Mesodermal ALK5 controls lung myofibroblast versus lipofibroblast cell fate

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Abstract

Background: Epithelial-mesenchymal cross talk is centerpiece in the development of many branched organs, including the lungs. The embryonic lung mesoderm provides instructional information not only for lung architectural development, but also for patterning, commitment and differentiation of its many highly specialized cell types. The mesoderm also serves as a reservoir of progenitors for generation of differentiated mesenchymal cell types that include αSMA-expressing fibroblasts, lipofibroblasts, endothelial cells and others. Transforming Growth Factor β (TGFβ) is a key signaling pathway in epithelial-mesenchymal cross talk. Using a cre-loxP approach we have elucidated the role of the TGFβ type I receptor tyrosine kinase, ALKS, in epithelial-mesenchymal cross talk during lung morphogenesis.

Results: Targeted early inactivation of Alk5 in mesodermal progenitors caused abnormal development and maturation of the lung that included reduced physical size of the sub-mesothelial mesoderm, an established source of specific mesodermal progenitors. Abrogation of mesodermal ALK5-mediated signaling also inhibited differentiation of cell populations in the epithelial and endothelial lineages. Importantly, Alk5 mutant lungs contained a reduced number of αSMApos cells and correspondingly increased lipofibroblasts. Elucidation of the underlying mechanisms revealed that through direct and indirect modulation of target signaling pathways and transcription factors, including PDGFRα, PPARγ, PRRX1, and ZFP423, ALKS-mediated TGFβ controls a process that regulates the commitment and differentiation of αSMApos versus lipofibroblast cell populations during lung development.

Conclusion: ALK5-mediated TGFβ signaling controls an early pathway that regulates the commitment and differentiation of αSMApos versus LIF cell lineages during lung development.

Keywords: Lipofibroblast, Lung development, Mesoderm, Myofibroblast, Pdgfra, Pparβ, Zfp423

Background

In mammals, the anterior foregut invades the surrounding splanchnic mesoderm to form the primordial lung. During embryogenesis, this bilayered structure undergoes a series of highly orchestrated morphogenetic steps to form a complex respiratory organ purported to include over 40 specialized cell varieties. Elucidating the mechanisms that govern commitment and differentiation of these cell types has been a major challenge. The bulk of the cell types studied has been of endodermal origin. Much less is known about the regulatory mechanisms that control the ontogeny and differentiation of the mesodermally-derived cell varieties.

In very broad terms, the lung mesenchyme can be divided into two histologically distinct cell populations that are detectable as early as E13.5 [1]. The 'sub-epithelial mesenchymal' cells wrap around the epithelial ducts, while the 'sub-mesothelial mesenchymal' (SMM) cells inhabit the area between the mesothelium and the subepithelial mesenchymal cells [1, 2]. SMM is a major site of Fgf10 expression. We and others have shown that Fgf10pos cells contribute, but are not the sole source of smooth muscle (SM) cells and lipofibroblasts (LIFs) [2, 3]. Viewed from the perspective of gene expression, mesodermal derivatives can be simply grouped into two molecularly defined cell populations; the αSMApos and αSMAneg groups. The primary αSMApos group comprises the fibroblasts in the peribronchial (airway) and perivascular SM...
layers as well as interstitial αSMApos myofibroblasts. Notably, the latter cells attain αSMApos status at different times during lung development. While airway and perivascular SM cells are αSMApos as early as E11.5, interstitial fibroblasts begin to display αSMA only in mid to late gestation. With that caveat in mind, in the present study we have opted to use the term ‘αSMApos cells’ in a ‘broad stroke’ to conveniently refer collectively to all cells that express this marker and not solely the ‘interstitial myofibroblasts’ noted routinely by other investigators.

Generation of mesodermal cell diversity occurs concurrently with the structural development of the lung. A central player is the reciprocal communication known as epithelial-mesenchymal interactions that occur between the foregut endoderm and the lateral plate mesoderm-derived splanchnic mesenchyme. This process works on a ‘signaling, transcription factors, signaling’ algorithm [4–6]. Additional integral components include the extracellular matrix, structural proteins and differentiation-specific proteins. A major signaling pathway in the lung and other mammalian organs is the transforming growth factor beta (TGFβ) family of secreted polypeptides.

The significance of TGFβ signaling during development and disease can be hardly overstated. TGFβ is the prototype of a family of secreted dimeric peptide growth factors that includes the TGFβs, activins, inhibins, and bone morphogenetic proteins [7]. In vertebrates, TGFβ regulates key processes in stem cell maintenance, organogenesis, wound healing, and homeostasis. Given this broad range of activity, it is not surprising that TGFβ dysregulation results in a spectrum of pathologies ranging from cancer to pulmonary fibrosis.

The TGFβ machinery has many ‘moving parts’. The ligands are produced as ‘latent’ peptides. Upon secretion and activation, all three TGFβs signal by engaging a specific receptor, composed of two related transmembrane serine/threonine kinases, called the type I and type II TGFβ receptors (TβR1, or ALK5 and TβR2) [8]. The basic mechanism of receptor activation involves binding of the ligand to TβR2, followed by recruitment of ALK5. Recruitment triggers ALK5 kinase activity, transducing the signal by phosphorylating and activating members of the SMAD family of transcription factors [8].

In various tissues, TGFβ response is remarkably cell type and context dependent. It is equally true that the downstream effects of TGFβ are transduced not simply via a single target, but may involve multiple nodes. Given the multicomponent nature of the pathway, there is a wide spectrum of versatility and selectivity in TGFβ biologic functions. Some are ostensibly paradoxical. For example, TGFβ acts as both a cancer promoter and suppressor [9]. Selective utilization of receptors is a potential mechanism for generating versatility in TGFβ function. TGFβ expression is thought to be ubiquitous throughout the lung with both endodermal and mesodermal cells displaying ALK5 and TβR2. Elucidating the function of each receptor in specific cell types and in particular in endodermal versus mesodermal cells of the lung is a necessary step towards unlocking the precise roles of TGFβ in development and disease. Attempts to define the specific role played by each of the receptors using targeted germline deletions of either TβR2 or ALK5 have not been fully successful due to early embryonic lethality [10, 11]. Using conditional inactivation, we have shown that lack of mesodermal-specific TβR2 causes embryonic lethality, while endodermal TβR2 inactivation is not only tolerated and viable, but protects against experimentally induced fibrosis and bronchopulmonary dysplasia [12, 13]. A systematic approach involving abrogation of each receptor on a cell-type specific basis has the promise of revealing the precise roles of each receptor during embryonic development.

In this study, Alk5 was specifically inactivated in the early embryonic multipotent mesoderm that is the origin of lung mesodermal progenitors. Alk5Dermo1flox/flox mice displayed multiple abnormalities and postnatal lethality. Their lungs were structurally immature (pulmonary hypoplasia), characterized by thickened mesenchymal walls around significantly reduced alveolar spaces. Importantly, the studies revealed a novel role for ALK5-mediated TGFβ signaling that regulates the balance between αSMApos and LIF cell fate commitment and differentiation during lung development. The precise balance between these key cell populations is critical to normal lung development and its disruption underlies the pathobiology of serious pulmonary disorders.

Results

Mesodermal-specific Alk5 inactivation

The localization and efficiency of LoxP-dependent excision in Dermo1-cre (aka, Twist2-cre) mouse lungs has been previously reported [14, 15]. Dermo1-driven, Cre-mediated recombination occurs early and exclusively throughout the tracheal and pulmonary mesoderm, but not in the epithelium (Additional file 1A–C). Accordingly, we generated triple transgenic Dermo1-cre;Alk5flox/flox;mTmG mice (Methods) in which exon 3 of the Alk5 gene is excised in early pulmonary mesodermal progenitors (Additional file 1D). Over 800 fetuses at various embryonic stages were genotyped and analyzed (Additional file 1E).

Heterozygous Alk5flox/wt;Dermo1-Cre;mTmG mice were born alive and had no discernable abnormalities in development, growth, or reproduction. Homozygous Alk5flox/flox;Dermo1-Cre;mTmG (hereafter Alk5Dermo1) embryos examined at E11.5 showed no obvious morphological defects. Defects in body-wall closure in Alk5Dermo1 embryos, as previously reported [16], were
evident at E12.5 (data not shown). Embryonic lethality occurred between E14.5 and E18.5. Occasionally, mutant embryos with a less severe phenotype survived to birth but died immediately afterwards.

To confirm deletion of Alk5 from the pulmonary mesoderm, expression of ALKS, phospho-SMAD2 (p-SMAD2), and PAI-1 were assessed by immunoblotting. The latter is expressed by mesenchymal cells in response to canonical TGFβ signaling. Compared to controls, ALK5 was decreased by nearly 85% (0.15 ± 0.013), p-SMAD2 by 73% (0.23 ± 0.02), and PAI-1 expression reduced by 83% (0.17 ± 0.05) (Additional file 1F). Immunohistochemistry (IHC) for ALK5 and PAI-1 on E15.5 lung sections confirmed decreased mesenchymal and intact epithelial ALK5 immunoreactivity (Additional file 1G–I). While these results confirm abrogation of canonical TGFβ signaling, the source of the residual activity can be certainly attributed to intact signaling in the lung epithelium.

Lung immaturity and blocked epithelial and endothelial cell differentiation in Alk5Dermo1 lungs

The lungs of E18.5, Alk5Dermo1 embryos were smaller and poorly expanded, but the number of lobes appeared normal (Additional file 2A–D and data not shown). The alveolar diameters were decreased and septal thicknesses increased relative to control lungs (Additional file 2E–I). Glycogen stores, which are an epithelial marker of immature lungs [17], were also increased in Alk5Dermo1 lungs (Additional file 2G,J). IHC using antibodies against acetylated tubulin, CC10, pro-SPC, and T1a (specific to ciliated cells, Clara cells, alveolar epithelial type II cells, and alveolar epithelial type 1 cells, respectively) showed reduced differentiation of all four major epithelial cell types in E18.5 Alk5Dermo1 lungs (Additional file 3A–H). These findings were also examined by quantitative PCR (Q-PCR) (Additional file 3I). Cumulatively, these results suggest that lung maturation and epithelial cell differentiation are delayed by mesenchymal Alk5 inactivation.

The lung mesoderm serves as the origin of endothelial cells that form the pulmonary vasculature. Flk1, expressed by vascular endothelial progenitors, was reduced in E13.5 Alk5Dermo1 lungs as was the fraction of CD34pos cells (Additional file 3J–M). As lung development progressed, PECAM1, a vascular endothelial differentiation marker, was also reduced. The distal capillary plexus adjacent to the airway epithelium was less dense compared to age-matched controls (Additional file 3N–Q). However, the large blood vessels were intact (Additional file 3P, Arrows). Q-PCR of multiple vascular markers showed that in Alk5Dermo1 lungs, Pecam-1 mRNA was 0.58 ± 0.19 (P < 0.05), Flk1 was 0.63 ± 0.18 (P < 0.05), and Flt-1 was 0.67 ± 0.15 (P < 0.05) of controls (Additional file 3R). However, mutant and control lungs had similar Flt-4 (0.83 ± 0.23, P > 0.05) and Vegfa (0.78 ± 0.23, P > 0.05) mRNAs.

Mesodermal-specific Alk5 inactivation reduces the sub-mesothelial mesenchyme

Structurally, the SMM [15] of Alk5Dermo1 lungs was smaller and more irregularly shaped compared to control lungs (Fig. 1a–e and Additional file 4). Anti-phospho-histone H3 labeling revealed fewer mitotic cells within the distal mesenchyme of mutant lungs (2.02 % vs. 0.6 %). The SMM regulates epithelial, smooth muscle, and vascular development by producing factors such as FGF10 [18]. FGF10 mRNA in mutant lungs was 0.63 ± 0.02 of controls (Fig. 1g). In addition, Fgf9 made by the mesothelium/epithelium and Fgf7 expressed by the mesenchyme were respectively expressed at 0.84 ± 0.04 and 0.58 ± 0.05 of controls (Fig. 1g). To assess the functional significance of the reduced Fgf10 and Fgf9 mRNAs, we examined expression of their target genes. Immunoblotting revealed decreased SPRY2 and SPRY4 in Alk5Dermo1 lungs relative to controls, confirming functionally reduced FGF10 signaling (Fig. 1h).

Similarly, there was profound decrease in the homeodomain transcription factor PITX2, a major target of FGF9 (Fig. 1h). These data suggest that mesodermal Alk5 inactivation reduces SMM volume by decreasing overall functional FGF signaling.

Inhibition of airway smooth muscle development

FGF10pos cells contribute to differentiated peribronchial smooth muscle (PBSM) cells [3]. By IHC, layers of αSMApos PBSM were thinner in diameter and included large gaps in E18.5 mutant lungs (Fig. 2a–h). However, αSMA expression around large blood vessels was similar in mutant and control lungs (Fig. 2i, Arrowheads). Consistent with the IHC, Q-PCR showed reduced mRNA for multiple myofibroblast markers in Alk5Dermo1 lungs (Fig. 2i). These included αSMA (0.56 ± 0.85), Calponin (0.67 ± 0.016), SM-MHC (0.63 ± 0.016), and SM22a (0.71 ± 0.18). In addition, there was reduced mRNA for both Pdgfra (0.45 ± 0.021) and Pdgfrβ (0.625 ± 0.073). Transcripts for NOGGIN, an airway smooth muscle cell marker [19, 20], were also decreased (Fig. 2j, 0.39 ± 0.1 of controls, P < 0.05) while those for Heyl, a vascular smooth muscle marker [20, 21], were only slightly reduced (Fig. 2j, 0.71 ± 0.078 of controls, P < 0.05). Thus, the diminished αSMApos cells in Alk5Dermo1 lungs were primarily lost from the PBSM layers. In addition, there was decreased mRNA for paired-homebox transcription factor PRRX1 (0.7 ± 0.056 of controls, P < 0.05) and the extracellular matrix glycoprotein TENASCIN-C (TNC; 0.53 ± 0.047 of controls, P < 0.01), both of which promote myofibroblast growth and differentiation (Fig. 2k). The effects of ALK5 abrogation on αSMApos...
cell commitment was also assessed by analysis of PDGFRα protein (Fig. 3a–f), which is regulated by TNC and highly expressed on αSMA pos cell precursors [22]. The fraction of PDGFRα pos cells surrounding the airway epithelium was significantly reduced in Alk5 Dermo1 lungs (Fig. 3d–f), and western blot analysis confirmed the decrease in αSMA and PDGFRα (Fig. 3g, i). Consistent with this observation, phosphorylation of AKT, the canonical substrate of PDGFA-activated PDGFRα [23–25], was reduced in both Alk5 Dermo1 lung homogenates (Fig. 3g) and isolated primary Alk5 Dermo1 mesenchymal cells (Fig. 3h). Thus, ALK5-mediated TGFβ signaling is required in lung αSMA pos cell development, and early defects in this pathway lead to decreased populations of cells expressing αSMA or PDGFRα.

**Lipofibroblast (LIF) hyperplasia in Alk5 Dermo1 lungs**

In addition to αSMA pos and endothelial cells, the multipotential lung mesoderm gives rise to LIFs. In the rat, LIFs are detected on embryonic day E16, increase during gestation, and decline after birth [26]. Oil Red O staining of Alk5 Dermo1 lungs revealed overwhelmingly abundant LIFs within the developing mesoderm, relative to controls (Fig. 4a–d and Additional file 5). To confirm that observation, Q-PCR was used to quantify the mRNA for multiple LIF markers, including adipocyte transcription factors PPARγ and CEBPα, the lipid droplet-associated Adipose Differentiation-Related Protein (ADRP), and adipocyte Fatty Acid Binding Protein 4 (FABP4). All were elevated in Alk5 Dermo1 lungs (Fig. 4e). Immunoblots of total Alk5 Dermo1 lung homogenates confirmed that ADRP, CEBPα, and PPARγ proteins were more abundant compared to controls. Furthermore, p-AKT and p-ERK were decreased while PTEN, a TGFβ-repressed phosphatase associated with LIF hyperplasia [27], was increased in Alk5 Dermo1 lungs (Fig. 4f). Importantly, Zfp423, which encodes a zinc finger transcription factor that regulates PPARγ in pre-adipocytes [28, 29], as well as WISP2, a matricellular protein that is highly expressed in adipocyte precursors [30, 31], were increased in Alk5 Dermo1 lungs (Fig. 4g). These findings indicate that abrogation of ALK5-mediated signaling increases LIF commitment and differentiation.

**Establishment of Alk5-deficient mesodermal cell lines**

The aggregate data presented above suggests that inactivation of Alk5 in multipotential lung mesoderm promotes LIF commitment and inhibits SM differentiation. To examine the underlying mechanisms, we isolated mesodermal cells from Alk5 Dermo1;mTmG and Alk5 flox/flox;mTmG mouse lungs. These Alk5−/− and Alk5+/− cells were immortalized by transfection with an SV40 plasmid and purified by FACS (Methods). TGFβ-induced SMAD2 phosphorylation was nearly absent in Alk5−/− cells, confirming abrogation of ALK5-mediated canonical TGFβ signaling (Fig. 5a).
Other mediators of TGFβ signaling were also affected. Baseline p-p38, p-AKT and p-ERK were high in Alk5+/+ control cells but TGFβ stimulation minimally induced p38 activation in mutant cells. TGFβ also had little impact on p-AKT in the mutant cells, but total AKT was reduced. Importantly, baseline ERK phosphorylation was elevated in Alk5–/– cells, and TGFβ induced a robust response in these cells compared to Alk5+/+ controls (Fig. 5a). These findings indicate the critical and complex role of ALK5 activity in the TGFβ signal transduction pathway.

ALK5-mediated TGFβ represses the LIF transcription factor ZFP423 in vitro

We also evaluated the impact of TGFβ on the isolated cells. Treatment with recombinant TGFβ (4 ng/mL for 48 hours, Materials & Methods) initiated myogenic differentiation as assessed by increased aSMA and Prx1 transcripts in Alk5+/+ but not Alk5–/– mesenchymal cells (Fig. 5b–f). In contrast, Tnc induction by TGFβ was unaffected by Alk5 inactivation, suggesting that its regulation is independent of ALK5 and the canonical TGFβ pathway. Conversely, Alk5–/– cells displayed a significantly high rate of spontaneous adipogenic differentiation in non-induced, minimum standard culture medium (Fig. 5g, h). This did not occur in Alk5+/+ controls, and was verified by assessing the expression of adipogenic markers. After 5 days in culture, lipogenic mediators, including Adrp, Cebpα and Pparγ mRNA, increased by 1.57 ± 0.34-, 3.02 ± 0.7-, and 3.57 ± 1.1-fold, respectively in the Alk5+/+ cells compared to 3.95 ± 0.68-, 4.4 ± 1.0-, and 8.58 ± 2.1-fold in the Alk5–/– cells (Fig. 5i). Likewise, Zfp423 mRNA was 11-fold higher and Wisp2 mRNA was 17-fold higher in Alk5–/– cells relative to Alk5+/+ cells (Fig. 5j). To determine the relationship between TGFβ and Zfp423, we treated Alk5+/+ and Alk5–/– cells with recombinant TGFβ ligand (4 ng/mL...
Fig. 3 Mesodermal Alk5 deficiency reduces smooth muscle precursors. (a–f) Immunohistochemistry showed reduced αSMA (green) and PDGFRα (red) in Alk5^{Dermo1} lungs throughout lung development. (g) Western blot analysis showed decreased αSMA, PDGFRα and p-AKT protein in E15.5 Alk5^{Dermo1} whole lung tissue. β-ACTIN was used as control. n = 3 separate lungs. (h) Western blot analysis showed decreased p-AKT protein in primary mesenchymal cells isolated from E15.5 Alk5^{Dermo1} lungs. n = 3 pairs of separate lungs; β-ACTIN was used as control. (i) Densitometric analysis of western blot results in (g). Error bars show standard deviation. *P < 0.05. Scale bar: f = 20 μm

Fig. 4 Lipofibroblast hyperplasia in Alk5^{Dermo1} lungs. (a–d) Oil Red O-stained cells are increased in E18.5 Alk5^{Dermo1} lungs. Dotted lines indicate basement membrane between epithelium and surrounding mesenchyme. (e) Quantitative PCR (Q-PCR) showed mRNA for LIF markers increased in E18.5 Alk5^{Dermo1} lungs. n = 3 pairs of independent lungs. Error bars show standard deviation. *P < 0.05. (f) Western blot analysis confirmed increased LIF markers, n = 6 pairs of lungs. Western blot analysis also showed decreased p-AKT and p-ERK, and increased PTEN. β-ACTIN was used as control. n = 3 pairs of lungs. (g) Q-PCR showed increased Zfp423 and Wisp2 mRNA in Alk5^{Dermo1} lungs compared to controls. n = 5–6 pairs of independent lungs. Error bars show standard error of the mean. *P < 0.05. Scale bar: d = 10 μm
for 48 hours, Materials & Methods). Controls were treated exactly the same with the exception of bovine serum albumin (BSA) replacing TGFβ. TGFβ repressed Zfp423 mRNA levels and this activity was dependent on ALK5 (Fig. 5k, l). Taken together, these data indicate a novel mechanism by which abrogation of ALK5-mediated canonical TGFβ signaling, through regulation of Zfp423, promotes LIF versus αSMApos cell differentiation in lung mesodermal cells.

The role of PDGFRα
As noted above, PDGFRα and signaling were decreased in Alk5Dermo1 lungs (Fig. 3). Although PDGFRαpos cells can differentiate into either αSMApos cells or LIFs, their role in determining mesodermal commitment has not been elucidated. We therefore sought to determine whether PDGFA signaling through PDGFRα is necessary for Alk5 to induce αSMApos cell differentiation. TGFβ is known to induce Pdgfra; however, its role in regulating Pdgfra remains unknown. Because PDGFRα is decreased in Alk5Dermo1 mutant lungs, its possible regulation by TGFβ was evaluated by TGFβ treatment (4 ng/mL, Materials & Methods) for 48 hours. Controls were treated exactly the same with the exception of BSA replacing TGFβ. TGFβ increased both Pdgfrα and Pdgfrβ mRNAs in Alk5+/+ cells but not in Alk5−/− cells (Fig. 6a) indicating that TGFβ regulation of Pdgfrα and, hence, αSMApos cell differentiation, requires ALK5 activity. Surprisingly, Pdgfrα mRNA increased during spontaneous differentiation of Alk5−/− cells to LIFs over a 5 day period (Fig. 6b). In addition, PDGFRα protein was consistently higher in Alk5−/− compared to Alk5+/+ cells on
both day 2 and day 5 of culture (Fig. 6c). These observations suggest the possibility of alternative or additional mechanisms that may regulate PDGFRα expression in these cells.

The need for signaling through PDGFRα during αSMApos cell differentiation was examined in vitro and in vivo. Application of the kinase inhibitor Imatinib to Alk5+/+ cells reduced the mRNA for known PDGFA targets Arid5b, Tiparp, and Schip1 (Fig. 6d), thereby validating its use. Importantly, Imatinib reduced the expression of myogenic markers (Fig. 6e) but, of the LIF markers, only Pparγ was strongly increased. Adrp was modestly increased by higher Imatinib doses (Fig. 6f). These results suggest that Pdgfra inactivation in mesodermal precursors prevents αSMApos cell differentiation but is insufficient to promote LIF commitment.

To confirm this finding in vivo, genetically engineered mice were generated in which Pdgfrα was inactivated in mesodermal tissues by Dermo1-cre. The resulting PdgfraRasfl/fl.Dermo1-cre mice (PdgfraDermo1) survived to birth at predicted Mendelian ratios but were 22% smaller than control littermates (1.45 ± 0.03 g vs. 1.14 ± 0.04 g, P < 0.001). PdgfraDermo1 mutant mice also developed spina bifida as reported in mice deficient in PDGFRα-stimulated phosphatidyl-inositol 3’ kinase activity [23]. IHC on E18.5 control lungs localized PDGFRα-expressing cells to the stroma and to areas surrounding the airways and blood vessels (Fig. 7a). These cells were absent or significantly reduced in PdgfraDermo1 lungs (Fig. 7b and Additional file 6). Q-PCR showed reduced mRNA for PDGFA targets, including Zfand5, Myo1e, Arid5b, Tiparp, and Schip1 [32], confirming functionally repressed PDGFA signaling in PdgfraDermo1 lungs.
αSMA-positive cell layers in mutant lungs were reproducibly thinner compared to controls, with gaps in αSMA expression (Fig. 7b, Arrows and Additional file 6). Q-PCR of E18.5 PdgfraDermo1 lungs showed decreased mRNA for multiple myofibroblast markers, including Prrx1 (0.45 ± 0.01 of control), SM22α (0.75 ± 0.02), and αSMA (0.79 ± 0.03) (Fig. 7d). Myofibroblast marker mRNAs were similarly decreased in E14.5 PdgfraDermo1 lungs (Additional file 7B). Similar analysis of adipogenic-related genes in PdgfraDermo1 lungs at E18.5 showed increased Pparγ mRNA but only modest increases in Adrp, Fabp4, and Cebpα (Fig. 7e). The balance between αSMA-positive cells and LIF phenotypes in PdgfraDermo1 lungs was less severely distorted than those in Alk5Dermo1 mice. In sum, these data support a model whereby ALK5-mediated TGFβ signaling regulates αSMA-positive versus LIF cell differentiation only partly through the PDGFA/PDGFRα pathway and that inhibition of this pathway is insufficient to promote LIF differentiation.

**Discussion and conclusions**

The present study provides genetic, histologic, and molecular evidence that mesodermal inactivation of Alk5 in the mouse lung restricts αSMA-positive cell fate and promotes LIF differentiation. In this context, ALK5 meets the definition of a molecular binary switch that functions in determination and subsequent specialization of at least two key lung mesodermal cell lineages.

An early morphological phenotype of Alk5Dermo1 embryonic lungs is the reduced physical size of the SMM. This was associated with decreased proliferation of the lung mesoderm and reduction in Fgf9. Fgf9 is expressed by both the mesothelium and the epithelial layers in the lung [1, 2]. Dermo1-cre is not known to be active in the mesothelium, although the origin of some mesothelial cells may be traced to the pulmonary mesoderm [33]. Whether reduced Fgf9 is due to direct impact of Alk5 inactivation or a result of mesoderm-mesothelial cross communication remains unknown. FGF9 signaling through FGFR1 and FGFR2 is both necessary and sufficient for SMM growth. Although Alk5 inactivation modestly decreased Fgf9, PITX2, a homeodomain transcription factor that is induced by FGF9, was strongly inhibited (Fig. 1k). SMM volume is profoundly sensitive to FGF9 signaling, being completely absent in Fgf9−/− lungs and enlarged in Fgf9 gain-of-function mutants [1]. The reduced SMM size and mitotic index indicate that ALK5 is required for either mesothelial Fgf9 expression or FGF9 responsiveness. Ligand-dependent TβRII signaling has been shown to regulate FGF9- and PITX2-mediated cell proliferation in the palatal mesenchyme [34]. Our observations suggest that ALK5 may similarly...
mediate TGFβ-mediated control of FGF9 signaling within the SMM.

The \(\text{Alk}5^{\text{Dermo1}}\) lungs also displayed defective epithelial differentiation. As Dermo1 is exclusively mesodermal, the abnormalities in epithelial cell differentiation presumably represent altered mesenchymal regulation of epithelial progenitors. Previous studies have suggested that FGF7, which is reduced in \(\text{Alk}5^{\text{Dermo1}}\) lungs, participates in mesenchymal-epithelial cross-talk [2]. Alternatively, TGFβ also regulates epithelial differentiation by altering the composition of the underlying matrix [35]. Finally, TUNEL\(^{\text{pos}}\) cells were found scattered in the parenchyma of late stage (i.e. E18.5) \(\text{Alk}5^{\text{Dermo1}}\) lungs (Additional file 8). Although these cells are not localized to the airways, the possibility that some of the changes in airway gene expression may be due to increased apoptosis cannot be presently ruled out.

The loss of \(\text{Alk}5\) in mesodermal progenitors also disrupted pulmonary vasculogenesis. Flk1, also known as vascular endothelial growth factor receptor 2, is expressed by endothelial progenitors [36] and was strongly decreased in E13.5 \(\text{Alk}5^{\text{Dermo1}}\) lungs. This likely accounts for the simplified vasculature observed in E18.5 mutant lungs. Since Dermo1 may not be expressed in endothelial progenitors [37, 38], it is unclear whether \(\text{Alk}5\) directly participates in vascular endothelial cell commitment. In addition, reduced Fgf9 in \(\text{Alk}5^{\text{Dermo1}}\) lungs might also contribute to the observed vascular phenotype. Increased Fgf9 in vivo is sufficient to expand the network of Tie2-lacZ and PECAM\(^{\text{pos}}\) cells through stimulation of undifferentiated mesenchyme and/or vascular progenitor cells [1].

The key finding of the present study is that inactivation of \(\text{Alk}5\) inhibits αSMA\(^{\text{pos}}\) and promotes LIF differentiation, both in vivo and in cultured lung mesodermal cell lines. Markers of myofibroblast differentiation, including αSMA, SM22α, and calponin, were reduced, while mediators of lipogenic commitment, such as ADRP, were robustly increased. The mechanism underlying this phenotype is complex. Since TGFβ signaling is known to induce αSMA\(^{\text{pos}}\) cell differentiation, its inhibition is readily explicable by ALK5 deficiency. In addition, FGF10\(^{\text{pos}}\) cells are known to contribute to αSMA\(^{\text{pos}}\) cells during lung development [3]. FGF10 mRNA was reduced as was the physical size of SMM, the site of FGF10\(^{\text{pos}}\) cells. In contrast to this finding, we had previously found increased Fgf10 mRNA and SHH signaling in mesodermally-targeted \(\text{T}β\text{R2}^{\text{Dermo1}}\) lungs [39]. The discrepancy between the previous and present observations on FGF10 provides an excellent illustration of differential TGFβ signaling via receptor selectivity as described in the Background section. Thus, receptor selectivity (TβR2 vs. ALK5) represents an important mechanism by which TGFβ can impose a vast spectrum of physiologic changes on different (or same) tissues and cell types. In the present study, when the observation on reduced Fgf10 levels in the \(\text{Alk}5^{\text{Dermo1}}\) lungs is combined with a diminished mitotic cell index in the SMM compartment, the data suggest a decreased number of FGF10\(^{\text{pos}}\) cells and hence reduced αSMA\(^{\text{pos}}\) cell differentiation. Consistent with this conclusion, histological analysis of \(\text{Alk}5^{\text{Dermo1}}\) lungs revealed reduced αSMA\(^{\text{pos}}\) cell differentiation in the PBSM but not in perivascular smooth muscle cells (Fig. 2f). It is unclear whether PBSM cells and vascular smooth muscle cells have common or distinct developmental origins.

PDGFRA is also expressed by αSMA\(^{\text{pos}}\) progenitors and was similarly and profoundly reduced in \(\text{Alk}5^{\text{Dermo1}}\) lungs. Its functional abrogation was confirmed by reduced phosphorylation of AKT, its principle intracellular substrate (Fig. 3). Our study showed that the TGFβ-ALK5 axis regulates Pdgfra mRNA expression (Fig. 6a). Therefore, it was surprising to find that Pdgfra mRNA increased during spontaneous differentiation of \(\text{Alk}5^{-/-}\) cells to LIFs (Fig. 6b). It is likely that in these isolated cells alternative or additional mechanisms may exist which regulate Pdgfra expression, independent of the TGFβ-ALK5 axis. One potential mechanism may be increased spontaneous phosphorylation of ERK found in \(\text{Alk}5^{-/-}\) cells compared to \(\text{Alk}5^{+/+}\) cells and \(\text{Alk}5^{\text{Dermo1}}\) lungs (Figs. 4 and 5). The underlying cause of increased p-ERK in cells lacking ALK5 activity is outside the scope of the present study, but is being currently investigated in separate studies. What remains firmly established from these in vitro studies is that inhibition of PDGFRA by Imatinib reduced expression of myofibroblast markers but did not fully promote LIF differentiation; of the lipogenic genes, only Ppary was increased. This finding was validated by genetic targeting of Pdgfra in vivo (Fig. 7). Inactivation of Pdgfra in early mesodermal progenitors (Pdgfra\(^{\text{Dermo1}}\)) robustly inhibited the expression of myofibroblast-specific genes (Fig. 7d), while, compared to \(\text{Alk}5^{\text{Dermo1}}\) lungs, the impact on LIF differentiation was subtle, despite strong activation of Ppary in cultured cells. Thus, while PDGFRA is required for full expression of the myofibroblast-specific gene battery, its absence is capable of initiating, but not completing, the LIF differentiation program.

LIFs are critical regulators of alveologenesis and as-similate and transfer neutral lipids to adjacent type II cells, thereby facilitating surfactant formation [40]. LIF commitment may be favored by increased expression of PTEN, a phosphatase that regulates the fate of mesodermal progenitors (Fig. 4f). This finding recapitulates our previous study in which Dermo1-cre-mediated inactivation of Pten profoundly reduced ADRP [14]. Pten was originally identified as a negatively regulated target of TGFβ signaling [41].
Based on the collective observations in this study we propose a model whereby multiple signaling pathways, controlled by ALK5-mediated TGFβ signaling, converge on transcriptional factors that regulate the cell lineage determination of two key mesodermal specialized cell types (Fig. 7f). The two major transcription factor targets of this process include the adipogenic zinc finger transcription factor ZFP423, and the myogenic transcription factor PRRX1. Activation of Zfp423 through knockdown of Zfp521, its negative regulator, is capable of altering mesodermal cell fate from osteogenic to lipogenic lineage [42]. We found increased LIFs in Alk5\textsuperscript{Dermo1} lungs associated with increased Zfp423 mRNA. Likewise, Zfp423 increased robustly in cultured Alk5\textsuperscript{−/−} cells undergoing spontaneous LIF differentiation (Fig. 5). Importantly, TGFβ1 strongly repressed Zfp423, and only in presence of functional ALK5 (Fig. 5l). Thus, while ALK5 activity is required for αSMA\textsuperscript{pos} cell lineage commitment, its inactivation de-represses Zfp423 favoring early LIF lineage commitment. In support of this conclusion, we found profoundly increased expression of Wisp2, known to be expressed at high levels in committed adipocyte precursors. In contrast, PRRX1 is a mesodermal transcription factor [43] that promotes differentiation of mesenchymal precursors into αSMA\textsuperscript{pos} cells. PRRX1 inhibits adipogenesis while its stable knockdown enhances adipogenesis, as shown by increased Ppary, Cebpa, and Fabp4 expression [44]. In Alk5\textsuperscript{Dermo1} lungs, Prx1 decreased as Zfp423 increased, indicating a key mechanism by which Alk5 repression facilitates LIF commitment while inhibiting αSMA\textsuperscript{pos} cell differentiation.

In conclusion, these data establish that ALK5 has a regulatory role in the control of early mesodermal progenitor commitment between two alternative cell lineages. This role is consistent with previous in vitro studies wherein genome-wide pathway analysis identified members of the TGFβ superfamily as modulators of adipocyte/myocyte differentiation [45]. αSMA\textsuperscript{pos} cell determination is regulated by TGFβ signaling and requires further signaling by FGF9 and PDGFRα. In the wild type multipotential mesoderm, activation of the αSMA\textsuperscript{pos} cell pathway, mediated by the transcription factor PRRX1 also limits the ontogeny of LIFs. Blocking ALK5-mediated TGFβ signaling in Alk5\textsuperscript{Dermo1} mesoderm inhibited αSMA\textsuperscript{pos} cell differentiation and increased (de-repressed) the expression of Zfp423, the key LIF transcription factor [28]. Therefore, it appears that, while αSMA\textsuperscript{pos} cell lineage is actively determined by ALK5-mediated TGFβ signaling, LIF determination represents a default position on a biological binary switch. This means that LIF can be activated only when αSMA\textsuperscript{pos}-promoting stimuli are diminished or eliminated.

### Methods

#### Animals

The Alk5\textsuperscript{flax/flox} (C57BL6 background) and Dermo1-cre mice were described previously [39, 46]. Pdgfra\textsuperscript{flax/flox} mice were purchased from the Jackson Laboratory. Mesodermal progenitor specific knockout mutants, Alk5\textsuperscript{Dermo1} (Alk5\textsuperscript{flax/flox};Dermo1-cre) or Pdgfra\textsuperscript{Dermo1} (Pdgfra\textsuperscript{flax/flox};Dermo1-cre) were generated by crossing Alk5\textsuperscript{flax/flox} or Pdgfra\textsuperscript{flax/flox} homozygous females with Alk5\textsuperscript{flax/+};Dermo1-cre or Pdgfra\textsuperscript{flax/+};Dermo1-cre double heterozygous males in a pure C57BL6 background. Alk5\textsuperscript{flax/flox} or Pdgfra\textsuperscript{flax/flox} mice were used as control. Triple transgenic Alk5\textsuperscript{flax/flox};Dermo1-cre;mTmG mice (simply Alk5\textsuperscript{Dermo1};mTmG) were generated as previously reported [47]. Genotyping of the Dermo1-cre mice containing Alk5\textsuperscript{flax}, Alk5\textsuperscript{Δ}, and Alk5\textsuperscript{wt} alleles was as previously described [39, 46]. All animal procedures were performed according to approved University of Southern California Institutional Animal Care and Use Committee regulations.

#### Tissue collection and Oil Red O staining

Embryonic lungs from control and mutant lungs were collected at various embryonic stages from E11.5 to E18.5. The embryos or lungs were dissected and fixed overnight in 4 % paraformaldehyde at 4 °C. They were then dehydrated through increasing ethanol concentration and embedded in paraffin or OCT compound. Sections of 5-μm thickness were used for histological analysis. Frozen lung sections or cultured cells were stained with Oil Red O to detect LIFs according to the manufacturer’s protocol (Poly Scientific, Bayshore, NY, USA). The experiments were repeated using three mice within each genotype group (n = 3). For raw data pertaining to quantification of Oil Red O positive cells in Additional file 5, please see Additional file 9.

#### TUNEL assay

Apopotic cells were detected by using TUNEL detection kit (In Situ Cell Death Detection Kit, Roche Diagnostics, Indianapolis, IN, USA). Briefly, tissue sections were deparaffinized, rehydrated, and washed with distilled-deionized water. After treatment with proteinase K (Invitrogen, Carlsbad, CA, USA), fragmented DNA was labeled with fluorescein-dUTP, using terminal transferase (Roche Diagnostics). Slides were mounted with VECTASHIELD containing DAPI (Vector Laboratories, Burlingame, CA, USA). The apoptotic percentage was obtained as previously described [12] by manual counting of TUNEL\textsuperscript{pos} cells in groups of 4,000 or more cells.

#### Immunohistochemistry (IHC)

Routinely prepared histological sections were deparaffinized with xylene and rehydrated through an alcohol
gradient series to water. Antigens were retrieved and endogenous peroxidase activity was quenched using 3% hydrogen peroxide. After normal serum blocking, the sections were incubated with a primary antibody at 4°C overnight. Impress-anti-rabbit or anti-mouse or anti-goat IgG (Vector Laboratories) were applied for 50 min at room temperature. Staining was visualized by Peroxidase Substrate Kit DAB (Vector Laboratories). For immunofluorescence staining, the sections were incubated with primary antibodies overnight at 4°C. After washing steps, the sections were reacted with a mixture of DyLight*549-conjugated donkey anti-rabbit or anti-mouse IgG (H+L) or DyLight*488-conjugated donkey anti-mouse IgG or anti-goat IgG (H+L) (all from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h in the dark at room temperature. After thorough rinses with PBS containing 0.1% Triton X-100, the sections were mounted with VECTASHIELD mounting medium containing DAPI (to visualize nuclei). Primary antibodies used are described in Additional file 10. The experiments were repeated in more than three mice within each genotype group (n > 3).

**Mesenchymal cell isolation, SV40 transformation, Fluorescence Activated Cell Sorting (FACS) and treatment**

Primary mesenchymal cells were isolated by differential adherence [48]. Briefly, fetuses were dissected from pregnant control and mutant mice at E15.5 or E16.5. Six pairs of lungs were pooled for each experiment (n = 6). Lungs were dissociated with 0.025% trypsin/EDTA in DMEM (Gibco) at 37°C. The cells were filtered and plated in 75 cm² tissue culture flasks for 1 h, after which the non-adherent cells were discarded. Adherent cells were then washed and grown for 24 h. The primary cells were subsequently transformed by SV40 infection and subjected to FACS based on GFP activity using AriAII (Becton Dickinson). Gates were set according to unstained controls. After FACS, GFP⁺ cells were either plated on 6-well plates in DMEM, containing 10% fetal bovine serum, FBS (Gibco) for subsequent analysis, or directly subjected to Q-PCR.

For TGFβ1 (R&D Systems, Minneapolis, MN, USA) or Imatinib (Cayman Chemical Co. Ann Arbor, MI, USA) treatment, the purified Alk5+/+ or Alk5−/− cells were plated at a density of 5 × 10⁶ cells/cm² into 6-well tissue culture plates in DMEM with 10% FBS. Cells were serum-starved overnight before treatment with TGFβ1 (4 ng/mL according to the manufacturer’s recommendation) or imatinib (5 μL and 10 μL). TGFβ treatment was for 48 h; Imatinib treatment was for 5 days. Controls were treated exactly the same with the exception of BSA in place of TGFβ and DMSO in place of Imatinib. All conditions in Fig. 6a (Control, Alk5+/+, Alk5−/−) were initiated and analyzed after the same incubation period.

For adipogenesis differentiation, low-passage (<6 passages) Alk5+/+ and Alk5−/− cells were plated at a density of 5 x 10⁴ cells/cm² in DMEM containing 10% FBS. At 80% confluence, old medium was aspirated and replaced with fresh DMEM containing 2% FBS. After 2, 5, or 10 days of incubation, the cells were stained with Oil Red O according to the manufacturer’s protocol (Poly Scientific, Bayshore, NY, USA) or prepared for RNA and protein analysis. Alk5+/+ and Alk5−/− cells at 80% confluence were used as a control. All experiments were performed in duplicate three times (n = 3).

**Western blot analysis**

Total protein was extracted with RIPA buffer (Sigma, St. Louis, MO, USA) from embryonic lung tissues or cultured cells. Protein concentrations were determined by a BCA Protein Assay kit (Thermo Scientific, Grand Island, NY, USA); 15–30 μg of protein was loaded onto 3–8% NuPAGE gels and transferred to Immobilon-P membranes (both from Millipore Corp, Billerica, MA, USA). Membranes were then blocked with 5% milk in Tris-buffered saline and incubated with primary antibodies overnight. The secondary antibodies, goat-anti-rabbit or goat-anti-mouse IgG-HRP (both from Pierce, Thermo Scientific), were applied at a 1:1000 dilution for 30 min. Membranes were reacted with chemiluminescence reagent ECL (Amersham Biosciences, GE Healthcare Life Sciences, PA, USA) and exposed to photographic film (Amersham Hyperfilm ECL). The primary antibodies used are described in Additional file 10.

**Quantitative Polymerase Chain Reaction (Q-PCR)**

DNase-free RNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. After DNase treatment, total RNA (2 μg) was reverse-transcribed to cDNA using the Superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen), according to the manufacturer’s instructions. The cDNA was subjected to Q-PCR using SYBR Green PCR Master Mix with a LightCycler (Roche) as previously described [47]. Gene expression was normalized to β-actin or Tbp. All primers for Q-PCR were designed by using the program of Universal Probe Library Assay Design Center from Roche Applied Sciences. Specificity of each PCR reaction was verified by electrophoresis on 2% gel.

Relative quantification analysis was carried out with LightCycler software, Version 4 (Roche). The results were expressed as a normalized ratio (fold). β-actin or Tbp were used as reference genes for normalization. Samples from control lungs served as ‘Calibrators’. For raw data pertaining to Q-PCR studies, please see Additional file 9.
Statistical analysis
In total, the lungs or embryos from 98-pair Alk5Dermo1 mutant and control mice were analyzed using different techniques. For Q-PCR and western blot analysis, most experiments were performed two or three times, and the samples in each experiment were analyzed in triplicates or duplicates. All data are represented as mean ± standard error or standard error of the mean. The significance of differences between two sample means was determined by the two-tailed Student t-test, which is a common and well established statistical method to ascertain differences between two samples. Level of significance was denoted by P < 0.05.

Availability of supporting data
Data supporting the results of this article are available in Additional file 9.

Additional files

Additional file 1: Dermo1-cre-mediated mesodermal progenitor-specific deletion of Alk5. A–C, Dermo1-cre expression pattern in E14.5 Dermo1-cre;M132/Gfp lungs. Dermo1-cre-mediated recombination (green fluorescence) was located in the mesenchyme surrounding the trachea (A), bronchi (B), bronchioles and blood vessels (C), not in the epithelial cells. D. Deletion of Alk5 exon 3 by crossing Alk5floxed to Dermo1-cre mice. E. Deletion of Alk5 was validated by PCR with genomic DNA. F. Western blot analysis using total protein from E16.5 control and Alk5Dermo1 lungs, n = 3. β-actin was used as a control. G–I. Immunohistochemistry for Alk5 (G and I) and P51 (H and J) staining showed lost or decreased Alk5 and P51 (arrows in I and J) in Alk5Dermo1 lungs. Scale bars: C, J = 20 μm. (PPTX 925 kb)

Additional file 2: Mesodermal-specific Alk5 inactivation causes pulmonary hypoplasia. A–D. Gross morphology of E16.5 control (A and B) and Alk5Dermo1 (C and D) lungs. E–J. PAS (E, H, G and J) and H&E (F and I) staining of E18.5 control (E–G) and Alk5Dermo1 (H–J) lungs. Arrows in I indicate a thicker alveolar wall in Alk5Dermo1 lungs; Arrows in J indicate robust PAS staining in Alk5Dermo1 lungs. Scale bars: H = 100 μm; I = 10 μm; J = 20 μm. (PPTX 1736 kb)

Additional file 3: Mesodermal Alk5 deficiency inhibits epithelial cell differentiation and disrupts vasculogenesis. A–H. Immunohistochemistry (IHC) for Acetylated-tubulin (A and D), CC10 (B and F), pro-SPC (C and G) and T1a (D and H) in E18.5 control and Alk5Dermo1 lungs showing decreased expression of the latter genes in Alk5Dermo1 lungs. I. Q-PCR confirmed inhibition of epithelial cell markers in Alk5Dermo1 lungs, n = 2–9 pairs of separate lungs. J and L: IHC for GFP and FLK1 showing decreased GFP/FLK1+ cells in E14.5 Alk5Dermo1 lungs. K and M: IHC showed decreased CD34+ cells in E14.5 Alk5Dermo1 lungs. N–O: IHC showing decreased of PECAM1+ cells in E18.5 Alk5Dermo1 lungs. Arrows indicate large blood vessels appearing intact. R. Quantitative PCR confirmed decreased Pecam1, Flk1, and Flt1 mRNAs in E13.5 mutant lungs, n = 3 pairs of separate lungs. Error bars show standard deviation. *P < 0.05. Scale bars: H = 30 μm; M and Q = 20 μm. (PPTX 1560 kb)

Additional file 4: Quantification of sub-mesothelial mesenchyme (SMM) size. Hematoxylin and eosin-stained lung tissue sections were prepared and random images were collected under 40x objective from both control and Alk5Dermo1 embryos at E12.5 and E13.5. The various dimensions that were measured included boundaries of the SMM compartments, as delineated on each photomicrograph, from mesothelium to the mesenchymal wrapped around the epithelium (SEM). The regions encompassing the SMM were measured in Photoshop by dividing each region at random of six points to manually calculate the length from mesothelium to SMM (μm). Sample size, n = 3 separate lung tissue sections. Error bars show standard deviation. *P < 0.05. (PPTX 75 kb)

Additional file 5: Quantification of Oil Red O storage. Lung tissue sections from E18.5 control and Alk5Dermo1 lungs were analyzed by staining with Oil Red O for assessment of LIF differentiation. Oil Red O stained sections were viewed and random images were collected under 40x objective, and were analyzed by imaging using a previously described protocol [49], n = 3. The density of Oil Red O stained cells was then calculated and plotted as shown. Error bars show the standard deviation. *P < 0.05 (PPTX 43 kb)

Additional file 6: Quantification of smooth muscle (SM) thickness and areas covered by smooth muscle in control and PdgfraDermo1 lungs at E18.5. αSMA immunohistochemically stained lung sections were collected by random sampling using the 40x objective from three pairs of control and PdgfraDermo1 mutant lungs at E18.5. The thickness and coverage of SM were quantified. A. Quantification of SM thickness. The arithmetic mean thickness of the SM cell layer was determined by volume of αSMApos compartment, measured by counting all points intercepting the airway epithelium and αSMApos compartment. n = 3. B. Quantification of SM coverage. The percentage of SM airway coverage was determined by volume of αSMApos compartment in contact with the airway epithelium in circumference in contact with number of αSMApos cells. n = 3. Error bars show the standard deviation. *P < 0.05 (PPTX 54 kb)

Additional file 7: Mesodermal progenitor-specific deletion of Pdgfra. A. Quantitative PCR (Q-PCR) showed Pdgf signaling target genes are decreased, indicating an overall functional repression of Pdgf signaling pathway in E14.5 PdgfraDermo1 lungs, n = 2 pairs of separate lungs, repeated twice or thrice. B. Q-PCR showed repression of myofibroblast-related genes in PdgfraDermo1 lungs, n = 2 pairs of separate lungs, repeated thrice for each. Error bars show SEM. (PPTX 68 kb)

Additional file 8: Mesodermal Alk5 deficiency increased cell apoptosis. The number of TUNEL+ cells as determined by previously described methods [12], A–D. TUNEL assay showed increased number of TUNEL+ cells in the Alk5Dermo1 lungs at E18.5. E. Quantification of the relative number of TUNEL+ cells (green) per 4,000 total cells. Error bars show standard deviation. *P < 0.05. (PPTX 794 kb)

Additional file 9: Raw data. (XLXS 247 kb)

Additional file 10: Primary antibodies used in western blots and immunohistochemistry. (DOC 37 kb)

Abbreviations
FACS: Fluorescence Activated Cell Sorting; IHC: Immunohistochemistry; LIF: Lipofibroblasts; PBSM: Peribronchial smooth muscle; Q-PCR: Quantitative polymerase chain reaction; SM: Smooth muscle; SMM: Sub-mesothelial mesenchyme; TGFβ: Transforming growth factor beta.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AL and PM conceived the study and designed the experiments. AL, SM, SMS, and AF performed the experiments. AL, PM, CL, and SB analyzed data. AL and PM wrote the manuscript. MLK, ZB, CL, and SB discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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