungs (Fig. 7C). Meanwhile, fewer SSEA1-positive cells were found in *Pten*$^{\Delta/\Delta}$ lungs (see Fig. S6 in the supplementary material). 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 the *CMV-Pten* plasmid and efficiency of transfection (Fig. 7E). Endogenous *Hes1* mRNA and phospho-ERK levels were examined by real-time PCR and western blot, respectively. Compared with the *CMV-vector* transfection (control), *CMV-Pten* reduced *Hes1* mRNA levels (Fig. 7E) concomitant with blocking ERK phosphorylation (Fig. 7D). Thus, both in vitro and in vivo data are consistent with a role for *Pten* in regulating Clara cell lineage determination mediated by the TGFβ/*Alk5* pathway.

DISCUSSION

The purpose of the current study was to determine the precise role of endodermal-specific TGFβ 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 cell varieties. Endodermal deletion of *Alk5* resulted in abnormal lung morphogenesis, characterized by enlarged airways in E15.5 lungs. As development progressed, this

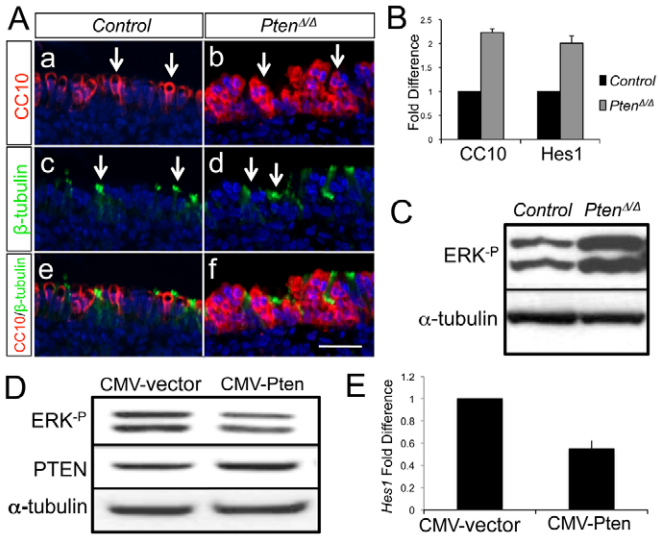

Fig. 7. Conditional deletion of *Pten* increases the Clara cell population in E18.5 lung; *Pten* negatively regulates *Hes1* expression and ERK phosphorylation in MLE15 cells. Lung epithelial deletion of *Pten* was accomplished by using an *Nkx2.1-Cre* driver line as described in the Materials and methods. (**Aa-f**) Immunolocalization for CC10 in control and $Pten^{\Delta/\Delta}$ lungs (a and b, respectively, arrows). Immunolocalization of β-tubulin in control and $Pten^{\Delta/\Delta}$ lungs (c and d, respectively, arrows). CC10/β-tubulin merge (e,f). (**B**) Real-time PCR analysis of *CC10* and *Hes1* mRNA in $Pten^{\Delta/\Delta}$ and control lungs. (**C**) ERK phosphorylation in $Pten^{\Delta/\Delta}$ and control lungs was determined by western blot. α-tubulin was used as the control. (**D**) MLE15 cells were transfected with either CMV-*Pten* or CMV-vector (control) plasmid. *Hes1* mRNA was measured by real-time PCR. (**E**) PTEN protein and ERK phosphorylation were examined in the same MLE15 cells as in D by western blot analysis. α-tubulin was used as the control. Scale bar: 30 μm.

phenotype evolved into immature alveoli and formation of a 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. By contrast, the 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, either the three epithelial cell types might originate from independent progenitor/precursor cells or that distinct pathways are involved in their differentiation or expansion.

Prior to the current report, it was generally 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 was 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 (Reynolds 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 2-4 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, but did not entirely eliminate, the Clara cell numbers in association with reduced *Hes1*. Analysis of embryonic lungs from $Hes1^{-/-}$ embryos also showed significant reduction of approximately 50% in the number of Clara cells (Ito et al., 2000). Comparison between the two studies suggests that abrogation of TGFβ/ALK5 signaling has a more profound and encompassing impact on epithelial cell differentiation compared with deletion of *Hes1* alone, as this gene is only one of perhaps many direct downstream targets of the TGFβ/ALK5 signaling pathway. *Hes1* mutants also exhibit changes in neuroendocrine cell populations which were not found in $Alk5^{\Delta/KO}$ lungs. Thus, *Hes1* does not simply act to mediate part of *Alk5* function. Instead, *Hes1* and *Alk5* have overlapping, but also distinct, functions. Furthermore, the observation that $Alk5^{\Delta/KO}$ lungs are not entirely devoid of Clara cells could suggest a number of potential interpretations. For example, 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 Fig. 1 where *lacZ* was uniformly expressed in what appears to be the entire epithelium. Alternatively, TGFβ signaling via ALK5 might be necessary for expansion rather than determination of Clara cell lineage. The finding that Hes1 is directly controlled by TGFβ via *Alk5* argues against the latter, but supports the former possibility, although this requires further validation. It is also possible, as suggested by a recent report, that the residual Clara cells in *Alk5* mutant lungs might be derived from other independent progenitors (Rock et al., 2009).

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, might either arise from different progenitor cells or, alternatively, differentiate via independent pathways. To investigate these possibilities, we examined the temporal and spatial pattern of SSEA1 expression, which had hitherto remained unknown, during lung development (Fig. 4). We found that the nascent airway epithelium comprised entirely what appears to be SSEA1-positive cells. Therefore, it is probable that SSEA1-positive cells serve as progenitors for all differentiated airway epithelial cells including Clara cells. Whether there are distinct sub-groups of cells within the SSEA1 population remains unknown. Given a model of a common SSEA1-positive progenitor cell population, each specialized cell type might 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, TGFβ1 signaling (Fig. 5). Thus, the role of TGFβ/ALK5 signaling might not be involved in regulation of ciliated cell differentiation.

TGFβ/ALK5-mediated signaling controls many processes during cell and tissue differentiation. Ligand-binding activates the TGFβ/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 TGFβ inhibition of cell differentiation and progenitor cell determination (Alliston et al., 2001). However, absence of Smad3 did not block the *Hes1* response to TGFβ1 treatment, indicating that the role of Smad3 in TGFβ1/ALK5-induced *Hes1* expression, and hence Clara cell differentiation, is at best limited. Although the role of TGFβ is probably not confined to differentiation of Clara cells alone, the role of *Alk5* appears to be specific, as epithelial deletion of the type II TGFβ receptor has been reported to have no impact on Clara cell differentiation (Chen et al., 2008).

TGFβ 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 TGFβ/ALK5-induced *Hes1* gene regulation in vitro. Blockade of ERK phosphorylation led to loss of *Hes1* response to TGFβ1 (Fig. 5D). *Hes1* induction by TGFβ1 was partially blocked also by treatment with an AKT inhibitor, suggesting a minor requirement for PI3/AKT activity (Fig. 5E). Recent findings in human neuroblastoma cells also contend that *Hes1* response is dependent on activation of ERK and AKT phosphorylation (Stockhausen et al., 2005). Consistent with our in vitro data, we found that deletion of ALK5 caused a 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 TGFβ 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 TGFβ1 in vitro (Fig. 6A,C). Moreover, *Pten* negatively regulates the phosphorylation of ERK in vivo (Fig. 7C) and in vitro (Fig. 7E). 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 (Fig. 8) whereby the impact of TGFβ via ALK5 on Clara cell ontogeny in the lung is mediated through regulation of *Pten* and phospho-ERK. A number of observations support the validity of this hypothetical model. First, TGFβ modulation of *Pten*, as the effect of TGFβ on *Hes1* occurs independently of Smad signaling (Chow et al., 2008). Second, and as we found in this study (Fig. 7), *Pten* is known to inhibit activation of the MEK/ERK pathway (Gu et al., 1998). TGFβ/ALK5 regulation of the Clara cell progenitor marker *Hes1* also involves MEK/ERK activation (Fig. 5). In addition, *Pten* is a negative regulator of AKT phosphorylation (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 (Fig. 7). In summary, the results of this study suggest that, during mouse lung development, TGFβ signaling via *Alk5* through a pathway that involves *Pten*-regulated ERK and AKT activation might be required for Clara cell ontogeny but not those of ciliated or pulmonary neuroendocrine cells.

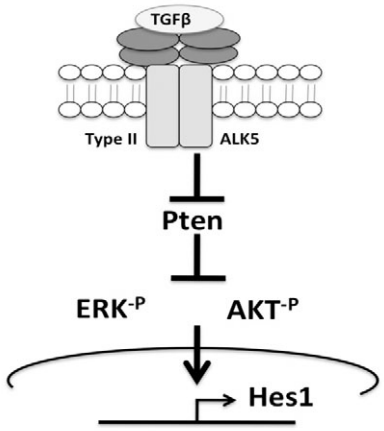

Fig. 8. A simplified model illustrating TGF-β/ALK5 signaling in lung airway cell differentiation. TGF-β signaling via ALK5 negatively regulates Pten expression, which in turn negatively regulates phosphorylated ERK and phosphorylated AKT. Phosphorylated ERK and AKT activate Hes1 expression. TGF-β/Alk5 signaling does not affect ciliated cell nor pulmonary neuroendocrine cell differentiation.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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