

Impaired Myofibroblast Proliferation is a Central Feature of Pathologic Post-Natal Alveolar Simplification

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This study provides **important** insights into postnatal lung development and the mechanisms underlying bronchopulmonary dysplasia (BPD), a condition with high morbidity and mortality in newborns. Through the use of neonatal hyperoxia, cell-type-specific inactivation of *Tgfr2*, and other injury models, the research focuses on the role of TGF- β signaling in BPD pathogenesis, highlighting impaired myofibroblast proliferation as a key factor. The inactivation of *Etc2* in *Pdgfra*-lineaged cells disrupts myofibroblast cytokinesis, leading to alveolar simplification and reduced cell numbers. The use of transgenic mice and single-cell transcriptomics offers a detailed and high-quality dataset, advancing our understanding of BPD and serving as a invaluable resource for developmental biology and neonatal pulmonary research. The study's comprehensive approach, robust data, and methodological rigor make it a **compelling** contribution to the field, providing both mechanistic insights and a resource for further research into BPD pathogenesis.

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Abstract

Premature infants with bronchopulmonary dysplasia (BPD) have impaired alveolar gas exchange due to alveolar simplification and dysmorphic pulmonary vasculature. Advances in clinical care have improved survival for infants with BPD, but the overall incidence of BPD remains unchanged because we lack specific therapies to prevent this disease. Recent work has suggested a role for increased transforming growth factor-beta (TGF β) signaling and myofibroblast populations in BPD pathogenesis, but the functional significance of each remains unclear. Here, we utilize multiple murine models of alveolar simplification and comparative single-cell RNA sequencing to identify shared mechanisms that could contribute to BPD pathogenesis. Single-cell RNA sequencing reveals a profound loss of myofibroblasts in two models of BPD and identifies gene expression signatures of increased TGF β signaling, cell cycle arrest, and impaired proliferation in myofibroblasts. Using pharmacologic and genetic approaches, we find no evidence that increased TGF β signaling in the lung mesenchyme

contributes to alveolar simplification. In contrast, this is likely a failed compensatory response, since none of our approaches to inhibit TGF β signaling protect mice from alveolar simplification due to hyperoxia while several make simplification worse. In contrast, we find that impaired myofibroblast proliferation is a central feature in several murine models of BPD, and we show that inhibiting myofibroblast proliferation is sufficient to cause pathologic alveolar simplification. Our results underscore the importance of impaired myofibroblast proliferation as a central feature of alveolar simplification and suggest that efforts to reverse this process could have therapeutic value in BPD.

Introduction

During the alveolar phase of lung development, alveolar walls protrude from secondary crests into the lumen of existing airways and form new alveoli to increase the lung's surface area for gas exchange ¹. Many premature infants are born prior to the onset of alveologenesis and develop bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurity caused by developmental arrest of secondary alveolar septation ². Over 50 years ago, Northway and colleagues described BPD as a heterogeneous pattern of severe lung injury with pathologic findings of severe inflammation, airway dysplasia, and fibrosis ³. Despite advances in neonatal care, the incidence of BPD remains unchanged as 30% of infants born before 30 weeks of gestation will develop BPD ^{2,4}. More infants are now surviving at earlier gestational ages, and the lungs of these infants have a “new” BPD phenotype characterized by a homogenous process of alveolar simplification, dysmorphic pulmonary vasculature, and mild airway thickening and fibrosis ^{1,2}. BPD is defined clinically by the need for prolonged respiratory support and supplemental oxygen, and infants with BPD suffer from long-term morbidity including severe respiratory infections, reactive airway disease, pulmonary hypertension, and neurodevelopmental impairment ^{1,2}.

The inflammatory response plays a significant role in BPD pathogenesis and results from a combination of perinatal infection, oxygen toxicity, and barotrauma from mechanical ventilation ¹. Several recent studies have suggested a role for the pro-fibrotic cytokine transforming growth factor-beta (TGF β) in BPD pathogenesis ^{4–9}. Infants with BPD have increased TGF β in serum and bronchoalveolar lavage fluid ^{10–13}. Experimentally, murine models of BPD show increased TGF β signaling and activation of downstream fibrotic pathways ^{7–9,14–17}. At the same time, TGF β plays an important role in normal development, and complete loss of TGF β signaling impairs either embryonic lung development or alveolar septation ^{18–29}. TGF β is a known regulator of myofibroblasts in fibrotic disease ³⁰, but it also drives mesenchymal cells to commit towards the myofibroblast lineage during embryonic lung development ²¹. Despite its essential role in normal lung development, the functional significance of increased TGF β signaling in BPD pathogenesis remains unclear.

Alveolar myofibroblasts are contractile mesenchymal cells that are critical for alveolar septation ³¹. They are characterized by the expression of platelet-derived growth factor receptor alpha (PDGFR α), alpha-smooth muscle actin (α -SMA), and the production of elastin ^{21,31,32–34}. Myofibroblasts are derived from PDGFR α + mesenchymal cells at birth and peak during alveologenesis when they colocalize with secondary septae ^{21,31,33,34–35}. Ablation of PDGFR α + cells in neonatal mice causes alveolar simplification, while neonatal hyperoxia treatment causes loss of PDGFR α + fibroblasts, dysregulated alveolar elastin deposition, and impaired PDGFR α + cell contractility ^{31,33,34,36}. Inhibition of myofibroblast contraction has been shown to lead to alveolar simplification ³⁷. These studies support a fundamental functional role for myofibroblasts during alveologenesis, but whether the number of myofibroblasts is reduced in BPD or experimental models of BPD remains controversial ^{31,34,38–40}.

To identify conserved mechanism of injury in BPD pathogenesis, we implemented a novel strategy to compare two murine models of alveolar simplification by single-cell RNA-sequencing (scRNA-seq): neonatal hyperoxia exposure and loss of epithelial TGF β signaling. Using flow cytometry and sequencing, we observed a dramatic reduction of myofibroblasts in both models of disease. Additionally, we found that increased TGF β signaling, decreased PDGFR α signaling, and impaired proliferation are hallmark features of injured myofibroblasts during alveologenesis. Blocking TGF β through several different approaches consistently worsened hyperoxia-induced disease, suggesting TGF β plays an essential homeostatic function in both normal alveolar development and hyperoxia-induced disease, but that increased TGF β signaling does not seem to be an important driver of alveolar simplification. We also demonstrated that PDGFR α + cells undergo robust proliferation during normal alveologenesis, but this proliferation is impaired in both hyperoxia-treated and TGF β -manipulated mice. We showed that modulation of PDGFR α + cell proliferation is sufficient to cause alveolar simplification, even in the absence of hyperoxic injury. These studies demonstrate that PDGFR α + cells are profoundly sensitive to injury during alveologenesis, they require TGF β signaling, and their proliferation is essential for normal alveolar development.

Results

Neonatal Hyperoxia Exposure and Loss of Epithelial TGF β Signaling Both Cause Alveolar Simplification

To identify conserved features of lung injury in bronchopulmonary dysplasia (BPD), we compared two different mouse models of lung injury known to cause alveolar simplification in mice. We first implemented the hyperoxia-induced model of BPD in which newborn mice are exposed to 75% oxygen from postnatal day (P) 0-10 and then recovered in room air until analysis (**Figure 1A**). This model takes advantage of the fact that mice are born during the sacular phase of lung development and undergo alveolarization from postnatal day P4 – P39. We harvested lungs to analyze alveolar architecture after completion of alveolarization at P40. Hyperoxia-treated mice showed significant alveolar airspace enlargement compared to controls (**Figure 1B**). Consistent with findings from other groups using similar protocols, we did not see evidence of fibrosis or scarring. In parallel, we used the flexiVent rodent ventilator to measure respiratory mechanics. Hyperoxia-treated mice had increased total lung capacity, increased compliance, and decreased elastance compared to control mice (**Figure 1C**). These findings are consistent with those of emphysematous lungs, and these physiologic changes align with the enlarged, simplified alveolar structures observed by histology.

For our second model of alveolar simplification, we generated mice which lack the critical TGF β receptor TGFBR2 in lung epithelium by crossing Nkx2.1-cre to the *Tgfb2* conditional allele (*Tgfb2*^{F/F};Nkx2.1-cre). These mice are viable at birth, have no respiratory distress, gain normal weight, and generate expected ratios of offspring with respect to each genotype (data not shown). Lungs from conditional knockout (cKO) mice show enlarged airspaces consistent with alveolar simplification, confirming earlier reports that TGF β signaling in lung epithelium is required for normal secondary septation. We then used the flexiVent system to analyze respiratory mechanics in cKO mice and observed increased compliance and decreased elastance comparable to what we saw in hyperoxia-exposed mice. Previous work demonstrated that *Tgfb2*^{F/F};Nkx2.1-cre mice are protected from lung disease caused by transgenic overexpression of TGF β 1 or neonatal hyperoxia treatment. To our surprise, *Tgfb2*^{F/F};Nkx2.1-cre mice were not protected from disease in our model of neonatal hyperoxia, but rather developed worse disease. These data suggest TGF β signaling to the lung epithelium is required for both normal alveologenesis and that it plays a protective role in the response to hyperoxia-induced injury.

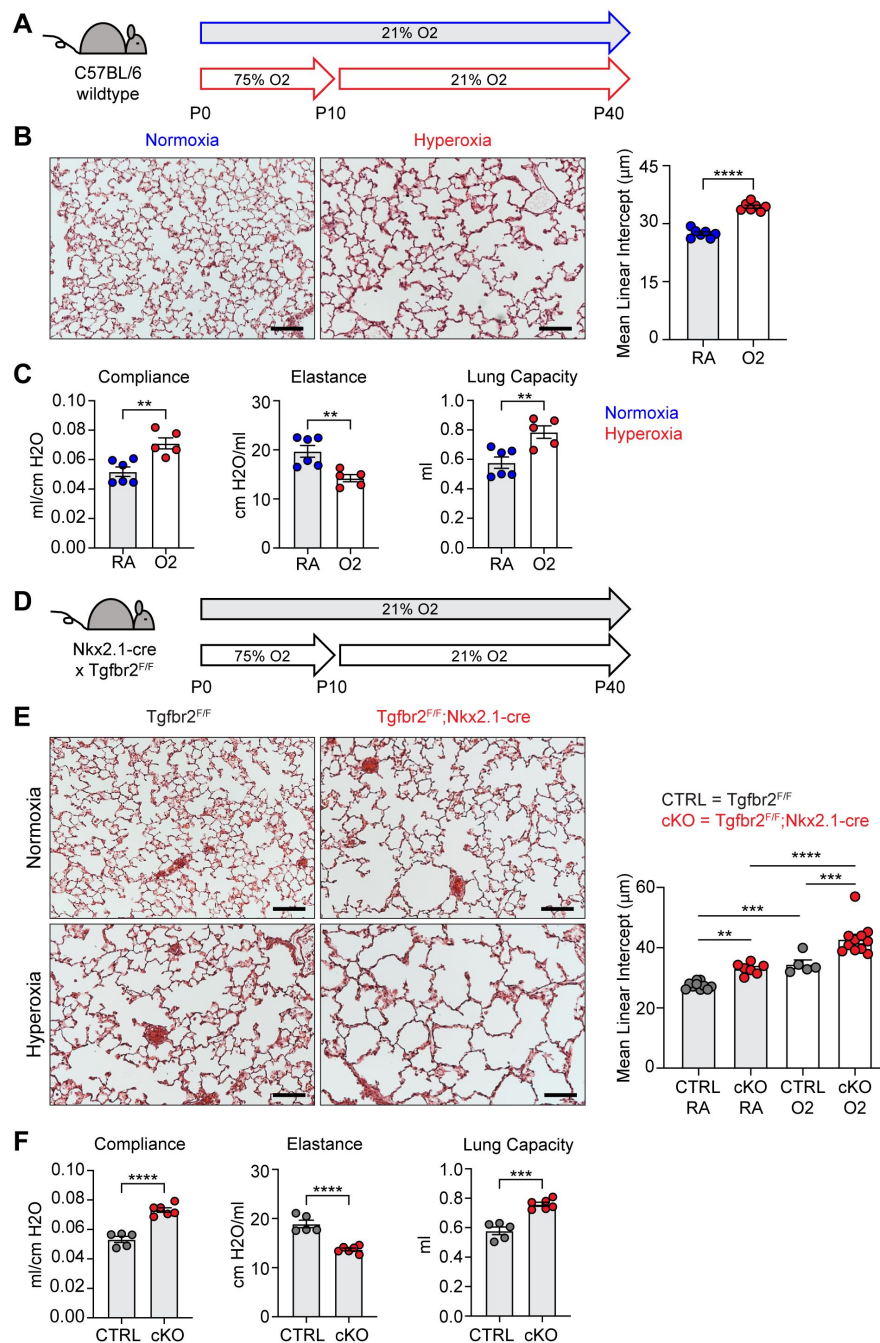


Figure 1.

Neonatal Hyperoxia Treatment and Loss of Epithelial TGF β Signaling Both Cause Alveolar Simplification.

(A) Wildtype C57BL/6 mice were treated in 75% hyperoxia versus normoxia from P0-P10 and recovered in room air until harvest at P40 for analysis by either histology or lung physiology. (B) H&E sections of representative lungs from (A) harvested at P40 (left) with quantification of mean linear intercept (right). (C) Mice treated as in (A) and harvested for lung physiology measurements of compliance, elastance and lung capacity. (D) *Tgfr2^{F/F}* and *Tgfr2^{F/F};Nkx2.1-cre* littermates were treated in hyperoxia versus normoxia from P0-P10 and recovered in room air until harvest at P40 for analysis by histology. (E) H&E sections of representative lungs from (D) harvested at P40 (left) with quantification of mean linear intercept (right). (F) Normoxia cohort treated as in (D) and harvested for lung physiology measurements of compliance, elastance and lung capacity. Data in (B), (C), and (F) compared by 2-tailed unpaired Student's t-test. Data in (E) compared by ANOVA with Fisher's post hoc test. Error bars depict mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars = 100 μ m.

Profound Loss of PDGFR α + Fibroblasts During Hyperoxia Treatment

To begin exploring the mechanisms underlying alveolar simplification in these models, we treated wildtype mice with hyperoxia from P0-P10 and analyzed dissociated lung cells by flow cytometry at P10. While we observed changes in many cell types of the lung with hyperoxia treatment, the most striking result was the profound loss of PDGFR α + fibroblasts (**Figure 2A-B**). Using flow cytometry, we found that both the percentage and absolute number of PDGFR α + fibroblasts were substantially reduced with hyperoxia treatment (**Figure 2B**). Next, we analyzed Tgfr2^{F/F};Nkx2.1-cre mice at P10 in normoxia and observed a similar phenotype of reduced PDGFR α + fibroblasts (**Figure 2C-D**). While the magnitude of reduction was less impressive than we observed in the hyperoxia model, the most significant change in cell numbers in Tgfr2^{F/F};Nkx2.1-cre mice was the reduction in PDGFR α + fibroblasts. Together, these results are consistent with earlier studies which showed a loss of PDGFR α + cells with neonatal hyperoxia treatment and reinforce the critical role of PDGFR α + fibroblasts in normal alveolar development ^{31,36}.

scRNA-seq Reveals Loss of Myofibroblasts in Both Models of Alveolar Simplification

To identify conserved molecular and cellular mechanisms underlying pathologic alveolar simplification, we performed single-cell RNA sequencing on lungs from Tgfr2^{F/F} (CTRL) and Tgfr2^{F/F};Nkx2.1-cre (cKO) mice in either normoxia or hyperoxia (**Figure 3A**). This approach allowed for simultaneous comparison of both injury models: normoxia versus hyperoxia in CTRL mice, and CTRL versus cKO mice in normoxia (**Figure 3B**). Given the reduction of PDGFR α + fibroblasts observed by flow cytometry in both models of lung injury, we hypothesized that changes in the lung mesenchyme might play a substantial role in the pathogenesis of alveolar simplification. To ensure sufficient mesenchymal cells in our analysis, we used flow cytometric sorting to enrich for mesenchymal and epithelial cells by limiting the input of hematopoietic and endothelial cells. After processing and combining all samples, we identified 24 clusters from 26,610 cells (**Figure S1A-C**). We focused our analysis on the lung mesenchyme in which we used the Seurat software package, differential gene expression, and comparison to published scRNA-seq data to assign identities to seven distinct mesenchymal clusters. (**Figures 3C** and **S2**) ⁴⁷⁻⁵⁰.

Although flow cytometry had already shown a profound loss of PDGFR α + cells in these two injury models, we could not attribute this loss to a specific fibroblast subset. Our single cell data enabled higher resolution assessment of the dynamics of fibroblast subsets (**Figure 3D**). In particular, we observed a significant loss of alveolar and ductal myofibroblast clusters in both models of injury (**Figure 3D-E**), suggesting that the reduction of PDGFR α + cells by flow cytometry was due to the loss of alveolar and ductal myofibroblasts. To externally validate these findings, we re-analyzed two recently published scRNA-seq studies in which mice were treated with 85% oxygen from P0-P14 ^{47,50}. Consistent with our own data, hyperoxia-treated mice in both studies showed a significant loss of myofibroblasts within the lung mesenchyme (**Figure S3**).

To gain insight into the molecular mechanisms driving the loss of myofibroblasts, we used Qiagen Ingenuity Pathway Analysis (IPA) to compare differential gene expression in the myofibroblasts in both models of injury ⁵¹. After filtering for upregulated pathways, we identified 32 predicted upstream regulators that were shared in both models (**Figure 3F**). Of these pathways, we noted several predictions corresponding to TGF β signaling, inhibitors of cell cycle, and Wnt signaling. These results are notable because TGF β is a known driver of myofibroblast differentiation and profibrotic programs in fibroblasts ^{21,30}, while its overall contribution to alveolar

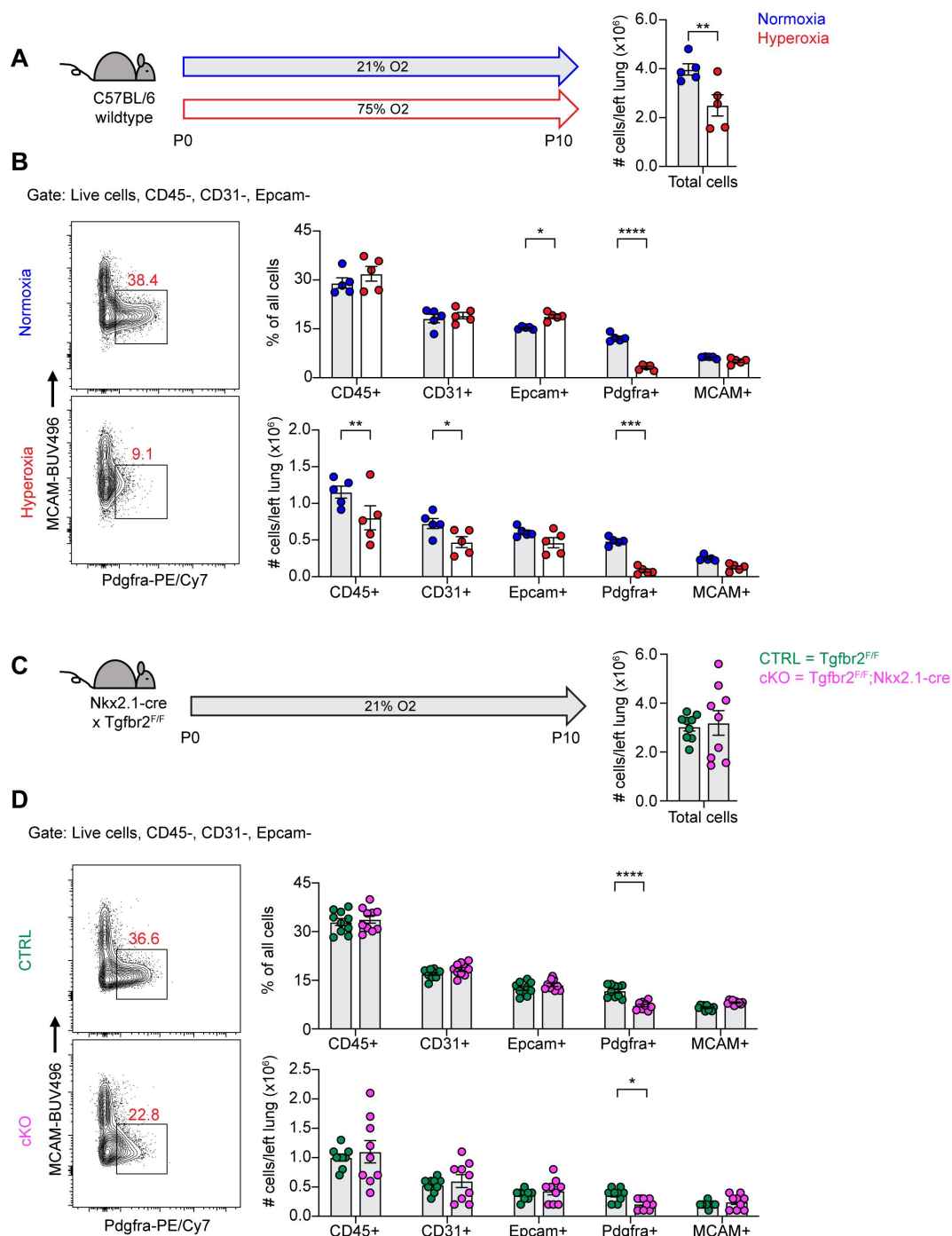


Figure 2.

Loss of PDGFR α + Cells With Neonatal Hyperoxia Treatment and Loss of Epithelial TGF β Signaling.

(A) Wildtype C57BL/6 mice were treated in 75% hyperoxia versus normoxia from P0-P10 and harvested on P10 for analysis by flow cytometry. Graph on right shows total cells per left lung as quantified by flow cytometry. (B) Representative flow cytometry plots of the lung mesenchyme (live, CD45-, CD31-, and Epcam-) with gates depicting PDGFR α + cells (left). Major cell populations of the lung were defined by the indicated cell surface markers and shown as either a percentage of all cells (top) or as absolute number (bottom). (C) Tgfb2^{F/F} and Tgfb2^{F/F};Nkx2.1-cre littermates were maintained in normoxic conditions from P0-P10 and harvested on P10 for analysis by flow cytometry. Graph on right shows total cells per left lung as quantified by flow cytometry. (D) Flow cytometry plots for (C) using the same gating strategy as described above. Data in (A-D) analyzed by 2-tailed unpaired Student's t-test. Error bars depict mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

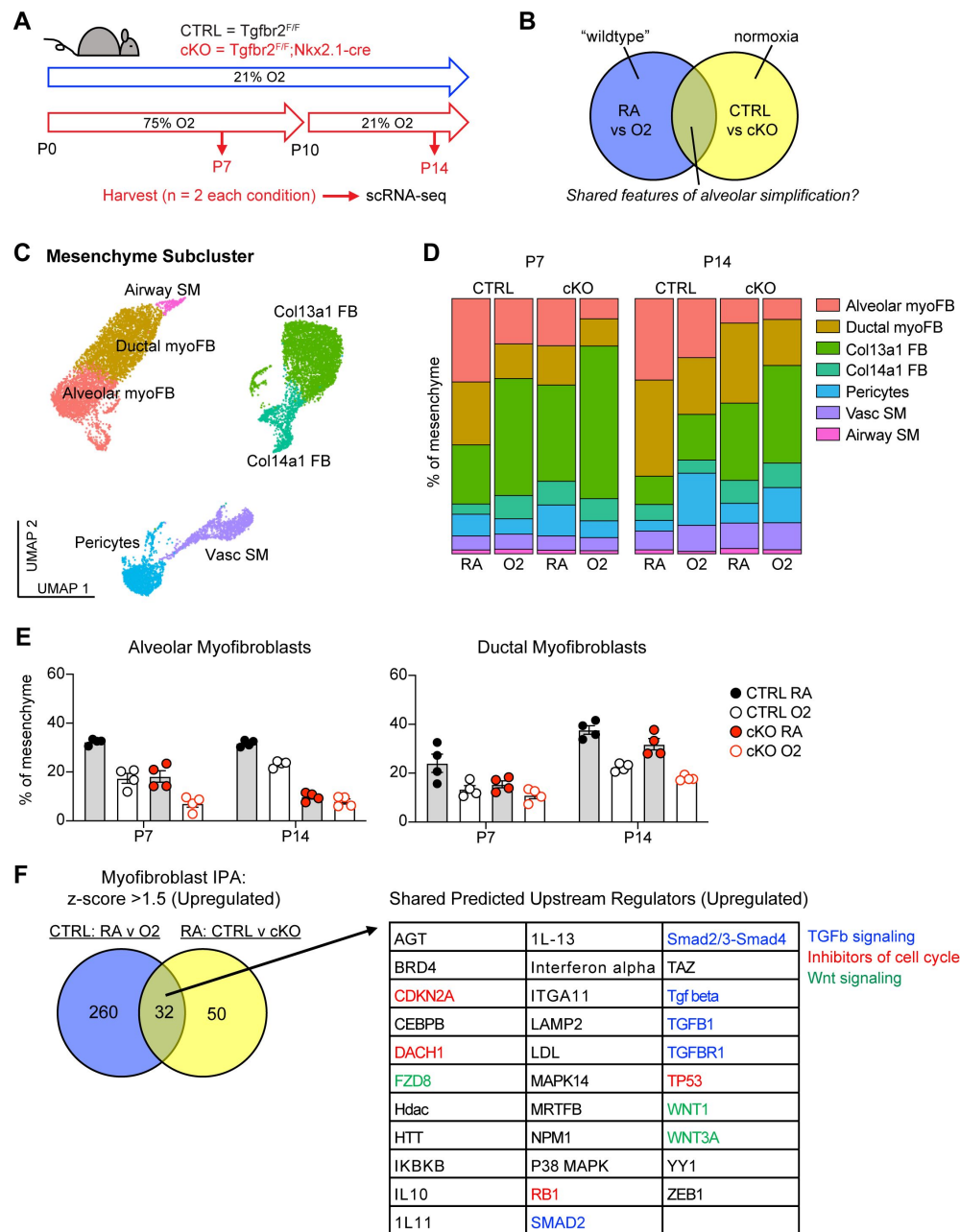


Figure 3.

scRNA-seq Reveals Loss of Myofibroblasts in Both Models of Alveolar Simplification.

(A) Schematic of scRNA-seq project showing CTRL (Tgfr2^{F/F}) and cKO (Tgfr2^{F/F};Nkx2.1-cre) littermates treated in 75% hyperoxia versus normoxia from P0-P10 and harvested on P7 or P14 for FACS-purification and analysis by scRNA-seq. n=2 mice harvested for each genotype, treatment condition and timepoint. (B) Venn-diagram depicting strategy to compare these two models to identify shared features of alveolar simplification. (C) UMAP projection of mesenchymal cells from scRNA-seq as outlined in (A). (D) Bar graphs depicting the frequency of each cell type by treatment condition, genotype, and timepoint. (E) The frequency of alveolar and ductal myofibroblasts within the mesenchyme as depicted in (D) with each data point representing either a biologic or technical replicate. Because the data depicts both biologic and technical replicates, no statistics were performed on this data set. (F) Differentially expressed genes in myofibroblasts were identified by comparing either CTRL RA vs O2 cells or RA CTRL vs cKO cells. These lists were subsequently analyzed by Qiagen IPA to identify predicted upstream regulators with z-score >1.5 (upregulated), while the table on right lists these 32 shared upstream regulators. Blue = TGFβ signaling, red = inhibitors of cell cycle, green = Wnt signaling.

simplification and BPD is unclear [53](#),[52](#). The prediction of increased cell cycle inhibition is also of interest because it suggests that the reduction of myofibroblasts in these models might be caused by their impaired proliferation.

We used the NicheNet software package to gain insight into the specific cell-cell interactions which might be impacting the myofibroblasts in these two models of lung injury [53](#). We reasoned that epithelial-myofibroblast signals would be particularly important, since the defect in the cKO model is restricted to epithelial cells, yet the most prominent phenotype is found in myofibroblasts. To explore this further, we used NicheNet to identify shared patterns of changes in ligand-receptor signaling originating from the lung epithelium to the myofibroblasts (**Figure 4A**). Here we again noted an increase in TGF β signaling to the myofibroblasts (**Figure 4B**). Of the decreased pathways, the most notable were Pdgfa-Pdgfra and Shh-Hhip, both of which are known to be critical for myofibroblast differentiation and function (**Figure 4C**) [29](#),[54](#). Additionally, the predicted reduction of Pdgfra signaling aligns with our flow cytometry data showing a loss of PDGFR α fibroblasts in both injury models as well as a reduction in the mean fluorescence intensity of PDGFR α antibody staining in these cells (**Figure 2B-D**). Together, these data suggest that epithelial dysfunction may cause myofibroblast defects through loss of supportive signals and gain of inhibitory signals.

TGF β Signaling Plays Homeostatic Role in Normal Alveolar Development and in Hyperoxia

Both IPA and NicheNet analyses predicted that TGF β signaling is activated in myofibroblasts in both models of alveolar simplification (**Figures 3** and **4**). Given the conflicting literature regarding the role of TGF β in normal lung development [53](#),[18](#),[52](#), we sought to determine the functional significance of TGF β signaling during postnatal alveologenesis in both normoxic and hyperoxic conditions. We hypothesized that if excessive TGF β signaling is a pathologic response in hyperoxia, titration of a pan-TGF β -blocking antibody (1D11) could identify a therapeutic window to protect from disease while permitting normal development under normoxic conditions. Alternatively, worsened disease with 1D11 treatment would suggest that increased TGF β is a compensatory response to injury rather than a primary driver of disease in the hyperoxia model. To test these hypotheses, we injected wildtype mice with 0, 10, 20 or 30 mg/kg of 1D11 from P2-P10 in either normoxia or hyperoxia and then harvested at P40 for analysis by histology (**Figure 5A**). To our surprise, we found that while only the highest dose of 1D11 treatment caused alveolar simplification in normoxia, all doses of 1D11 treatment caused worse alveolar simplification in hyperoxia compared to PBS (**Figure 5B**). Consistent with these histology results, lung physiology studies of 1D11-treated mice showed a pattern of emphysematous changes with increased compliance and decreased elastance (**Figure 5C**). These changes are similar to what we previously observed in hyperoxia-treated mice and in Tgfr2^{F/F};Nkx2.1-cre mice and complement the histologic findings of alveolar simplification in 1D11-treated mice. Together, these results support the alternative hypothesis that TGF β signaling plays a homeostatic role in both normal alveolar development and in hyperoxia.

Because 1D11 antibody treatment is systemic, we were unable to attribute the results of these studies to a specific cell type within the lung. Our current study, consistent with earlier reports, demonstrates that TGF β signaling to the epithelium is required for normal alveolar development as well as an adaptive response to hyperoxia [26](#). To interrogate the role of TGF β signaling to the lung mesenchyme, we sought to cross Tgfr2^{F/F} mice to either the Pdgfra-CreERT2 or Gli1-CreERT2 allele to target *Tgfr2* in myofibroblasts and mesenchymal cells during alveologenesis. While Pdgfra-CreERT2 has been used to specifically target PDGFR α cells [55](#), many studies have also used the broader Gli1-CreERT2 to target myofibroblasts during alveolar lung development [29](#),[54](#). Because the Pdgfra-CreERT2 and Gli1-CreERT2 alleles were generated using a knock-in/knock-out approach, mice carrying the CreERT2 allele are haploinsufficient for *Pdgfra* or *Gli1*,

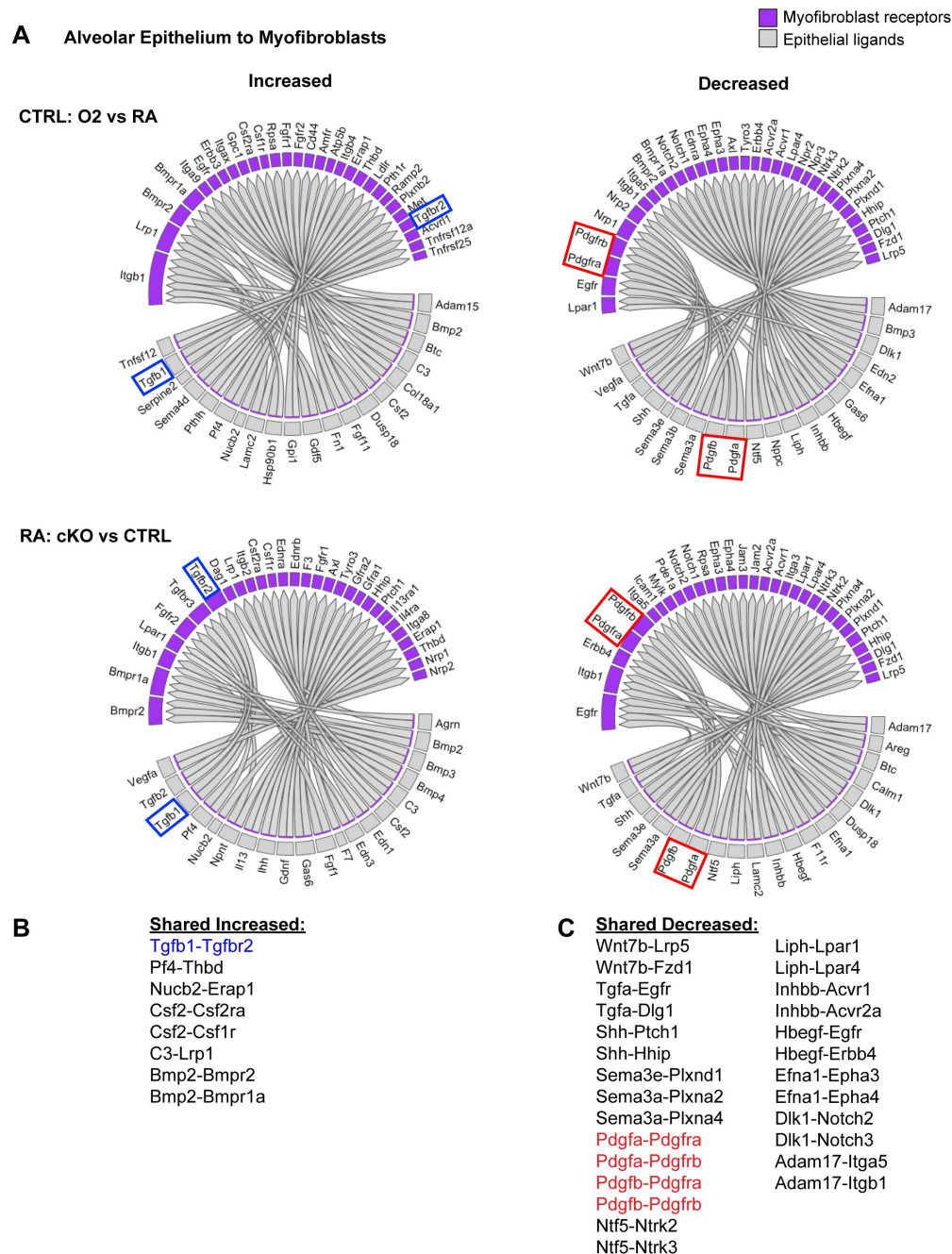


Figure 4.

NicheNet Ligand-Receptor Analysis of Epithelial-Mesenchymal Crosstalk in Both Models of Alveolar Simplification.

scRNA-seq data was analyzed using the NicheNet software package. **(A)** Alveolar epithelial clusters were pooled to define sender population and myfibroblast clusters were pooled to define receiver population. By analyzing differential expression of ligands, receptors, and downstream gene expression changes in myfibroblasts, NicheNet predicted increased (left) or decreased (right) ligand-receptor signaling in each comparison of interest. Gray boxes show ligands expressed on the epithelium and the purple-shaded boxes indicate the corresponding receptors expressed on myfibroblasts. Upper panels compare hyperoxia versus normoxia in CTRL cells. Lower panels compare CTRL versus cKO cells in normoxia. **(B)** List of ligand-receptor pairs predicted to be increased in both injury models. Tgfb1-Tgfb2 pairings highlighted in blue. **(C)** List of ligand-receptor pairs predicted to be decreased in both injury models. Pdgra-Pdgfra and Pdgb-Pdgfrb pairings highlighted in red.

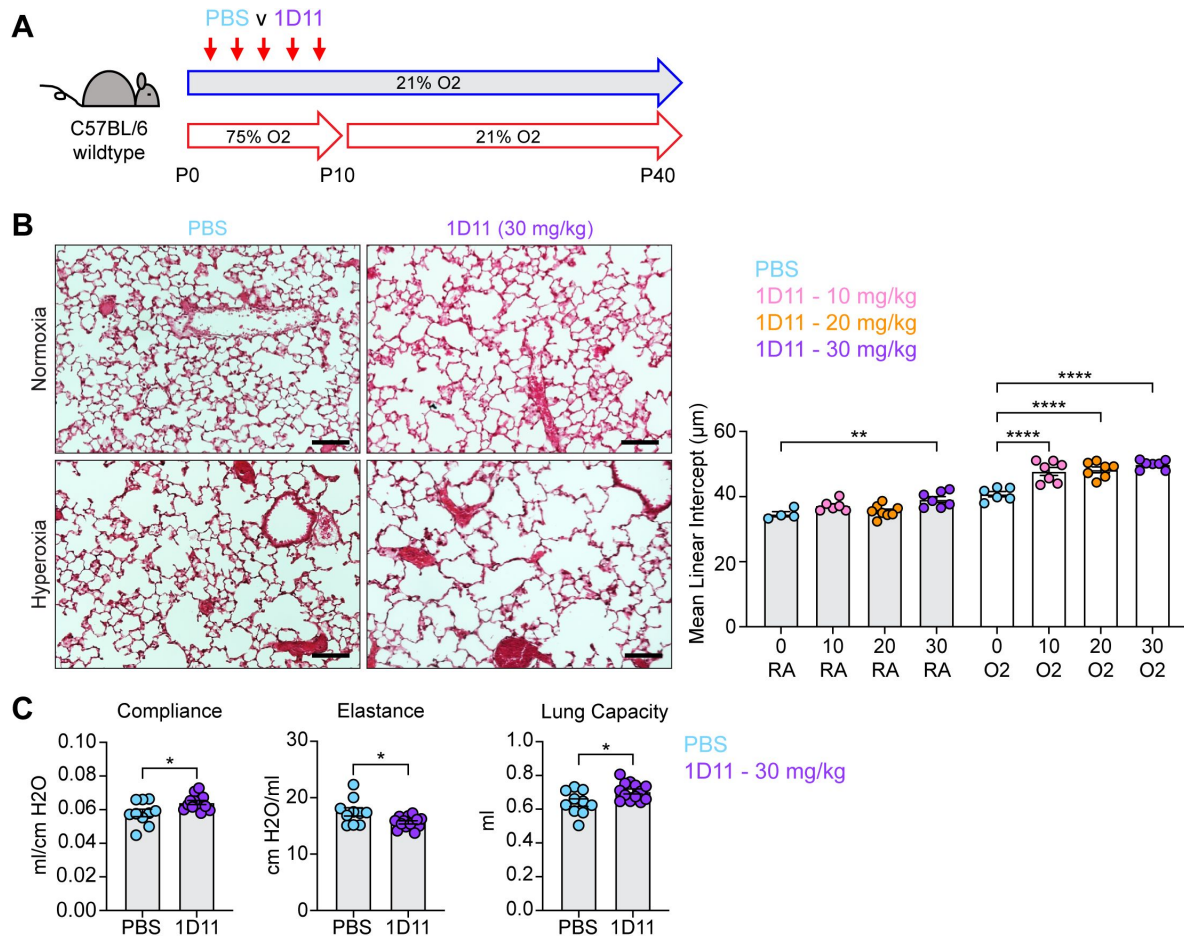


Figure 5.

Inhibiting TGFβ Disrupts Alveolar Development and Exacerbates Hyperoxia-induced Injury.

(A) Wildtype C57BL/6 mice were injected every other day from P2-P10 with PBS or 1D11 (pan-TGFβ-blocking antibody), treated in 75% hyperoxia treatment versus normoxia from P0-P10, and recovered in room air until harvest at P40 for analysis by either histology or lung physiology. (B) H&E sections of representative lungs from (A) harvested at P40 (left). Images shown are from PBS and 30 mg/kg 1D11 treatment groups. Mean linear intercepts calculated for all treatment groups (right). (C) PBS- and 30 mg/kg 1D11-treated mice treated in normoxic conditions as in (A) and harvested for lung physiology measurements of compliance, elastance and lung capacity at P40. Data in (B) compared by ANOVA with Fisher's post hoc test; for readability and limitations of graphing, only the statistical significance values within normoxia or hyperoxia cohorts are plotted. Data in (C) compared by 2-tailed unpaired Student's t-test. Error bars depict mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001. Scale bars = 100 μm.

respectively [55](#), [56](#). We conducted hyperoxia studies in each of these two lines and found that the Gli1-CreERT2 allele alone does not disrupt alveolar development in either normoxia or with hyperoxia treatment (**Figure S4A-B**). In contrast, mice with the *Pdgfra*-CreERT2 allele undergo normal development in normoxia, but they develop worse alveolar simplification with hyperoxia treatment compared to their cre-negative littermates (**Figure S4A-B**). These data support use of the Gli1-CreERT2 allele to target the lung mesenchyme in either normoxia or hyperoxia while limiting the use of *Pdgfra*-CreERT2 mice (and other *Pdgfra*-haploinsufficient alleles) to normoxic conditions.

Based on these validation studies, we generated *Tgfb2*^{F/F};Gli1-CreERT2 mice to study the effects of TGFβ signaling to the lung mesenchyme. Interestingly, we observed a similar pattern of lung disease as 1D11 treatment when deleting *Tgfb2* in the lung mesenchyme: *Tgfb2*^{F/F};Gli1-CreERT2 mice developed alveolar simplification in normoxia when compared to littermate controls, and they developed worse disease than their littermates with hyperoxia exposure (**Figure S5A-B**). Together, these results show that TGFβ signaling is required to both the lung epithelium and mesenchyme for normal development, that it plays a homeostatic role in response to neonatal hyperoxia treatment, and that all our efforts to inhibit TGFβ made hyperoxia-induced alveolar simplification worse.

To address mechanisms of TGFβ activation in normal alveolar development and in the hyperoxia model of alveolar simplification, we generated *Itgb6*^{F/F} mice and crossed them to the *Nkx2.1*-cre allele. The αvβ6 integrin is expressed predominantly in epithelial tissues, activates TGFβ *in vivo*, and has been shown to be a critical mediator of lung injury in models of acute lung injury and pulmonary fibrosis [57](#)–[62](#). *Itgb6*^{F/F};Nkx2.1-cre mice lack αvβ6 on lung epithelial cells and undergo normal alveolar development, but to our surprise, show no difference in disease severity with hyperoxia treatment (**Figure S5C-D**). Within the mesenchyme, αvβ1 and αvβ8 have been shown to activate TGFβ *in vivo* [62](#)–[65](#), so we next deleted all αv-integrins in the lung mesenchyme by crossing *Itgav*^{F/F} mice to the Gli1-CreERT2 allele. These mice develop spontaneous alveolar simplification in normoxic conditions, have lung physiology parameters consistent with an emphysematous phenotype, and develop worse lung disease with hyperoxia exposure (**Figure 6A-C**). These data suggest that epithelial αvβ6 does not play a role in TGFβ activation during normal development or neonatal hyperoxia, while αv-integrins in the lung mesenchyme are required for normal development and play a protective role in response to hyperoxia.

Impaired Proliferation of PDGFRα+ Fibroblasts with Hyperoxia Treatment

Flow cytometry and scRNA-seq both showed a reduction of PDGFRα+ fibroblasts in hyperoxia-treated mice, which suggests these cells are exquisitely sensitive to injury. To address whether this might be due to impaired proliferation as suggested by IPA analysis, we exposed wildtype mice to hyperoxia and treated them with the nucleoside analogue 5-ethynyl-2'-deoxyuridine (EdU) to identify proliferating cells. We found a decrease of PDGFRα+ cells in hyperoxia-treated mice by P8 both by percentage and by absolute number (**Figure 7A-C**), which persisted to P10 and continued even with 4 days of normoxic recovery. Using EdU analysis, we found hyperoxia caused a proliferative defect in PDGFRα+ cells as early as P4, preceding the decrease in cell number. This early impairment of proliferation by PDGFRα+ cells was the most striking change observed across all subsets in our EdU analysis, and suggests that a proliferative defect contributes to the decreased cell number.

After observing impaired proliferation of PDGFRα+ cells in the hyperoxia model of lung injury, we explored whether this mechanism is conserved across our other models of alveolar simplification. We used EdU treatment and flow cytometry to characterize cell subsets and proliferation in *Tgfb2*^{F/F};Nkx2.1-cre mice and 1D11-treated wildtype mice. Remarkably, both models showed a

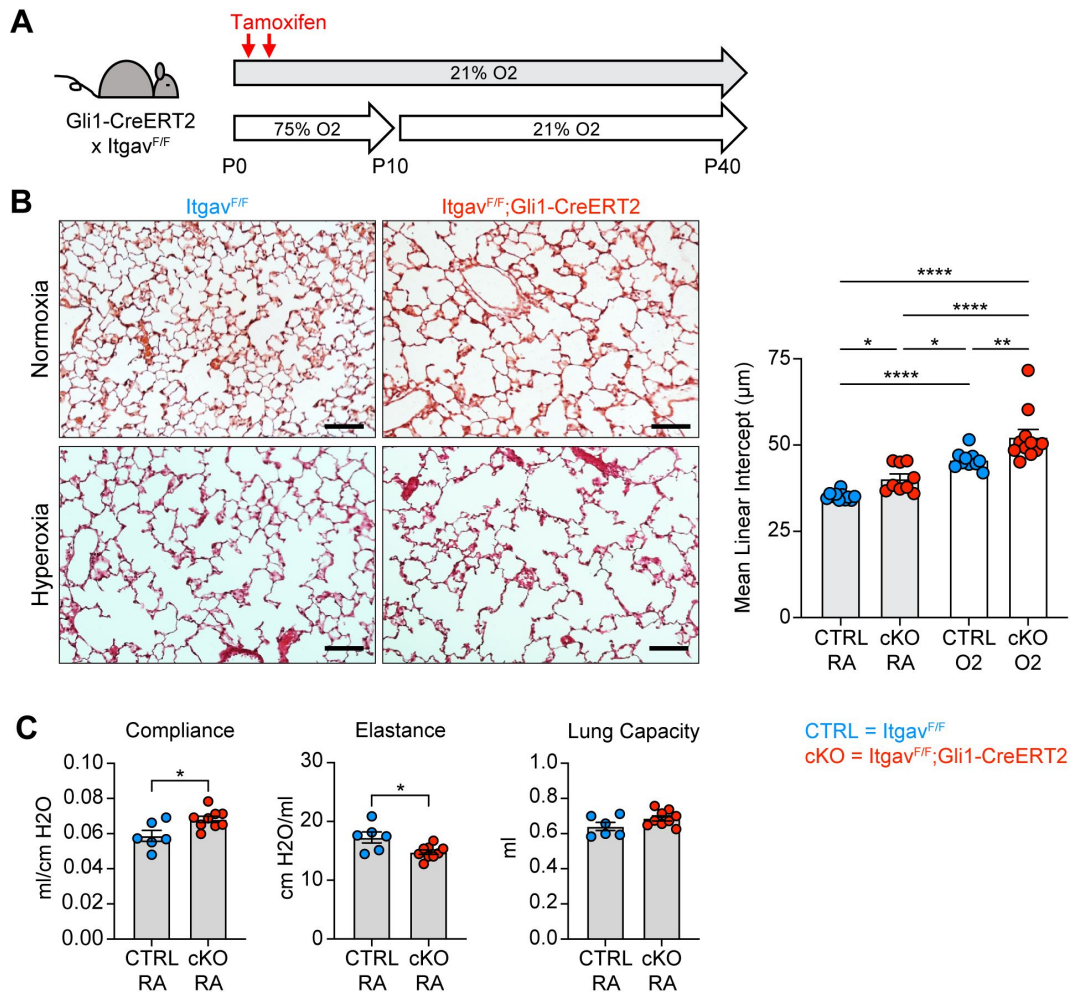


Figure 6.

Deletion of α v-integrins in Lung Mesenchyme Impairs Alveolar Development and Worsens Hyperoxia-induced Injury.

(A) Itgav^{F/F} and Itgav^{F/F};Gli1-CreERT2 littermates were injected with tamoxifen on P2 and P4, treated in 75% hyperoxia versus normoxia from P0-P10, and recovered in room air until harvest at P40 for analysis by either histology or lung physiology. (B) H&E sections of representative lungs from (A) harvested at P40 (left). Mean linear intercepts calculated for all treatment groups (right). (C) Normoxia cohort treated as in (A) and harvested for lung physiology measurements of compliance, elastance and lung capacity. Data in (B) compared by ANOVA with Fisher's post hoc test. Data in (C) compared by 2-tailed unpaired Student's t-test. Error bars depict mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Scale bars = 100 μ m.

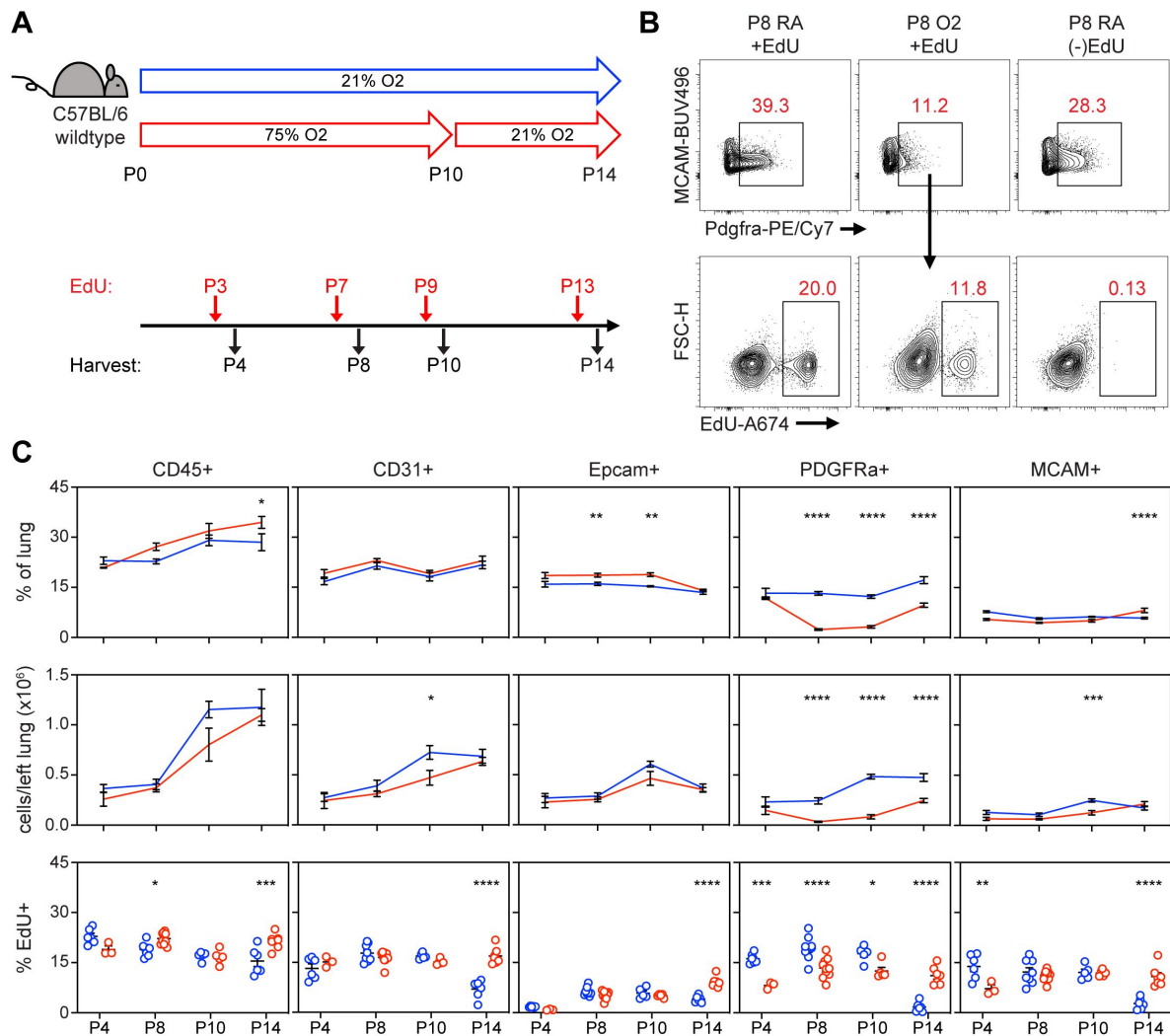


Figure 7.

Impaired Proliferation of PDGFR α + Fibroblasts With Neonatal Hyperoxia Treatment.

(A) Wildtype C57BL/6 mice were treated in 75% hyperoxia versus normoxia from P0-P10 and recovered in room air until indicated timepoints for analysis. Mice were injected with EdU 24 hours prior to each analysis timepoint. (B) Flow cytometry plots of lung mesenchyme (CD45-, CD31-, Epcam-, MCAM-) show gating of PDGFR α + cells (upper panels) and subsequent identification of EdU+/PDGFR α + cells (lower panels). Panels on far-right show an EdU-untreated littermate used to define EdU+ gate. (C) Time course graphs showing indicated populations of the lung as percent of lung (top), total cells in left lung (middle), and percent EdU+ cells (bottom). Data graphed as mean \pm with exception of EdU+ panel in which each animal is plotted individually. Data compared by 2-tailed unpaired Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

similar reduction of PDGFR α + cells along with decreased PDGFR α + cell proliferation as quantified by EdU uptake (**Figure 8A-D**). These results provide functional validation of our IPA analyses showing enrichment of cell cycle inhibitory pathways in injured myofibroblasts, and they suggest impaired PDGFR α + cell proliferation might be a conserved feature of developmental lung injuries which result in alveolar simplification.

Impaired Proliferation of PDGFR α + Fibroblasts Is Sufficient to Cause Alveolar Simplification

After observing impaired PDGFR α + cell proliferation in multiple models of alveolar simplification, we sought to determine whether inhibiting proliferation in these cells would be sufficient to cause disease. We generated mice with conditional deletion of *Ect2*, a protein required for cytokinesis⁶⁶, by crossing the *Ect2*^{F/F} mice with the *Pdgfra*-CreERT2 allele. We found earlier that PDGFR α + cells underwent robust proliferation through P10 in normoxic conditions (**Figure 7C**), so we analyzed *Ect2*^{F/F};*Pdgfra*-CreERT2 mice at P14 to characterize the impact of this mouse model on the cellular composition of the lung during early alveologenesis. Using flow cytometry, we observed a significant reduction in PDGFR α + cells within the lung by percentage and absolute number (**Figure 9A-B**). While this reduction in PDGFR α + cells was expected in *Ect2*^{F/F};*Pdgfra*-CreERT2 mice, it was interesting that *Pdgfra* expression was reduced in the remaining PDGFR α + cells as quantified by mean fluorescence intensity (MFI). Earlier studies found a correlation between *Pdgfra* expression levels and fibroblast proliferation⁶⁷, so we wondered whether this phenotype of reduced *Pdgfra* expression was conserved across the other injury models in this study which showed reduced PDGFR α + cell proliferation. Indeed, quantification of PDGFR α MFI by flow cytometry confirmed that *Pdgfra* expression is significantly reduced in the lung mesenchyme and specifically in PDGFR α + cells in each of the injury models we analyzed in this study (**Figure S6A**).

Next, we aged *Ect2*^{F/F};*Pdgfra*-CreERT2 mice until P40 to analyze their lungs by either morphometry or lung physiology. By histology, we observed enlarged alveolar airspaces and increased MLI's in conditional knockouts (*Ect2*^{F/F};*Pdgfra*-CreERT2) compared to controls (**Figure 7C**). Importantly, conditional heterozygous mice showed no difference in MLI compared to cre-negative mice, which confirms that haploinsufficiency of *Pdgfra* caused by using the *Pdgfra*^{CreER} allele in normoxic conditions is not responsible for this phenotype (**Figures 7C** and **S6A-B**). By lung physiology, *Ect2*^{F/F};*Pdgfra*-CreERT2 mice produced the same emphysematous phenotype of increased compliance and decreased elastance which we observed in several other models of alveolar simplification in this study (**Figure 7D**). Together, these data demonstrate that impaired proliferation of PDGFR α + cells is sufficient to cause alveolar simplification, even in the absence of environmental insult or modulation of TGF β signaling.

Discussion

Using a combination of flow cytometry, lung physiology, and scRNA-seq, we identified several core features of lung injury that are conserved across multiple mouse models of alveolar simplification. We first compared neonatal hyperoxia exposure (75% hyperoxia P0-P10) to the loss of epithelial TGF β signaling (*Tgfb2*^{F/F};*Nkx2.1*-cre). Flow cytometric analysis of both models showed a significant reduction of PDGFR α + cells, while scRNA-seq studies attributed these changes to a loss of alveolar and ductal myofibroblasts. Qiagen IPA and NicheNet ligand-receptor analyses identified several pathways that were upregulated in injured myofibroblasts in both models: increased TGF β signaling, enrichment of inhibitors of cell cycle, and decreased *Pdgfa*-*Pdgfra* signaling. Using a combination of pharmacologic and genetic approaches, we demonstrated that increased TGF β signaling in the mesenchyme does not seem to be a driver of alveolar simplification, but rather TGF β signaling is critical for both normal alveolar development and for

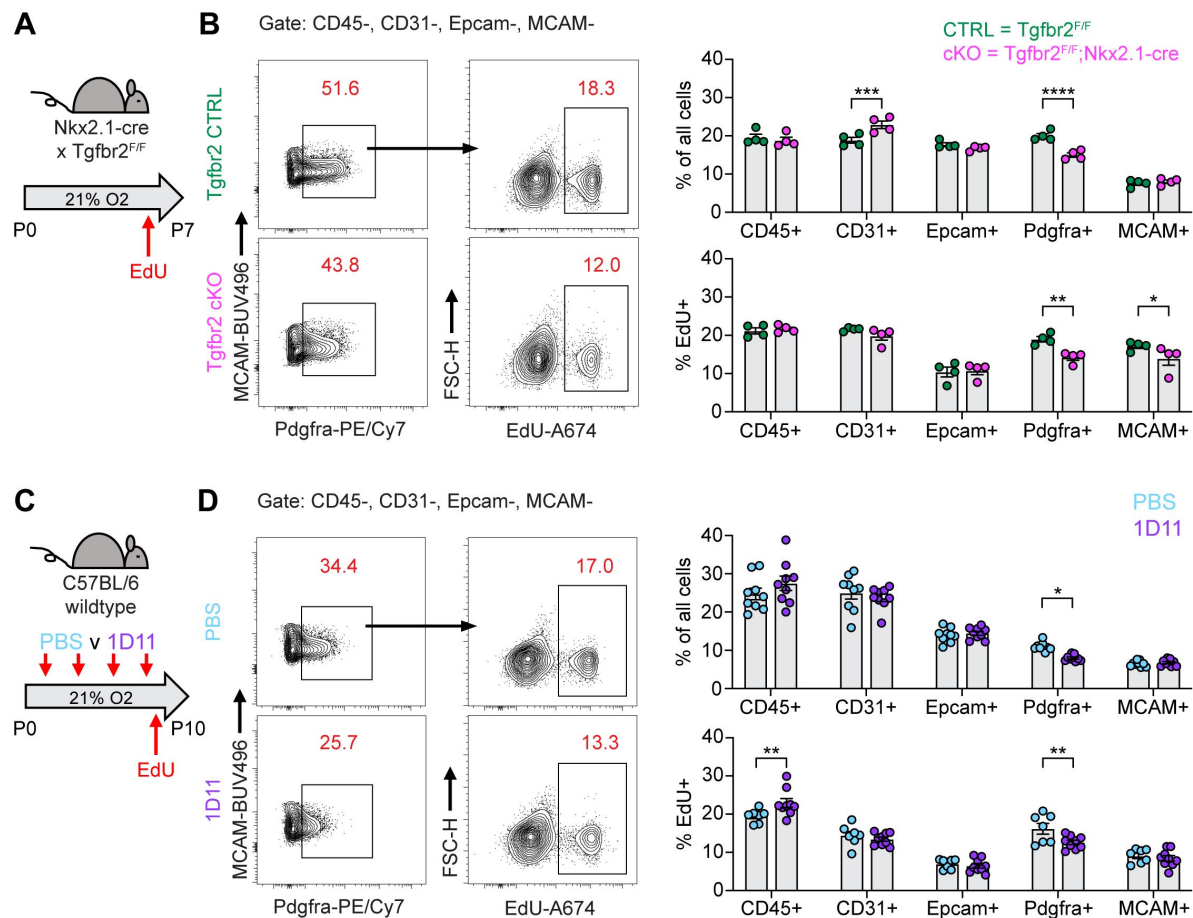


Figure 8.

Impaired PDGFRα⁺ Cell Proliferation is a Conserved Feature Across Multiple Models of Alveolar Simplification.

(A) Tgfb2^{F/F} and Tgfb2^{F/F};Nkx2.1-cre littermates were maintained in normoxic conditions from P0-P7, injected with EdU on P6, and harvested 24 hours later on P7 for flow cytometry. (B) Flow cytometry plots of lung mesenchyme (CD45⁻, CD31⁻, Epcam⁻, MCAM⁻) show gating of PDGFRα⁺ cells (left panels) and subsequent identification of EdU⁺/PDGFRα⁺ cells (right panels). Graphs on far right show major cell populations of the lung by percentage (upper graphs) and percent EdU-positive within each of these populations (lower graphs). (C) Wildtype C57BL/6 mice were injected every other day from P2-P8 with PBS or 30 mg/kg 1D11 (pan-TGFβ-blocking antibody) in normoxic conditions, injected with EdU on P9, and harvested 24 hours later on P10 for flow cytometry. (D) Flow cytometry plots of lung mesenchyme (CD45⁻, CD31⁻, Epcam⁻, MCAM⁻) show gating of PDGFRα⁺ cells (left panels) and subsequent identification of EdU⁺/PDGFRα⁺ cells (right panels). Graphs on far right show major cell populations of the lung by percentage (upper graphs) and percent EdU-positive within each of these populations (lower graphs). Data in analyzed by 2-tailed unpaired Student's t-test. Error bars depict mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

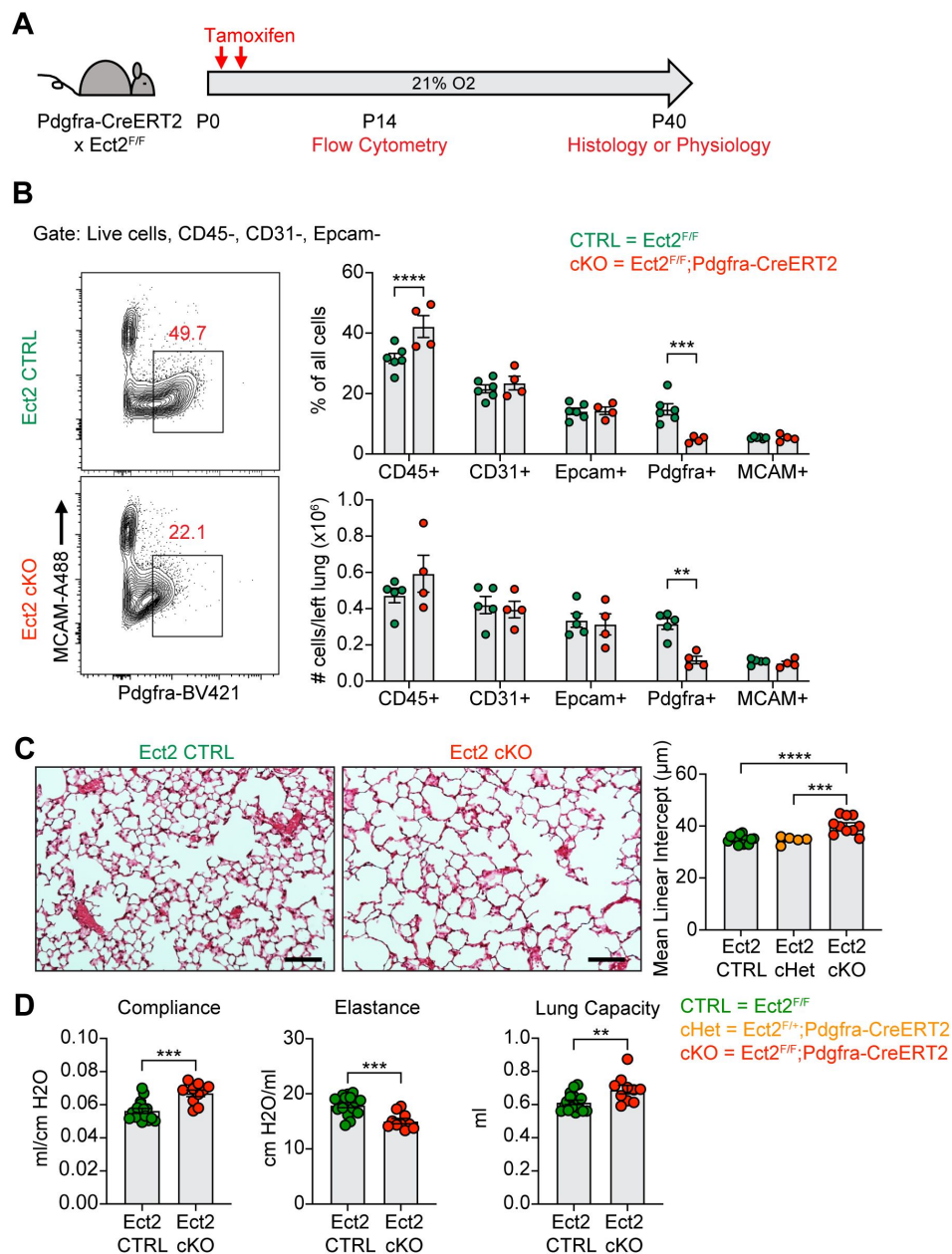


Figure 9.

Blocking Proliferation of PDGFR α + Fibroblasts is Sufficient to Cause Alveolar Simplification.

(A) Ect2^{F/F} and Ect2^{F/F};Pdgfra-CreERT2 littermates were injected with tamoxifen on P2 and P4 in normoxic conditions. Mice were analyzed by flow cytometry on P14 or aged until P40 for analysis by either histology or lung physiology. (B) Representative flow cytometry plots of the lung mesenchyme (live, CD45⁻, CD31⁻, and Epcam⁻) with gates depicting PDGFR α + cells (left). Major cell populations of the lung were defined by the indicated cell surface markers and shown as either a percentage of all cells (top) or as absolute number (bottom). (C) H&E sections of representative lungs from (A) harvested at P40 (left). Mean linear intercepts calculated for all treatment groups (right). (D) Mice treated as in (A) and harvested for lung physiology measurements of compliance, elastance and lung capacity. Data in (B) compared by 2-tailed unpaired Student's t-test. Data in (C) compared by ANOVA with Fisher's post hoc test. Error bars depict mean \pm SEM. **p<0.01, ***p<0.001, ****p<0.0001. Scale bars = 100 μ m.

protecting against impaired alveolar development in response to hyperoxia. In contrast, we show that PDGFR α + cell proliferation is reduced in multiple models of alveolar simplification, and that this impaired proliferation on its own is sufficient to cause alveolar simplification.

Several recent studies have identified lung mesenchymal cells as critical mediators of alveologenesis. Li *et al.* established the requirement of myofibroblasts during postnatal development by specifically ablating PDGFR α + cells at the onset of alveologenesis and showing that these mice developed alveolar simplification³¹. Other work has identified essential functions of myofibroblasts during alveolar development, including contractility³⁷ and mitochondrial energetics⁶⁸. Ricetti *et al.* used bulk RNA sequencing to show a skewing from myofibroblast to matrix fibroblast phenotypes after hyperoxia exposure along with reduced Ki-67 uptake and contractility in myofibroblasts³⁶. Our current study builds on this existing body of work by characterizing significant changes in PDGFR α + cell number in several models of developmental lung injury. By flow cytometry, we found reduction in the number of PDGFR α + cells in hyperoxia-treated mice and in Tgfr2^{F/F};Nkx2.1-cre mice during the early phase of alveologenesis. Using scRNA-seq, we attributed this reduction to the alveolar and ductal myofibroblast populations. Importantly, we identified myofibroblast proliferation as a critical feature of normal alveolar development and validated this observation in several experimental models.

NicheNet analysis of our scRNA-seq data predicted decreased Pdgfa-Pdgfra signaling from the epithelium to the myofibroblasts in both hyperoxia-treated and Tgfr2^{F/F};Nkx2.1-cre mice. This observation is interesting because several studies have focused on the requirement of Pdgfa-Pdgfra interactions for myofibroblast identity and function. Deletion of *Pdgfra* in mesenchymal populations of the neonatal lung has been shown to disrupt alveologenesis^{54,69}. Other studies have found lower *Pdgfra* expression in lung samples from BPD patients^{40,70}. In our current study we use flow cytometry to show a loss of PDGFR α + cells in multiple models of alveolar simplification: hyperoxia-treated mice, Tgfr2^{F/F};Nkx2.1-cre mice, 1D11-treated mice, and Ect2^{F/F};Pdgfra-CreERT2 mice. In addition to the loss of PDGFR α + cells, we also observed decreased *Pdgfra* expression within the remaining PDGFR α + cells (**Figure S6A**). Given that EdU uptake is also reduced amongst the remaining PDGFR α + cells, these results suggest Pdgfa-Pdgfra signaling might regulate myofibroblast proliferation in addition to their identity and function during neonatal lung development.

As part of our current studies, we conducted control experiments with the Gli1-CreERT2 and Pdgfra-CreERT2 alleles and discovered results with broad implications for others utilizing similar genetic tools for their work. Both alleles were generated using a knock-in/knock-out gene targeting approach which disrupts the native expression of *Gli1* and *Pdgfra*, respectively^{55,56}. Because Hedgehog and Pdgfa-Pdgfra signaling pathways are important for lung development^{71,72}, we sought to validate the effects of haploinsufficiency of *Gli1* and *Pdgfra* when using these alleles. Both Gli1-CreERT2 and Pdgfra-CreERT2 mice underwent normal alveolar development in normoxic conditions when compared to cre-negative littermates. In contrast, Pdgfra-CreERT2 mice developed worse alveolar simplification after hyperoxia treatment compared to their cre-negative littermates. The Gli1-CreERT2 results suggest this allele can be used for conditional knockout studies in either normoxic or hyperoxic conditions with little impact from *Gli1* haploinsufficiency. The Pdgfra-CreERT2 results, however, should serve as a caution to researchers utilizing genetic tools with *Pdgfra*-haploinsufficiency.

Recent work has established an essential role for TGF β signaling in normal alveolar development⁵², but the functional role of TGF β in models of BPD and alveolar simplification remains unclear. Our scRNA-seq data showed increased TGF β signaling in myofibroblasts in two injury models, so we hypothesized that excess TGF β signaling might be a driver of disease pathogenesis. To determine the functional significance of TGF β in the hyperoxia model, we treated mice with the pan-TGF β -blocking antibody 1D11. Because TGF β is required for normal alveologenesis, we

hypothesized a dose titration approach might identify a “sweet spot” to neutralize an excess of TGFβ in hyperoxia while preserving normal development in normoxia. Instead, we found that every dose of 1D11 treatment worsened hyperoxia-induced alveolar simplification while only the highest dose caused disease in normoxia. While the 1D11-normoxia results confirm an essential role for TGFβ in normal alveolar development, the 1D11-hyperoxia results suggest TGFβ plays a homeostatic function rather than a pathologic role in the hyperoxia model of injury. Because 1D11 treatment neutralizes TGFβ ligands systemically, we used *Nkx2.1-cre* and *Gli1-CreERT2* alleles to conditionally delete *Tgfr2* in the lung epithelium and mesenchyme, respectively. Confirming earlier reports, we found that disrupting TGFβ signaling to either of these populations causes alveolar simplification^{26,29}. Of significance, however, both *Tgfr2^{F/F};Nkx2.1-cre* and *Tgfr2^{F/F};Gli1-CreERT2* mice developed worse disease with neonatal hyperoxia treatment. Taken together with our 1D11-hyperoxia experiments, we conclude that TGFβ signaling to both the lung epithelium and mesenchyme is required for normal alveolar development and is protective rather than pathologic in hyperoxia-perturbed alveolar development.

Little is known about mechanisms of TGFβ activation in alveolar development and neonatal lung injury. TGFβ ligands are secreted as latent, inactive complexes that are anchored to the cell surface or the extracellular matrix⁶². These complexes prevent TGFβ from engaging its receptors, and therefore TGFβ activation is tightly regulated in tissues despite the high levels of latent TGFβ complex⁶². Integrins are cell-surface proteins that regulate interactions in the extracellular matrix, and several studies by our group have demonstrated that the epithelial integrin αvβ6 is critical for TGFβ activation in models of acute lung injury and pulmonary fibrosis^{58,60,73,74}. However, TGFβ activation by epithelial αvβ6 does not play a role in alveolar development in either normoxia or hyperoxia. We then deleted αv-integrins in the lung mesenchyme since αvβ1 and αvβ8 are the only other integrins known to activate TGFβ *in vivo*⁶². Interestingly, deletion of αv-integrins under normoxic conditions only produced a modest degree of alveolar simplification but clearly led to more severe alveolar simplification after hyperoxia.

We originally hypothesized that increased TGFβ signaling was a pathologic response to neonatal hyperoxia treatment and that targeting the TGFβ pathway might protect from disease pathogenesis. While our results disproved this hypothesis, they also seem at odds with published data where blocking TGFβ signaling was beneficial in various lung injury models. For example, it was unexpected that *Tgfr2^{F/F};Nkx2.1-cre* mice develop worse lung injury in our hyperoxia-induced model of BPD because earlier work showed these same mice to be protected from hyperoxia²⁶. We suspect this difference in outcome can be explained by the more aggressive models of lung injury used by this earlier study. Specifically, these authors used either transgenic overexpression of TGFβ or 100% hyperoxia exposure which both resulted in neonatal mortality and severe inflammation, neither of which are observed in our model of 75% hyperoxia. Additionally, many earlier studies focused on TGFβ activation in hyperoxia-induced BPD utilized chronic 85% hyperoxia such as P0-P14 or P0-P28, both of which result in inflammation and fibrosis^{8,14,16,75}. Similarly, one earlier study used transgenic expression of *Il-1b* in the developing airways to produce a BPD-like phenotype due to inflammation⁷⁶. Deletion of *Itgb6*, and therefore loss of epithelial αvβ6 integrins, conferred partial protection from disease in this injury model⁶¹. A common theme across these earlier studies implicating TGFβ in pathology is that they utilized aggressive models of lung injury which caused severe inflammation, fibrosis, and neonatal mortality, all of which recapitulate the histopathologic features of “old BPD” in the clinical setting. In contrast, the hyperoxia model used in our current study more closely represents the “new BPD” phenotype of emphysematous changes with simplified alveolar structures in the absence of lung scarring and fibrosis^{1,2}. Collectively, these data suggests that while inhibition of TGFβ may have some protective role in severe injury models associated with significant fibrosis, the major functions of TGFβ signaling during normal alveolar development and in response to moderate hyperoxia are homeostatic and protective.

There are several limitations to our current study. We used flow cytometry to phenotype population-level changes across several models of neonatal lung injury. While flow cytometry is a powerful tool to quantify population subsets and sort cells for scRNA-seq, it requires tissue digestion and generation of single-cell suspensions for downstream analysis. The cells which survive these protocols may represent a skewed population and may not be completely representative of *in vivo* conditions. For example, many fragile and dying/apoptotic cells do not survive this processing and would be excluded from analysis. Another drawback to flow cytometry is that our ability to define population subsets is limited by the knowledge and feasibility of using cell-surface markers to faithfully distinguish cell types within each population. Recent work by our group has identified cell-surface markers to characterize fibroblast subsets in the adult lung by flow cytometry, but we are unaware of similar protocols to address fibroblast heterogeneity in the neonatal lung ⁷⁷. We used scRNA-seq to characterize cell subsets within the lung mesenchyme, but the identification and validation of cell-surface proteins suitable for flow cytometric analysis of these same populations is beyond the scope of this current study. Our scRNA-seq data, together with emerging scRNA-seq from multiple other laboratories should provide a rich dataset for others to identify cell surface markers that allow more precise analysis and sorting of distinct mesenchymal populations in the developing lung.

Our current work highlights the essential role of myofibroblast proliferation and TGF β signaling in normal alveologenesis. We show that myofibroblasts are depleted during neonatal lung injury and that their loss is at least partially due to impaired proliferation and expansion during this critical window of development. We confirm the results of others who similarly observed increased TGF β signatures in the hyperoxia-injured neonatal lung and have generated interest in targeting this pathway as a therapeutic intervention for BPD. However, after conducting exhaustive studies targeting TGF β ligands, receptors and activating integrins, we conclude that increased TGF β signaling in myofibroblasts more likely represents a failed compensatory mechanism rather than a central driver of disease pathogenesis. Clinical BPD remains a heterogeneous disease encompassing both the severe inflammation, scarring and fibrosis of the “old BPD” phenotype as well as the increasingly prevalent “new BPD” phenotype of alveolar simplification and emphysematous changes ^{1,2}. Therefore, further work is necessary to contextualize our results with respect to these different clinical BPD phenotypes.

In summary, our results underscore the importance of impaired myofibroblast proliferation as a central feature of alveolar simplification in several models of murine lung injury. Further work is needed to validate these findings in human BPD. If validated, these results would suggest that efforts to prevent or reverse this process could have therapeutic value in BPD. Our sequencing data should provide useful insights to the broader community studying alveolar development and neonatal lung injury. While our current work focuses on a few of the genes and pathways highlighted by these data, we are optimistic that others will utilize this dataset to expand our understanding of the molecular mechanisms driving both normal and aberrant alveolar development.

Materials and Methods

Mice

C57BL/6 (Stock No. 000664), Nkx2.1-cre (Stock No. 008661), Pdgfra-CreERT2 (Stock No. 032770), and Gli1-CreERT2 (Stock No. 007913), and Itgav^{F/F} (Stock No. 032297) mouse lines were obtained from the Jackson Laboratory. Tgfb^{F/F} mice (exon 2 conditional allele) were described previously ⁷⁸. Ect2^{F/F} were described previously ⁶⁶, and were obtained from Dr. Alan Fields at Mayo Clinic. All lines were maintained on the C57BL/6 genetic background except for the Ect2^{F/F} line, which was

originally generated in BALB/c and backcrossed 5 generations to C57BL/6 for these studies. All animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center.

Neonatal Hyperoxia Treatment

The hyperoxia animal chamber (BioSpherix) was attached to a medical oxygen source controlled by a ProOx single gas controller (BioSpherix) set to maintain 75% oxygen under normobaric conditions. Birth was defined as <12 hours of life, and pups assigned to hyperoxia were transferred into the chamber with lactating dams and maintained from P0-P10 and then recovered in normoxia. During hyperoxia treatment, lactating dams were rotated between hyperoxia and normoxia to prevent maternal injury and to control for nutrition amongst both cohorts. Mice in both conditions were given nestlets and trail mix for additional enrichment. Mice in both conditions remained under typical 7a-7p light cycling, and chamber was checked daily to monitor temperature, humidity, and gas controller function.

Tamoxifen, Antibody, and EdU Treatments

Tamoxifen (Sigma) was dissolved in corn oil (Sigma) at 15 mg/ml, and 150 µg (10 µl) was administered via intraperitoneal (i.p.) injections on P2 and P4. Antibody clone 1D11 was used for pan-TGFβ-blocking studies [79](#), and was generated in our laboratory from a hybridoma obtained from ATCC. Antibody was diluted in PBS and administered via i.p. injections at 0, 10, 20 or 30 mg/kg on P2, P4, P6, P8 and P10. For proliferation studies, EdU (Thermo Fisher) was reconstituted in DMSO at 100 mg/ml, diluted in PBS to 5 mg/ml, and injected i.p. at 75 mg/kg 24 hours prior to harvest.

Generation of *Itgb6* Flox Mice

Itgb6 flox mice (*Itgb6*^{F/F}) were made by CRISPR/Cas9-aided homology-directed repair. Loxp sequences were inserted to flank exon 4 of *Itgb6*. Two guide RNA target sequences were chosen in the introns upstream and downstream of exon 4. crRNAs were obtained from IDT with input sequences CAGCTTATCATCCATCTAAA (upstream) and ACCTTCCTCTGACGCACTTT (downstream). Two 200bp donor DNAs were obtained from IDT. EcoRV sites (gatatc) were inserted following loxp sequences for screening purposes. The sequences of the donor DNAs are as follows:

Upstream donor DNA:

TAATCTCTCCTTTATTTGGCTCACCTTTTCTGCAACCACACACCAAGAAAGGGCAGCTTATCATCCA
TCTAAAATAACTTCGTATAGCATACATTATACGAAGTTATgatatcTGGATGCTACTTCTCCCTAGGAG
ATATAAAATATCCCAACATACACCTCCTTCTGTCCTTCAATCCTCAC

Downstream donor DNA:

TAACCTACATTTTTTCTCTGAGTTTTTCTATCAAAATAACAATTTTTGCACCTTCCTCTGACGCACT
TTATAACTTCGTATAGCATACATTATACGAAGTTATgatatcGGGAAATGTGGCTTCACTCATTGCTG
AGAGCAGCAGCCTTCATTGCAATTAAAGTCAAGAGGAAATGGG.

CRISPR/Cas9 complex (Cas9, crRNA, trRNA) and donor DNA were injected into C57BL/6 fertilized zygotes, which were then implanted into the oviducts of pseudopregnant female mice. 28 pups were born, and 6 of them had at least one allele with desired loxp insertion with two of them being homozygous for recombinant alleles. We picked one founder to expand the colony. Genotyping was performed with a forward primer 5'- CTGCAACCACACCAAGAA-3' and a reverse primer 5'- GCGTGACCTTATTAAGCTGCA-3', which provide 196bp bands for wild type alleles and 236bp bands for flox alleles.

Histology

For morphometry studies, lungs were inflated with ice-cold 4% paraformaldehyde (PFA) under constant pressure of 25 cm H₂O for 5 minutes. Lungs were carefully dissected of attached structures and transferred into vial containing cold 4% PFA. Samples were rocked in 4°C overnight, washed 3x with PBS, and dehydrated in a series of ethanol (30%, 50% and 70%). Tissues were submitted in 70% ethanol to the UCSF Gladstone Histology and Light Microscopy core for further processing for paraffin embedding and tissue blocks were sectioned for Hematoxylin and Eosin (H&E) staining. For quantification of mean linear intercept (MLI), 6-8 sections of 5 µm thickness were sampled at 20 µm levels through each set of lungs. Images were acquired on a Nikon Ti Inverted Microscope using a 10x objective and DS-Ri2 color camera. For each sample, approximately 30 images were acquired from which 12 were randomly selected for MLI quantification using ImageJ software as described previously⁸⁰. All samples were blinded during imaging and MLI quantification.

Lung Physiology

Pulmonary compliance and elastance were analyzed using the flexiVent system (SCIREQ) as previously described⁴⁵. Mice were anesthetized with ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine (3 mg/kg) before a tracheostomy was performed to cannulate the trachea with a 20-gauge catheter. Mice were then paralyzed with pancuronium (0.1 mg/kg) and analyzed using the flexiVent rodent ventilator. All mice were analyzed in a blinded fashion.

Tissue Dissociation

Mouse lungs were harvested after perfusion through the right ventricle with PBS. Lungs were dissected into individual lobes, minced with razor blades, and suspended in protease solution [0.25 % Collagenase A (Millipore Sigma), 1 U/ml Dispase II (Millipore Sigma), 2000 U/ml Dnase I (Millipore Sigma) in Dulbecco's Modified Eagle Medium (Thermo Fisher) containing 10 mM HEPES (Millipore Sigma) and 2% FBS (Millipore Sigma)]. The suspension was incubated in a 37°C water bath in a 15 ml conical for 25 minutes with aggressive trituration by glass Pasteur pipette every 8 minutes. The digestion was then quenched for 5 minutes in ice-cold PBS containing 5% FBS and 2 mM EDTA before passing cells through a 70 µm cell strainer. Cells were pelleted and resuspended in Red Blood Cell Lysing Buffer HybriMax (Sigma) for 5 minutes. RBC lysis was quenched dropwise with 5% FBS/PBS and cells were passed through a 40 µm cell strainer, washed, and resuspended in 5% FBS/PBS containing antibodies for FACS purification.

Flow Cytometry and Fluorescence-activated Cell Sorting (FACS)

After tissue dissociation, cells were resuspended in 5% FBS/PBS with Fc-blocking antibody (TruStain FcX; BioLegend) at 0.01 mg/ml for 5 minutes at room temperature. All antibodies were resuspended in Brilliant Stain Buffer (BD Biosciences) and added to Fc-blocked cells for final antibody concentration of 1:200. Cells were stained for 30 minutes on ice for surface antibody staining, washed, and then passed through another 40 µm before analysis and/or cell sorting. DRAQ7 (BioLegend) was used 1:1000 to identify dead cells. Flow cytometric cell counting was performed using CountBright Plus Absolute Counting Beads (Thermo Fisher). The following antibodies were used in this study: anti-CD9 (clone MZ3, PE; BioLegend), anti-CD31 (clone 390, A488, BV605; BioLegend, BD Biosciences), anti-CD45 (clone 30F-11, BV786, APC/Cy7, PE/Cy7; BioLegend, BD Biosciences), anti-Epcam (clone G8.8, FITC, PE, PE/Cy7; BioLegend), anti-I-A/I-E (clone M5/114.15.2, APC/Cy7, Spark UV 387; BioLegend), anti-Mcam (clone ME-9F1, A488, BUV496; BioLegend, BD Biosciences), anti-Pdgfra (clone APA5, APC, BV421, PE/Cy7; BioLegend). For proliferation studies, cells were stained for surface markers as described above and then washed, fixed, permeabilized, and processed for Click-iT EdU detection according to manufacturer's protocol (Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit; Thermo Fisher). All samples

were analyzed and sorted using an Aria Fusion (Becton Dickinson) with 85 μm nozzle except for scRNA-seq samples, which were collected using a FACS Aria III (Becton Dickinson) with 100 μm nozzle. Flow cytometry data were analyzed using FlowJo v10.8 (Becton Dickinson).

Single-cell RNA-seq Library Preparation and Sequencing

Lung tissues were harvested from $\text{Tgfr2}^{\text{F/F}}$ and $\text{Tgfr2}^{\text{F/F}};\text{Nkx2.1-cre}$ mice at P7 and P14 after hyperoxia or normoxia treatment. Two pups were harvested for each genotype, exposure, and timepoint for total of 8 mice on P7 and 8 mice on P14. Single cell suspension was obtained as described above. To enrich for epithelial and mesenchymal populations for sequencing input, 1×10^5 CD45+ cells, 1×10^5 CD31+ cells, and 1×10^6 CD45-/CD31-cells were sorted for each sample and collected in 10% FBS/PBS. Sorted cells were then counted and labeled with oligonucleotide tags for multiplexing using 10x Genomics 3' CellPlex Kit Set A. All 8 biologic samples for the P7 timepoint were pooled and 60,000 cells / lane were loaded onto 2 lanes of a Chromium Next GEM Chip (10x Genomics). The same workflow was used for P14 samples with all 8 biologic samples for P14 pooled together and loaded onto 2 lanes as well. Lanes 1 and 2 were therefore technical replicates for each biologic sample and similarly lanes 3 and 4 were also technical replicates. Chromium Single Cell 3' v3.1 (10x Genomics) reagents were used for library preparation according to the manufacturer's protocol. The libraries were sequenced on an Illumina NovaSeq 6000 S4 flow cell.

Sequencing Data Processing

Fastq files were uploaded to the 10x Genomics Cloud (<https://www.10xgenomics.com/products/cloud-analysis>) and reads were aligned to the mouse reference genome mm10. The data were demultiplexed, and cells with multiple oligonucleotide tags were identified as multiplets and removed by the 10x Genomics cloud analysis function with default parameters. Raw count matrices were imported to the R package Seurat v4.1.1⁸¹, and cells with fewer than 200 detected genes, more than 7500 detected genes, or more than 15% mitochondria genes were excluded. We used the DoubletFinder package⁸² for each sample using an estimated multiple rate of 1% remove doublets that were not detected upon alignment. We then merged all the sample objects, identified the top variable genes using Seurat's *FindVariableGenes* function, and integrated the samples using the *RunFastMNN* function of the SeuratWrappers R package⁸³. For visualization, Seurat's *RunUMAP* function was performed using MNN dimensional reduction.

Twenty-three clusters were initially identified from a total of 27,839 cells using Seurat's *FindNeighbors* and *FindClusters* functions with resolution = 0.3. The expression of canonical lineage markers (*Epcam*, *Col1a1*, *Pecam1*, *Ptpcr*, *Msln*) was used to define major cell types of the lung (epithelium, mesenchyme, hematopoietic, endothelium, mesothelium). Cluster 9 (729 cells) was identified as a contaminant and excluded from further analysis due to cells showing up in multiple locations across the UMAP embedding. While re-clustering and annotating epithelial, hematopoietic, and endothelial subpopulations we identified an additional 500 cells that clustered independently from known cell types within each subcluster, had less than 1000 detected genes, and were enriched for the expression of multiple canonical cell types, suggesting these cells were also contaminants. After removing these cells and cluster 9 cells, the remaining 26,610 cells were re-clustered with *FindVariableGenes*, *RunFastMNN*, *RunUMAP*, *FindNeighbors*, and *FindClusters* functions with clustering resolution = 0.8. Thirty clusters were identified at this stage and differentially expressed genes for each cluster were identified using *FindAllMarkers* focusing on genes expressed by more than 20% of cells (either within or outside of a cluster) and with a log fold change greater than 0.2. Using publicly available data, we were able to merge and annotate clusters to obtain the 25 clusters depicted in this study. Mesenchymal cells were re-clustered using the same workflow outlined above with clustering resolution = 0.3. Clusters were annotated based on a combination of three publicly available data sets for the neonatal lung. For Qiagen Ingenuity Pathway Analysis (Qiagen, <https://digitalinsights.qiagen.com/IPA>)⁵¹, differentially expressed genes in myofibroblasts were identified by comparing either CTRL RA vs O2 cells or RA CTRL vs cKO cells using Seurat's *FindMarkers* function. These lists were uploaded to Qiagen IPA to identify

predicted upstream regulators for each comparison. To identify shared predicted upstream regulators, lists were filtered for z-score >1.5 (upregulated) or z-score <-1.5 (downregulated), and the filtered results were compared by Venn diagram between the two comparisons.

NicheNet⁵³ [↗](#) was used to compare signaling between conditions, as described in the “Differential NicheNet analysis between conditions of interest” vignette on the GitHub repository. Standard NicheNet statistical thresholds described in the vignette were used in the analysis. Alveolar epithelial subtypes (AT2, AT2 Lyz1, AT2 Activated, AT1/AT2, and Prolif. AT2) were used as a combined sender cell population, and alveolar and ductal myofibroblasts were used as combined receiver cell populations. Differential signaling from sender-to-receiver cells was compared across two conditions, CTRL RA vs CTRL O2 and CTRL RA vs cKO RA, and the results were examined for pathways present in both comparisons.

Data Analysis

Mean linear intercept morphometry was quantified using ImageJ software as described previously⁸⁰ [↗](#). scRNA-seq data analysis was performed in R version 4.1.1. Statistical tests were performed in GraphPad Prism version 9.4.0.

Data Availability

The data that support this study are available upon reasonable request. Sequencing data and Seurat objects were deposited in the Gene Expression Omnibus Series GSE243129.

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Disclosures

D.S. is a founder of Pliant Therapeutics, a member of the Genentech Scientific Review Board, a member of the Amgen Immunology Scientific Advisory Board, and a member of the Scientific Review Board for Lila Biologics. No funding or reagents from any of these companies were used for this project. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

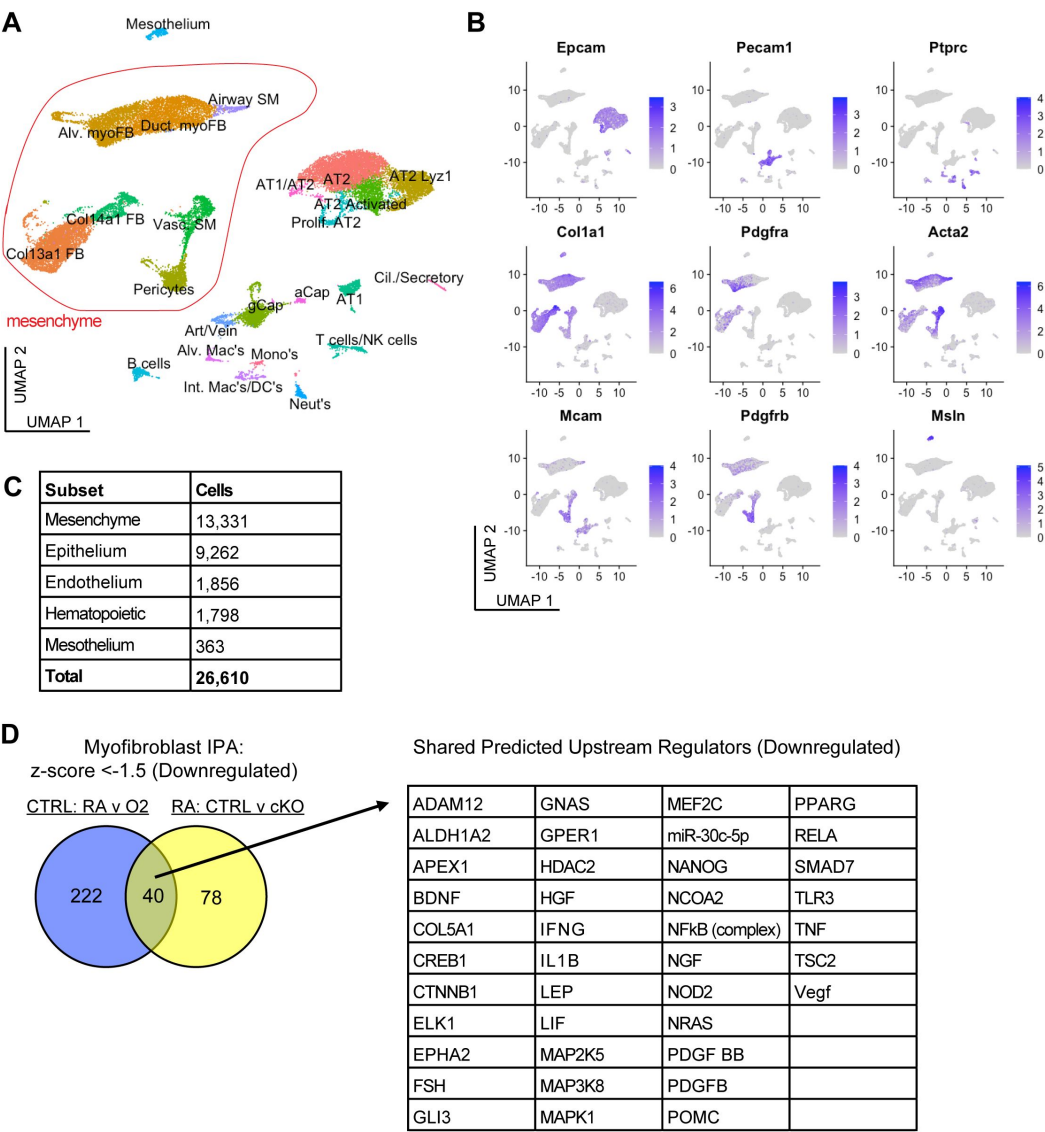
Author Contributions

I.S.K and D.S. conceived the study, interpreted the data, and wrote the manuscript. I.S.K., C.M., X.R., V.A., and M.C. performed the experiments and/or analyzed the data. T.T. and A.A. provided critical resources for this study. D.S. supervised the study.

Abbreviations

- α -SMA: alpha-smooth muscle actin
- BPD: bronchopulmonary dysplasia
- cHet: conditional heterozygous
- cKO: conditional knockout
- EdU: 5-ethynyl-2'-deoxyuridine
- PDGFR α : platelet-derived growth factor receptor alpha
- P: postnatal day
- scRNA-seq: single-cell RNA-sequencing
- TGF β : transforming growth factor-beta
- TGF β R2: transforming growth factor-beta receptor 2
- UMAP: Uniform Manifold Approximation and Projection.

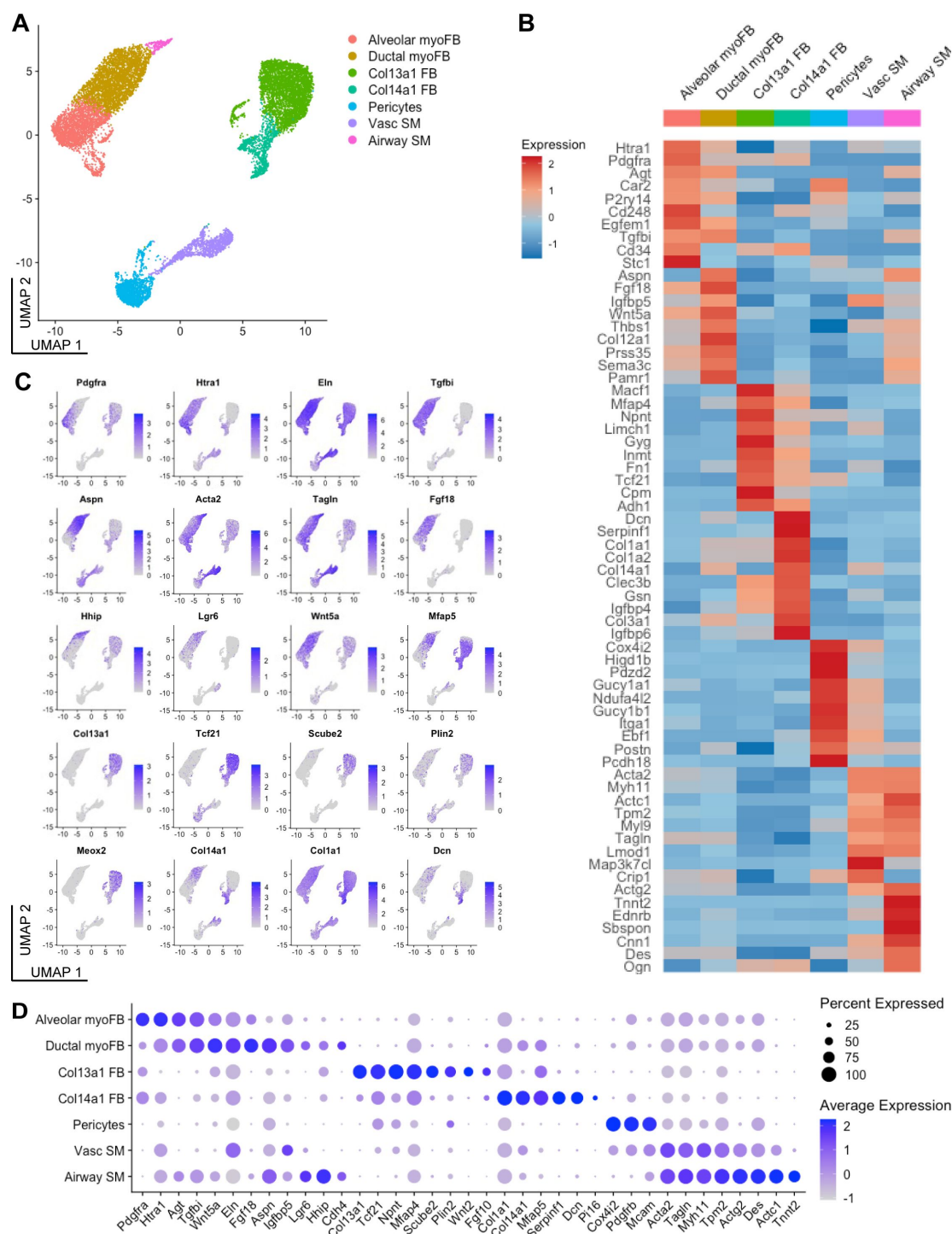
Supplementary Figure Legend



Supplementary Figure 1.

scRNA-seq of Murine Lungs With Neonatal Hyperoxia Treatment or Loss of Epithelial TGFβ Signaling.

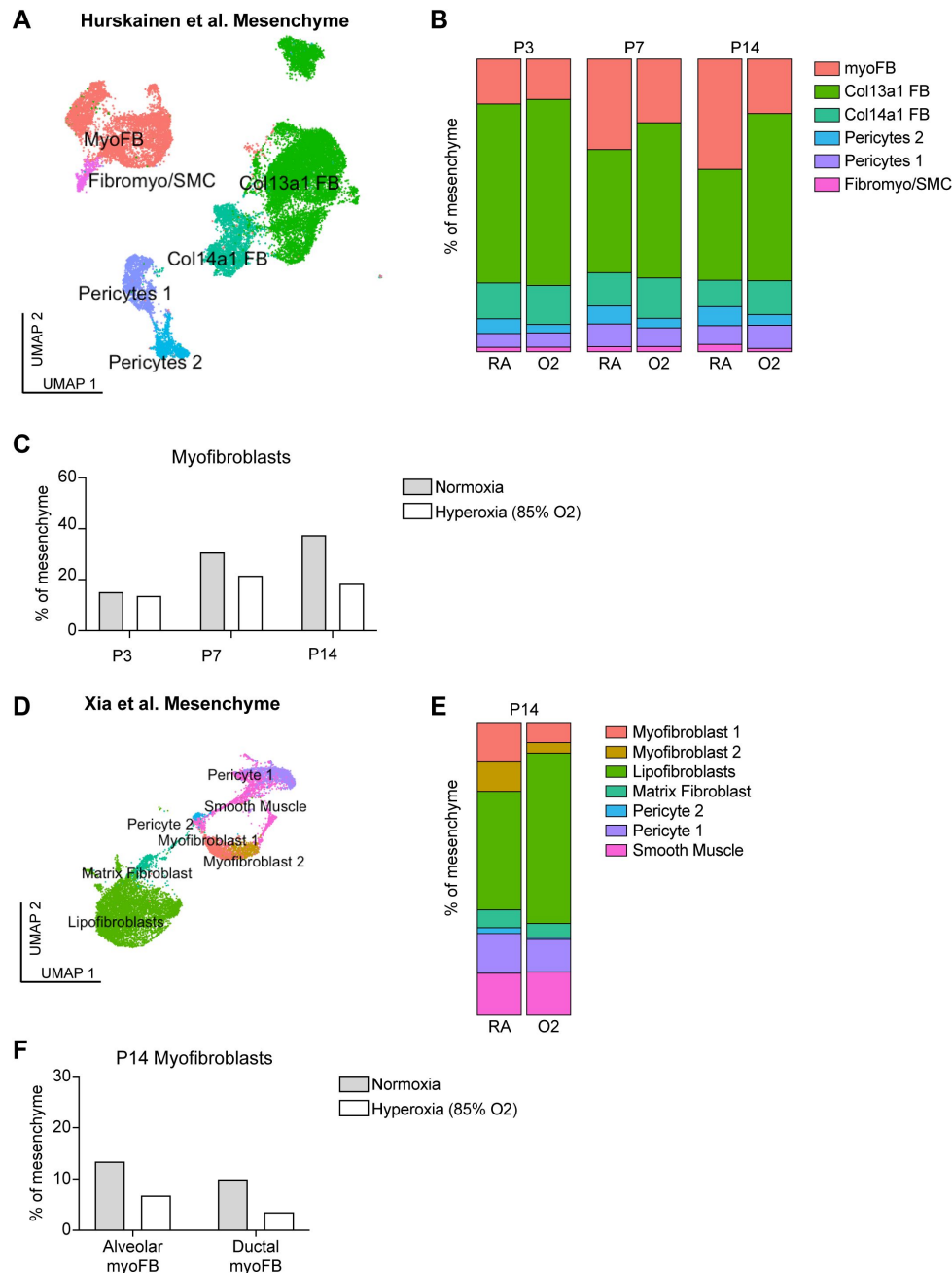
(A) UMAP projection of all scRNA-seq data. Outlined in red are the mesenchymal cell populations. (B) UMAP plots showing expression levels of canonical markers for epithelial, endothelial, hematopoietic, mesenchymal, and mesothelial populations. (C) Total cell number within each of the indicated major lung populations. (D) Differentially expressed genes in myofibroblasts were identified by comparing either CTRL RA vs O2 cells or RA CTRL vs cKO cells. These lists were subsequently analyzed by Qiagen IPA to identify predicted upstream regulators for each comparison. The Venn-diagram on left depicts the number of overlapping predicted upstream regulators with z-score <-1.5 (upregulated), while the table on right lists these 40 shared upstream regulators.



Supplementary Figure 2.

Characterization of Mesenchymal Cell Clusters by scRNA-seq.

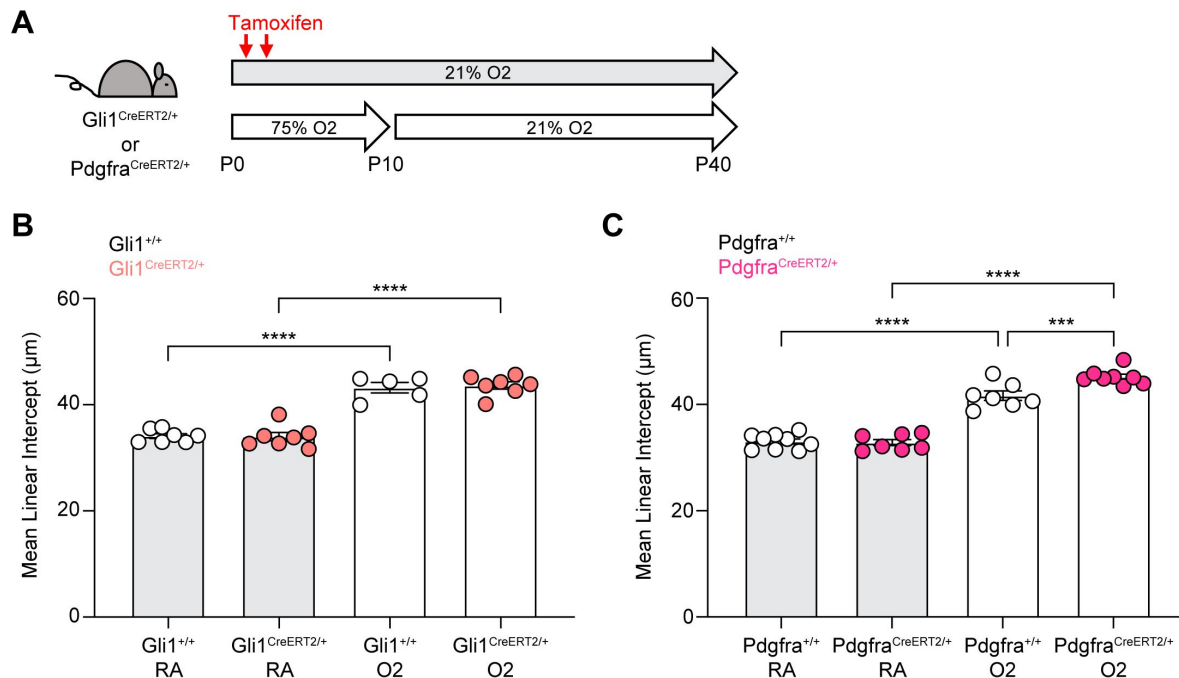
(A) UMAP projection of mesenchymal cells from scRNA-seq data as outlined in [Figure S1](#). (B) Heatmap of the top ten most differentially expressed genes across mesenchymal clusters. The intensity of expression is indicated as specified by the color legend. (C) UMAP plots showing expression levels of select canonical markers for alveolar myofibroblast, ductal myofibroblast, Col13a1 fibroblast, and Col14a1 fibroblast populations as depicted by recent work by Hurskainen *et al.* and Narvaez Del Pilar *et al.* (D) Dot plot showing selected markers for each cluster within the mesenchyme.



Supplementary Figure 3.

Re-analysis of Published Data Confirms Loss of Myofibroblasts With Neonatal Hyperoxia Treatment.

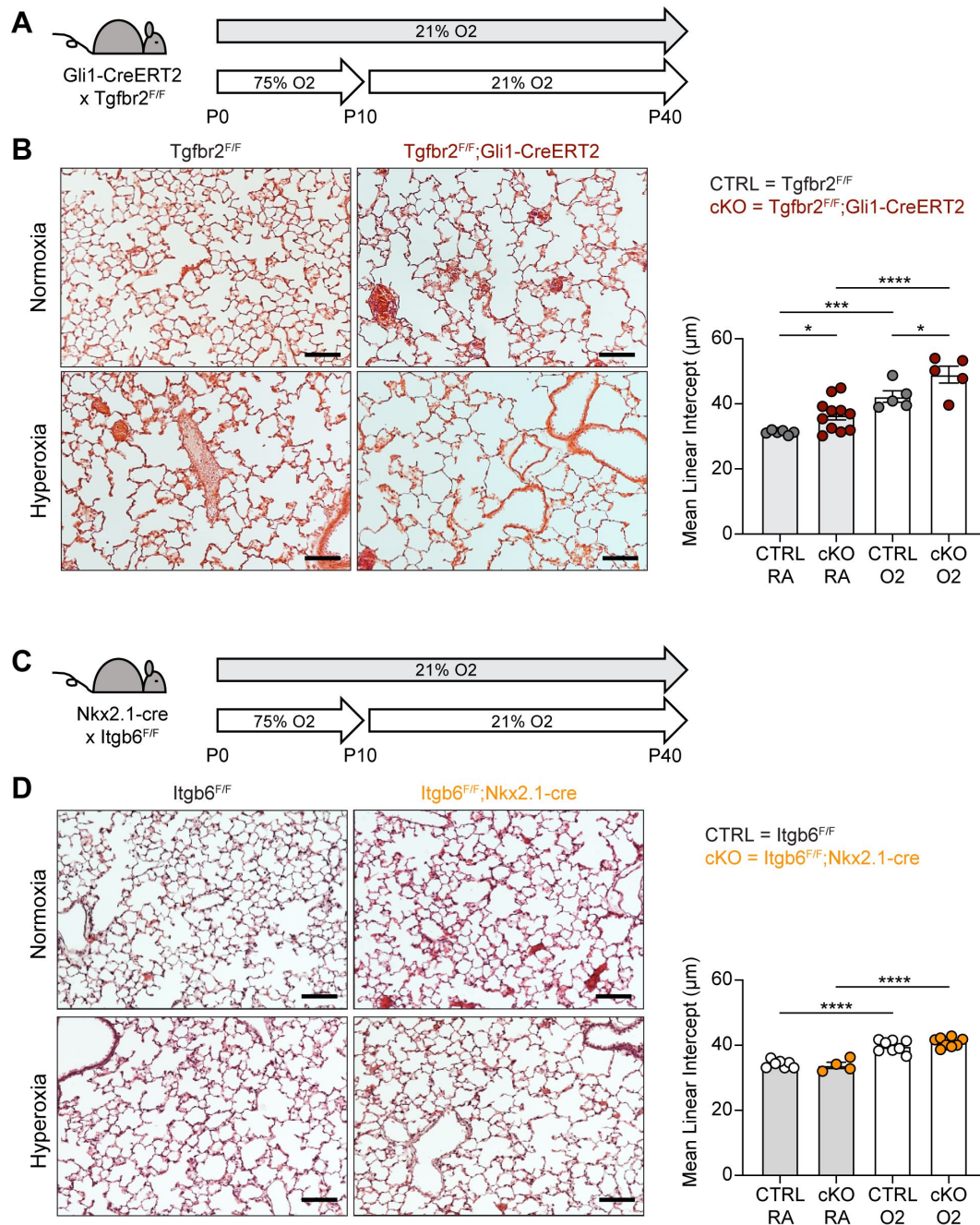
(A) Hurskainen *et al.* treated C57BL/6 wildtype mice with 85% hyperoxia versus normoxia from P0-P14 and analyzed the lungs by scRNA-seq at P3, P7, and P14 ⁴⁷. The Seurat object used for publication was provided by the authors. UMAP projection shows the mesenchymal populations as defined by Hurskainen *et al.* (B) By using metadata within the Seurat object, we graphed the frequency of each mesenchymal population by treatment condition and time point. (C) The frequency of myofibroblasts within the mesenchyme as depicted in (B). (D) Xia *et al.* treated C57BL/6 wildtype mice with 85% hyperoxia versus normoxia from P0-P14 and analyzed the lungs by scRNA-seq at P14 ⁵⁰. The Seurat object used for publication was provided by the authors. UMAP projection shows the mesenchymal populations as defined by Xia *et al.* (E) By using metadata within the Seurat object, we graphed the frequency of each mesenchymal population by treatment condition and time point. (F) The frequency of alveolar and ductal myofibroblasts within the mesenchyme as depicted in (E). Graphs in (C) and (F) depict one value for each condition because we were unable to extract replicate values from the data provided.



Supplementary Figure 4.

Gli1-CreERT2 Allele Does Not Disrupt Alveolar Development, But Pdgfra-CreERT2 Allele Worsens Hyperoxia-induced Injury.

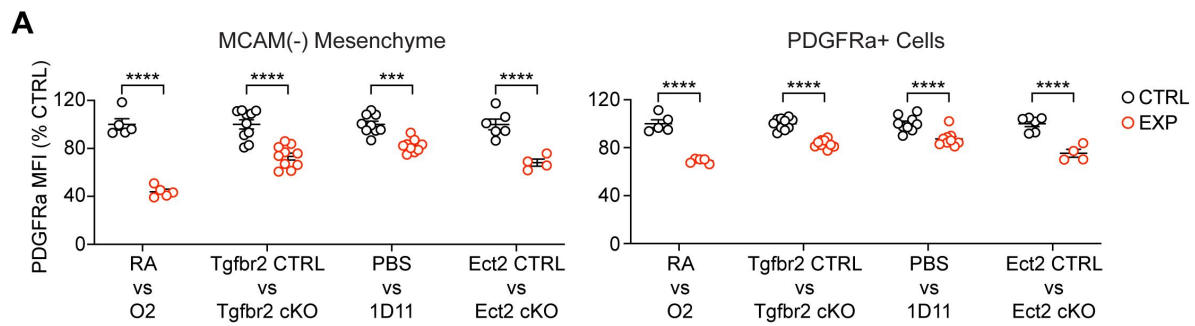
(A) Either Gli1-CreERT2 or Pdgfra-CreERT2 mice and their cre-negative littermates were injected with tamoxifen on P2 and P4, treated in 75% hyperoxia versus normoxia from P0-P10, and recovered in room air until harvest at P40 for analysis by histology. (B) Mean linear intercepts of Gli1^{CreERT2/+} and Gli1^{+/+} mice treated as outlined in (A) and harvested at P40. (C) Mean linear intercepts of Pdgfra^{CreERT2/+} and Pdgfra^{+/+} mice treated as outlined in (A) and harvested at P40. Data compared by ANOVA with Fisher's post hoc test. Error bars depict mean ± SEM. ***p<0.001, ****p<0.0001.



Supplementary Figure 5.

Loss of TGFβ Signaling to Lung Mesenchyme Causes Worse Disease in Hyperoxia While Itgb6 Plays No Role in Alveolar Development.

(A) Tgfr2^{F/F} and Tgfr2^{F/F};Gli1-CreERT2 littermates were injected with tamoxifen on P2 and P4, treated in 75% hyperoxia versus normoxia from P0-P10, and recovered in room air until harvest at P40 for analysis by histology. (B) H&E sections of representative lungs from (A) harvested at P40 (left). Mean linear intercepts calculated for all treatment groups (right). (C) Itgb6^{F/F} and Itgb6^{F/F};Nkx2.1-cre littermates were treated in 75% hyperoxia versus normoxia from P0-P10, and recovered in room air until harvest at P40 for analysis by histology (D) H&E sections of representative lungs from (C) harvested at P40 (left). Mean linear intercepts calculated for all treatment groups (right). Data compared by ANOVA with Fisher's post hoc test. Error bars depict mean ± SEM. *p<0.05, ***p<0.001, ****p<0.0001. Scale bars = 100 μm.



Supplementary Figure 6.

Decreased PDGFRα Mean Fluorescence Intensity Across Multiple Models of Alveolar Simplification.

(A) Mean fluorescence intensity (geometric mean, MFI) of PDGFRα antibody staining on MCAM-negative mesenchymal cells (CD45-, CD31-, Epcam-) and PDGFRα+ cells (CD45-, CD31-, Epcam-, MCAM-, PDGFRα+) as quantified by flow cytometry. Each column represents an experiment shown earlier in this study: normoxia vs hyperoxia at P10 (**Figure 2**), Tgfr2^{F/F} vs Tgfr2^{F/F};Nkx2.1-cre at P10 (**Figure 2**), PBS vs 1D11 at P10 (**Figure 8**), Ect2^{F/F} vs Ect2^{F/F};Pdgfra-CreERT2 at P14 (**Figure 9**). To compare values across multiple experiments, MFI's were normalized to the control condition within each experiment. Data compared by ANOVA with Fisher's post hoc test. Error bars depict mean ± SEM. ***p<0.001, ****p<0.0001.

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Reviewer #1 (Public review):

Summary:

In this study, the authors used both the commonly used neonatal hyperoxia model as well as cell-type-specific genetic inactivation of *Tgfb2* models to study the basis of BPD. The bulk of the analyses focus on the mesenchymal cells. Results indicate impaired myofibroblast proliferation, resulting in decreased cell number. Inactivation of *Ect2* in *Pdgfra*-lineaged cells, preventing cytokinesis of myofibroblasts, led to alveolar simplification. Together, the findings demonstrate that disrupted myofibroblast proliferation is a key contributor to BPD pathogenesis.

Strengths:

Overall, this comprehensive study of BPD models advances our understanding of the disease. The data are of high quality.

Comments on latest version:

In the revision, the authors addressed all critiques.

<https://doi.org/10.7554/eLife.94425.2.sa3>

Reviewer #2 (Public review):

Summary:

In this study the authors systematically explore mechanism(s) of impaired postnatal lung development with relevance to BPD (bronchopulmonary dysplasia) in two murine models of 'alveolar simplification', namely hyperoxia and epithelial loss of TGF β signaling. The work presented here is of great importance, given the limited treatment options for a clinical entity frequently encountered in newborns with high morbidity and mortality that is still poorly

understood, and the unclear role of TGF β signaling, its signaling levels, and its cellular effects during secondary alveolar septum formation, a lung structure generating event heavily impacted by BPD. The authors show that hyperoxia and epithelial TGF β signaling loss have similar detrimental effects on lung structure and mechanical properties (emphysema-like phenotype) and are associated with significantly decreases numbers of PDGFR α -expressing cells, the major cell pool responsible for generation of postnatal myofibroblasts. They then use a single-cell transcriptomic approach combined with pathway enrichment analysis for both models to elucidate common factors that affect alveologenesis. Using cell communication analysis (NicheNet) between epithelial and myofibroblasts they confirm increased projected TGF β -TGF β R interactions and decreased projected interactions for PDGFA-PDGFR α , and other key pathways, such as SHH and WNT. Based on these results they go on to uncover in a sequela of experiments that surprisingly, increased TGF β appears reactive to postnatal lung injury and rather protective/homeostatic in nature, and the authors establish the requirement for α V integrins, but not the subtype α V β 6, a known activator of TGF β signaling and implied in adult lung fibrosis. The authors then go beyond the TGF β axis evaluation to show that mere inhibition of proliferation by conditional KO of *Ect2* in *Pdgfra* lineage results in alveolar simplification, pointing out the pivotal role of PDGFR α -expressing myofibroblasts for normal postnatal lung development.

Strengths:

- (1) The approach including both pharmacologic and mechanistically-relevant transgenic interventions both of which produced consistent results provides robustness of the results presented here.
- (2) Further adding to this robustness is the use of moderate levels of hyperoxia at 75% FiO₂, which is less extreme than 100% FiO₂ frequently used by others in the field, and therefore favors the null hypothesis.
- (3) The prudent use of advancement single cell analysis tools, such as NicheNet to establish cell interactions through the pathways they tested and the validation of their scRNA-seq results by analysis of two external datasets. Delineation of the complexity of signals between different cell types during normal and perturbed lung development, such as attempted successfully in this study, will yield further insights into the underlying mechanism(s).
- (4) The combined readout of lung morphometric (MLI) and lung physiologic parameters generates a clinically meaningful readout of lung structure and function.
- (5) The systematic evaluation of TGF β signaling better determines the role in normal and postnatally-injured lung.

Weaknesses:

- (1) While the study convincingly establishes the effect of lung injury on the proliferation of PDGFR α -expressing cells, differentiation is equally important. Characterization of PDGFR α expressing cells and tracking the changes in the injury models in the scRNA analysis, a key feature of this study, would benefit from expansion in this regard. PDGFR α lineage gives rise to several key fibroblast populations, including myofibroblasts, lipofibroblasts, and matrix-type fibroblasts (Collagen13a1, Collagen14a1). Lipofibroblasts constitute a significant fraction of PDGFR α ⁺ cells, and expand in response to hyperoxic injury, as shown by others. Collagen13a1-expressing fibroblasts expand significantly under both conditions (Fig.3), and appear to contain a significant number of PDGFR α -expressing cells (Suppl Fig.1). Effects of the applied injuries on known differentiation markers for these populations should be documented. Another important aspect would be to evaluate whether the protective/homeostatic effect of TGF β signaling is by supporting differentiation of myofibroblasts. Postnatal Gli1 lineage gains expression of PDGFR α and differentiation markers, such as Acta2 (SMA) and Eln (Tropoelastin). Loss of PDGFR α expression was shown

to alter Elastin and TGFb pathway related genes. TGFb signaling is tightly linked to the ECM via LTBP, Fibrillins and Fibulins. An additional analysis in the aforementioned regards has great potential to more specifically identify the cell type(s) affected by the loss of TGFb signaling and allow analysis of their specific transcriptomic changes in response and underlying mechanism(s) to postnatal injury.

[The authors have added in detailed transcriptomic description of the fibroblast populations.]

(2) Of the three major lung abnormalities encountered in BPD, the authors focus on alveolarization impairment in great detail, to very limited extent on inflammation, and not on vascularization impairment. However, this would be important not only to better capture the established pathohistologic abnormalities of BPD, but also is needed since the authors alter TGFb signaling, and inflammatory and vascular phenotypes with developmental loss of TGFb signaling and its activators have been described. Since the authors make the point about absence of inflammation in their BPD model, it will be important to show the evidence.

[While this an important question, assessment of these components goes beyond the scope of this paper.]

(3) Conceptually it would be important that in the discussion the authors reconcile their findings in the experimental BPD models in light of human BPD and potential implications it might have on new ways to target key pathways and cell types for treatment. This allows the scientific community to formulate the next set of questions in a disease relevant manner.

[The authors have amended the discussion in this regard.]

Comments on latest version:

This reviewer would like to thank the authors for their efforts to address the concerns, in particular the better transcriptomic description of the fibroblast populations. The reviewer is well aware of the issues with PDGFRa antibodies that work on mouse tissue and also the problem with available reporters and lineage tracers in terms of haploinsufficiency.

There are no further concerns from this reviewer's side.

<https://doi.org/10.7554/eLife.94425.2.sa2>

Reviewer #3 (Public review):

This paper seeks to understand the role of alveolar myofibroblasts in the abnormal lung development after saccular stage injury.

Strengths:

(1) Multiple models of neonatal injury are used, hyperoxia and transgenic models that target alveolar myofibroblasts.

(2) The authors integrate their data with prior published single-cell data from neonatal hyperoxia injury models and demonstrate concordant findings.

Weaknesses:

(1) As the authors acknowledge in the discussion, there are no spatial and temporal validation data of the single-cell findings. As the ductal myofibroblasts has many overlapping genes, localizing and quantifying the loss of these cells in injury as a plausible mechanistic driver would greatly strengthen the conclusion.

(2) As they note in their response, this proved to be technically difficult and current Pdgfra-lineage trace tools are not without their own limitations.

Summary:

Taken together, this manuscript provides a rich data set from a model of irreversible neonatal lung injury. The single-cell analysis methods are well-articulated and the limitations are acknowledged, allowing this paper to provide a foundation for future work to spatially and temporally validate these claims.

<https://doi.org/10.7554/eLife.94425.2.sa1>

Author response:

The following is the authors' response to the original reviews.

We have responded to these criticisms below and have revised the main text and figures. Here, we outline the major points of our responses:

(1) The reviewers asked for more clarification regarding cell type annotation in the lung mesenchyme as shown in Figure 3C. We have included a new supplementary figure (Supplementary Figure 2) which shows differentially expressed genes amongst these mesenchymal cell subsets using a variety of visualization tools including a heatmap, UMAP plots, and the dotplot which was originally shown in Supplementary Figure 1D. The other supplemental figures have been re-numbered.

(2) We acknowledge the lack of consensus in the field regarding the nomenclature of fibroblast subsets in the developing mouse lung. We are not attempting to define new subsets, but rather we adopted annotations based on previously published work. Specifically, we used Seurat to define mesenchymal cell clusters and then compared the gene expression patterns of these clusters to published work by Hurskainen et al. (Bernard Thebaud's group) and Narvaez Del Pilar et al. (Jichou Chen's group). We acknowledge these annotations might conflict with other published data, but any approach to choosing a cell label would be subject to scrutiny. For example, Col13a1 fibroblasts share markers with cells which have been defined by others as lipofibroblasts or alveolar fibroblasts. Similarly, Col14a1 fibroblasts appear to share markers with matrix fibroblasts. Further work is clearly needed to address these discrepancies, and we hope that making our data publicly available will help that effort.

(3) The reviewers asked us to interrogate changes in canonical markers of fibroblast subsets (i.e. lipofibroblasts, matrix fibroblasts) to address whether the apparent loss of myofibroblasts could be explained by a change in myofibroblast specification/differentiation. We have included these data in the responses, but because we are unable to draw any clear conclusions from these results, we do not feel these data warrant inclusion in the manuscript/figures.

(4) As highlighted in the eLife assessment, our study does not include tissue validation (i.e. immunohistochemistry) of myofibroblast markers to distinguish whether the loss of myofibroblasts is attributable to lack of proliferation and/or changes in differentiation/specification. We spent considerable time over the past few months attempting to address these questions, however we were unable to produce convincing PDGFRa staining on tissues that we had collected during our original studies. Without PDGFRa staining, we regretfully could not co-stain for other useful markers to assess proliferation (EdU), apoptosis (TUNEL or caspase), or fibroblast function/specification (ACTA2, SM22a/TAGLN, ADRP, etc). We suspect that these experiments would require optimization of

tissue fixation/processing at the time of harvest or the inclusion of a *Pdgfra* lineage tool for better identification of these cells by immunohistochemistry. Given that the majority of *Pdgfra* lineage tools require a knock-in/knock-out approach, data generated using these tools should be interpreted with caution given our results here show that *Pdgfra*-haploinsufficiency alone worsens disease outcomes after hyperoxia exposure.

In summary, we have addressed several concerns raised by the reviewers and have attempted to perform some of the additional experiments suggested.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

*In this study, the authors used both the commonly used neonatal hyperoxia model as well as cell-type-specific genetic inactivation of *Tgfr2* models to study the basis of BPD. The bulk of the analyses focus on the mesenchymal cells. Results indicate impaired myofibroblast proliferation, resulting in decreased cell number. Inactivation of *Ect2* in *Pdgfra*-lineaged cells, preventing cytokinesis of myofibroblasts, led to alveolar simplification. Together, the findings demonstrate that disrupted myofibroblast proliferation is a key contributor to BPD pathogenesis.*

Strengths:

Overall, this comprehensive study of BPD models advances our understanding of the disease. The data are of high quality.

Weaknesses:

The critiques are mostly minor and can be addressed without extensive experimentation.

Reviewer #2 (Public Review):

Summary:

In this study, the authors systematically explore the mechanism(s) of impaired postnatal lung development with relevance to BPD (bronchopulmonary dysplasia) in two murine models of 'alveolar simplification', namely hyperoxia and epithelial loss of TGF β signaling. The work presented here is of great importance, given the limited treatment options for a clinical entity frequently encountered in newborns with high morbidity and mortality that is still poorly understood, and the unclear role of TGF β signaling, its signaling levels, and its cellular effects during secondary alveolar septum formation, a lung structure generating event heavily impacted by BPD. The authors show that hyperoxia and epithelial TGF β signaling loss have similar detrimental effects on lung structure and mechanical properties (emphysema-like phenotype) and are associated with significantly decreased numbers of PDGFR α -expressing cells, the major cell pool responsible for generation of postnatal myofibroblasts. They then use a single-cell transcriptomic approach combined with pathway enrichment analysis for both models to elucidate common factors that affect alveologenesis. Using cell communication analysis (NicheNet) between epithelial and myofibroblasts they confirm increased projected TGF β -TGF β R interactions and decreased projected interactions for PDGFA-PDGFR α , and other key pathways, such as SHH and WNT. Based on these results they go on to uncover in a sequela of experiments that surprisingly, increased TGF β appears reactive to postnatal lung injury and rather protective/homeostatic in nature, and the authors establish the requirement for α V integrins, but not the subtype α V β 6, a known activator of TGF β signaling and implied in adult lung fibrosis. The authors then go beyond the TGF β

axis evaluation to show that mere inhibition of proliferation by conditional KO of *Ect2* in *Pdgfra* lineage results in alveolar simplification, pointing out the pivotal role of PDGFR α -expressing myofibroblasts for normal postnatal lung development.

Strengths:

(1) The approach including both pharmacologic and mechanistically-relevant transgenic interventions both of which produced consistent results provides robustness of the results presented here.

(2) Further adding to this robustness is the use of moderate levels of hyperoxia at 75% FiO₂, which is less extreme than 100% FiO₂ frequently used by others in the field, and therefore favors the null hypothesis.

(3) The prudent use of advanced single-cell analysis tools, such as NicheNet to establish cell interactions through the pathways they tested and the validation of their scRNA-seq results by analysis of two external datasets. Delineation of the complexity of signals between different cell types during normal and perturbed lung development, such as attempted successfully in this study, will yield further insights into the underlying mechanism(s).

(4) The combined readout of lung morphometric (MLI) and lung physiologic parameters generates a clinically meaningful readout of lung structure and function.

(5) The systematic evaluation of TGF β signaling better determines the role in normal and postnatally-injured lungs.

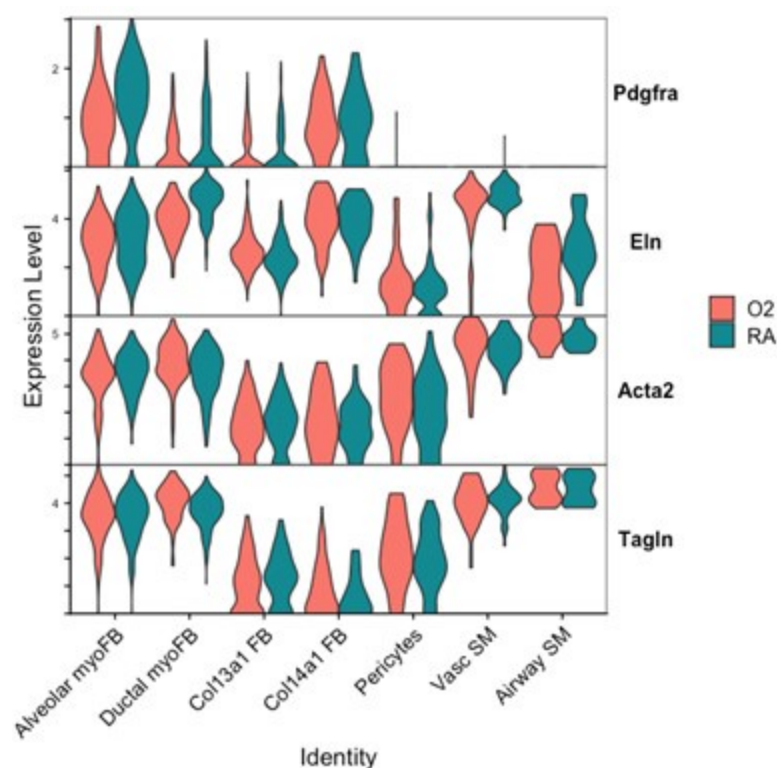
Weaknesses:

(1) While the study convincingly establishes the effect of lung injury on the proliferation of PDGFR α -expressing cells, differentiation is equally important. Characterization of PDGFR α expressing cells and tracking the changes in the injury models in the scRNA analysis, a key feature of this study, would benefit from expansion in this regard. PDGFR α lineage gives rise to several key fibroblast populations, including myofibroblasts, lipofibroblasts, and matrix-type fibroblasts (Collagen13a1, Collagen14a1). Lipofibroblasts constitute a significant fraction of PDGFR α + cells, and expand in response to hyperoxic injury, as shown by others. Collagen13a1-expressing fibroblasts expand significantly under both conditions (Figure 3), and appear to contain a significant number of PDGFR α -expressing cells (Suppl Fig.1). Effects of the applied injuries on known differentiation markers for these populations should be documented. Another important aspect would be to evaluate whether the protective/homeostatic effect of TGF β signaling is supporting the differentiation of myofibroblasts. Postnatal Gli1 lineage gains expression of PDGFR α and differentiation markers, such as Acta2 (SMA) and Eln (Tropoelastin). Loss of PDGFR α expression was shown to alter Elastin and TGF β pathway-related genes. TGF β signaling is tightly linked to the ECM via LTBP, Fibrillins, and Fibulins. An additional analysis in the aforementioned regard has great potential to more specifically identify the cell type(s) affected by the loss of TGF β signaling and allow analysis of their specific transcriptomic changes in response and underlying mechanism(s) to postnatal injury.

We attempted to conduct additional analyses on our sequencing data to evaluate the impact of lung injury on the differentiation of *Pdgfra*-expressing cells towards other fibroblast lineages. To specifically address the impact of hyperoxia on fibroblast differentiation, we subsetting wildtype cells collected at the P7 timepoint (while pups were still undergoing hyperoxia treatment) from the larger data set. Shown below are several Violin Plots comparing gene expression between RA and O₂ conditions across the mesenchymal populations.

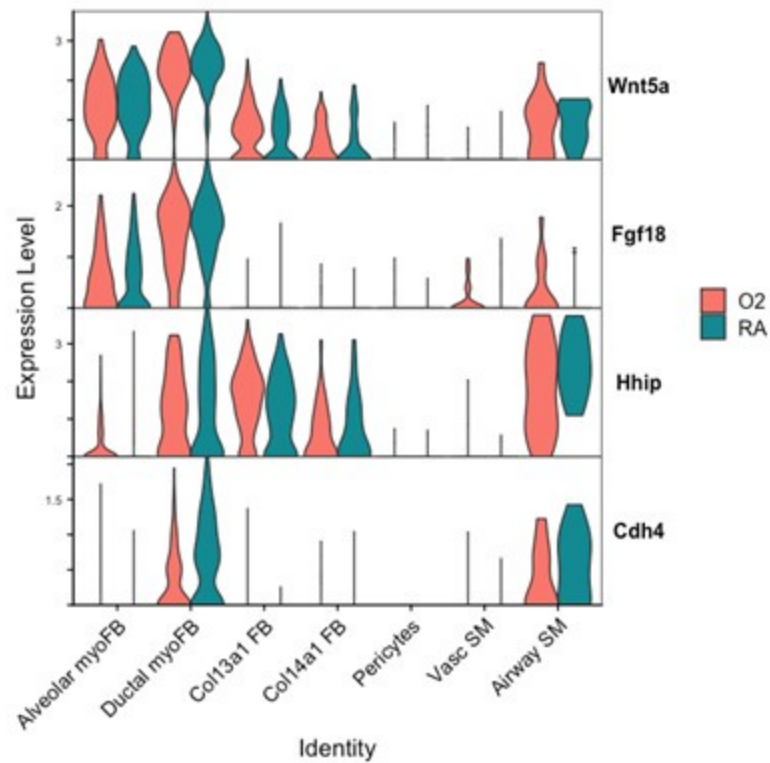
Although there are some interesting observations in this analysis, we could not identify a consistent theme from these data which could clearly answer the reviewers' questions. We see a clear reduction of *Pdgfra* and *Eln* in both myofibroblast subsets with hyperoxia, which support our findings of reductions in the myofibroblast subsets. *Acta2* and *Tagln* appear slightly lower in alveolar myofibroblasts, but both are higher in ductal myofibroblasts. Interestingly, both *Acta2* and *Tagln* are higher in Col14a1 fibroblasts with hyperoxia. The functional relevance of these data are unclear because there appears to be higher per-cell expression of *Acta2* in ductal myofibroblasts while the relative contribution of these cells is reduced (Figure 3D-E). Col14a1 fibroblasts show increased *Acta2* and *Tagln* expression and are slightly increased in proportion at P7 with hyperoxia treatment (Figure 3D), albeit to a much lesser degree compared to Col13a1 fibroblasts.

Author response image 1.

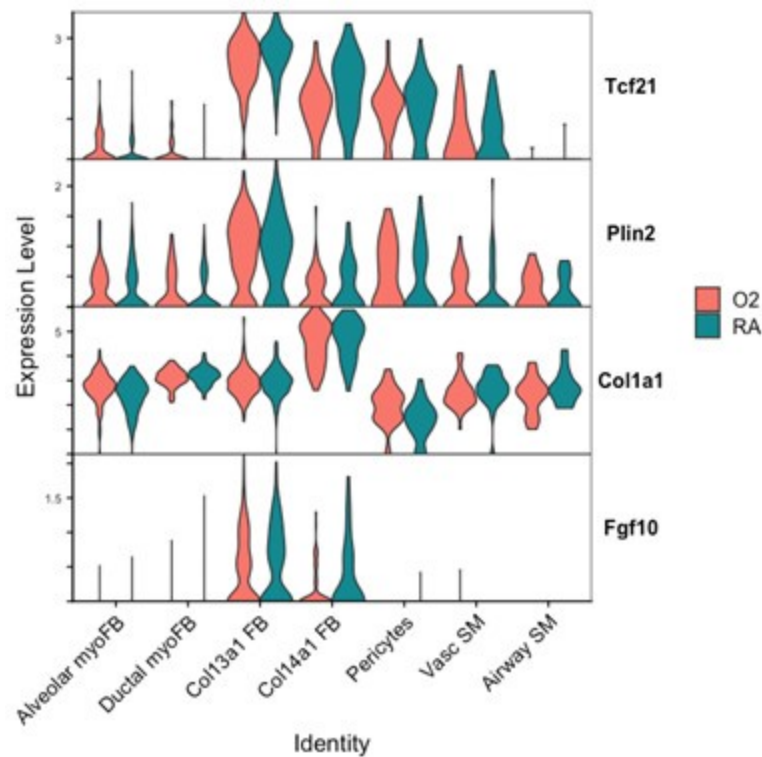


Markers of ductal myofibroblasts including *Hhip*, *Cdh4*, and *Aspn* all appear lower with hyperoxia. Interestingly *Plin2* expression is only slightly increased in Col13a1 fibroblasts with hyperoxia treatment, and there is also increased expression in alveolar myofibroblasts. *Tcf21* is another marker commonly used to identify lipofibroblasts and its expression is similarly increased in myofibroblasts during hyperoxia, although its expression is conversely lower in Col13a1 and Col14a1 fibroblasts in our data. Overall, these data would appear consistent with recently published data by Ricetti *et al.* in which the authors observed an increase in lipofibroblast gene signatures and reduced myofibroblast gene signatures with hyperoxia treatment.

Author response image 2.



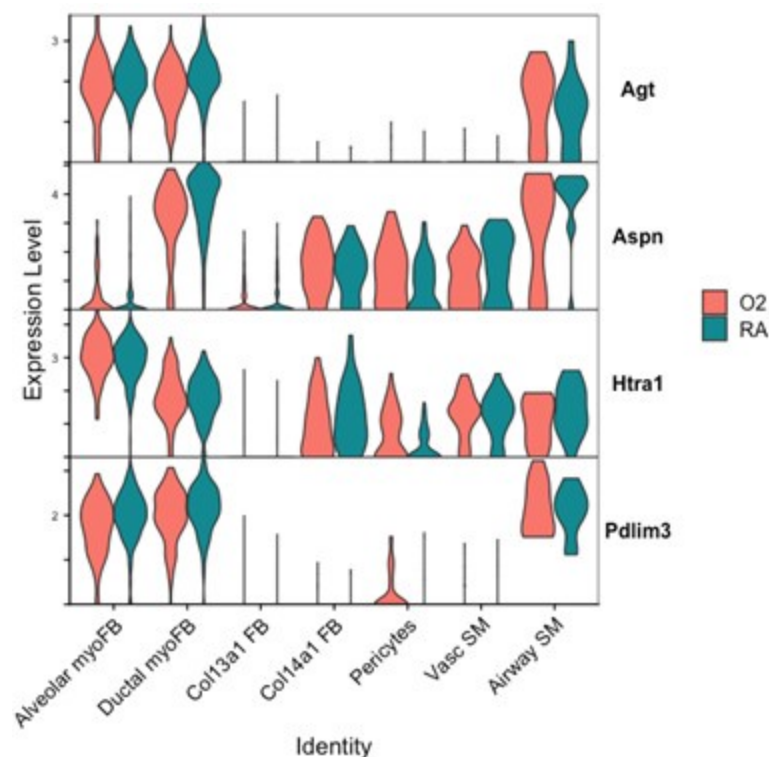
Author response image 3.



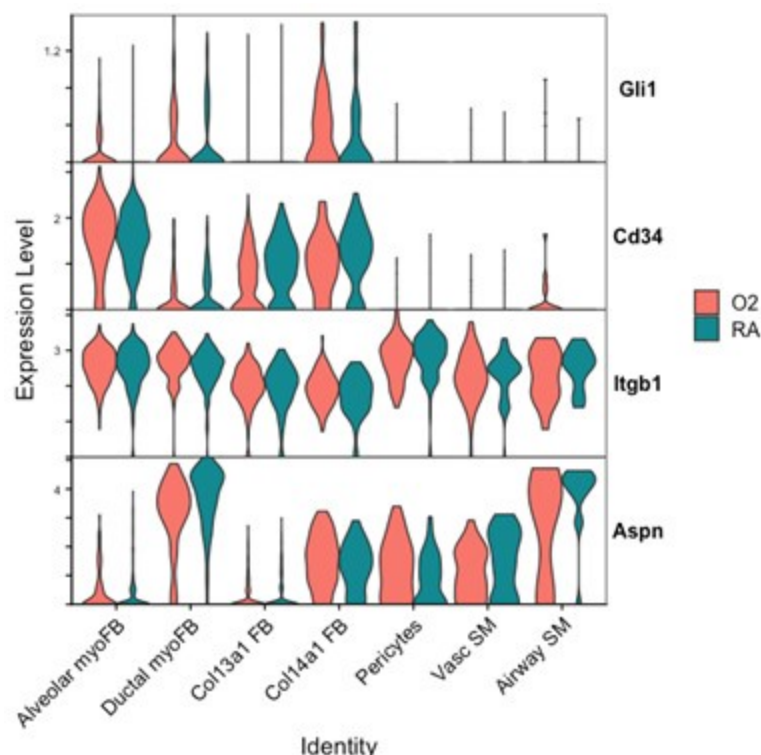
The ability of our data to clearly identify changes in cell fate differentiation is limited by our use of Seurat to define cell clusters because these methods are likely to mask subtle gene expression changes in a small number of cells nested within a parent cluster. In the example above with *Plin2*, the change in *Plin2* expression within myofibroblasts is not significant enough for Seurat to pull these cells out from their parent clusters to define a different lineage, nor are these cells similar enough in their current moment in time to be considered *Col13a1* fibroblasts or lipofibroblasts. Increasing the dimensions used to define Seurat clusters might be sufficient to identify this subset of cells as a distinct cluster, however this approach would come at the expense of creating several more cell subsets with increasingly small populations which would be difficult to further analyze.

One alternative approach to address these questions regarding differentiation might include using pseudo-time analysis of our sequencing data to predict cell lineage. Unfortunately, these analyses are beyond the scope of our current study, but we hope that our public data set can be used by investigators hoping to utilize this approach. Another method to address these questions could utilize a pulse-chase lineage experiment where one could label *Pdgfra*-expressing cells at the onset of injury and compare the differentiation of these labeled cells following injury. Li *et al.* conducted a similar experiment with hyperoxia in which *Pdgfra*-expressing cells were labeled during embryonic development and then postnatally following hyperoxia exposure. The authors noted a decrease in both lineaged myofibroblasts and lineaged lipofibroblasts and concluded that *Pdgfra*-lineaged cells were lost with hyperoxia treatment rather than undergoing aberrant differentiation. While these experiments likely have their own caveats related to the timing and efficiency of labeling, they represent a more conclusive approach to addressing differences in cell specification as compared to our sequencing- and flow cytometry-based approaches.

Author response image 4.



Author response image 5.



(2) Of the three major lung abnormalities encountered in BPD, the authors focus on alveolarization impairment in great detail, to a very limited extent on inflammation, and not on vascularization impairment. However, this would be important not only to better capture the established pathohistologic abnormalities of BPD, but also it is needed since the authors alter TGF β signaling, and inflammatory and vascular phenotypes with developmental loss of TGF β signaling and its activators have been described. Since the authors make the point about the absence of inflammation in their BPD model, it will be important to show the evidence.

We acknowledge that vascular changes significantly contribute to BPD pathogenesis, however our study was not designed to adequately characterize changes in vascular/endothelial cells. We were motivated to focus on the lung mesenchyme after observing a dramatic loss of PDGFR α + cells with our initial characterization of the hyperoxia injury model (Figure 2). At the onset of our study, the existing publicly available data did not contain enough mesenchymal cells for in-depth analysis. To generate new observations and hypotheses within the lung mesenchyme we enriched our single cell prep for mesenchymal cells at the time of FACS-sorting to ensure we would have sufficient cell numbers for downstream analysis.

(3) Conceptually it would be important that in the discussion the authors reconcile their findings in the experimental BPD models in light of human BPD and the potential implications it might have on new ways to target key pathways and cell types for treatment. This allows the scientific community to formulate the next set of questions in a disease-relevant manner.

We have edited text in the discussion to address this point.

Reviewer #3 (Public Review):

Summary:

This paper seeks to understand the role of alveolar myofibroblasts in abnormal lung development after saccular stage injury.

Strengths:

Multiple models of neonatal injury are used, including hyperoxia and transgenic models that target alveolar myofibroblasts.

Weaknesses:

There are several weaknesses that leave the conclusions significantly undersupported by the data as presented:

(1) There is no validation of the decreased number of myofibroblasts suggested by flow cytometry/scRNAseq at the level of the tissue. Given that multiple groups have reported increased myofibroblasts (aSMA+ fibroblasts) in humans with BPD and in mouse models, demonstrating a departure from prior findings with tissue validation in the mouse models is essential. There are many reasons for decreased numbers of a subpopulation by flow cytometry, most notably that injured cells may be less likely to survive the cell sorting process.

Unfortunately, we were unable to produce convincing PDGFRa staining on tissues that we had collected during our original studies. Without PDGFRa staining, we regretfully could not co-stain for other useful markers to assess proliferation (EdU), apoptosis (TUNEL or caspase), or fibroblast function/specification (aSMA/ACTA2, SM22a/TAGLN, ADRP, etc). We suspect that these experiments would require optimization of tissue fixation/processing at the time of harvest or the inclusion of a *Pdgfra* lineage tool for better identification of these cells by immunohistochemistry. Given that the majority of *Pdgfra* lineage tools require a knock-in/knock-out approach, data generated using these tools should be interpreted with caution given our results here show that *Pdgfra*-haploinsufficiency alone worsens disease outcomes after hyperoxia exposure.

Our single cell data show that there is increased expression of *Acta2* and *Tagln* shown in the plots which might be consistent with the increased aSMA staining which others have observed in these settings. Interestingly, the transcripts of both genes are reduced in alveolar fibroblasts while increased in ductal myofibroblasts, Col13a1 fibroblasts, Col14a1 fibroblasts, and vascular smooth muscle. We did not include aSMA antibody staining in our flow cytometry experiments, but this would certainly add value to future attempts to characterize the phenotypic changes occurring during these injury models.

(2) The hallmark genes used to define the subpopulations are not given in single-cell data. As the definition of fibroblast subtypes remains an area of unsettled discussion in the field, it is possible that the decreased number by classification and not a true difference. Tissue validation and more transparency in the methods used for single-cell sequencing would be critical here.

See response above and new Supplemental Figure 2.

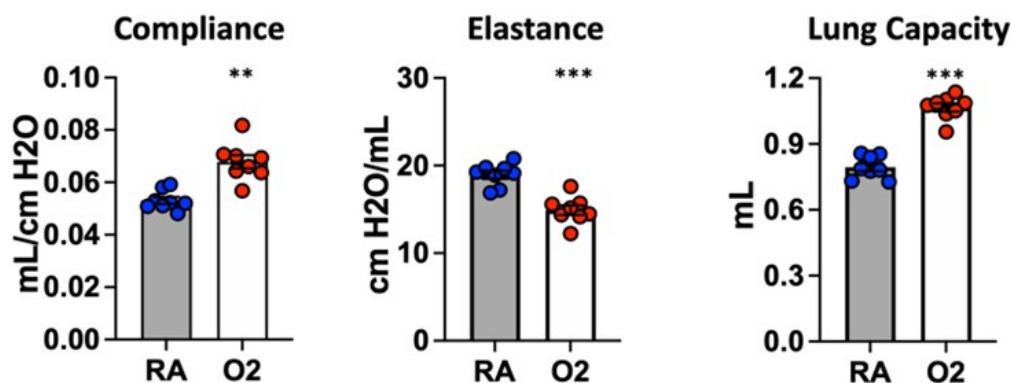
(3) There is an oversimplification of neonatal hyperoxia as a "BPD model" used here without a reference to detailed prior work demonstrating that the degree and duration of hyperoxia dramatically change the phenotype. For example, Morty et al have shown that hyperoxia of 85% or more x 14 days is required to demonstrate the septal

thickening observed in severe human BPD. Other than one metric of lung morphometry (MLI), which is missing units on the y-axis and flexivent data, the authors have not fully characterized this model. Prior work comparing 75% O₂ exposure for 5, 8, or 14 days shows that in the 8-day exposed group (similar to the model used here), much of the injury was reversible. What evidence do the authors have that hyperoxia alone is an accurate model of the permanent structural injury seen in human BPD?

At the onset of our studies, we noted that several groups were using widely variable protocols ranging from 60-100% O₂ exposure. Morty *et al.* have indeed conducted thorough experiments to characterize various different hyperoxia exposure protocols. In their 2017 study (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5312005/>) they showed that 85% O₂ from P1-P7 was sufficient to produce increased septal thickness compared to control mice, and this change was comparable to P1-P14 exposure with 85% O₂. Interestingly, they also noted that some therapeutic interventions could rescue disease caused by 60% O₂ but not 85% O₂ exposure. Our criteria in choosing a treatment protocol were: (1) nursing dams and pups survived hyperoxia exposure, (2) injury was reproducible across cohorts, and (3) injury was not reversible simply by recovering in room air. We found that recent work utilizing 75% O₂ exposure was sufficient to cause the alveolar simplification phenotype which we sought to investigate. In our hands, we did not observe mortality of nursing dams or pups except for litters lost to cannibalism/failure of cross-fostering.

We are confident that the injury caused by our hyperoxia protocol is not reversible simply by recovering mice in room air. Several groups have phenotyped mice at P4, P10, or P14 immediately following the conclusion of hyperoxia treatment. To ensure that we were studying a lasting, irreversible phenotype, we conducted our endpoint studies (morphometry and lung physiology) at P40. Because mice continue to undergo alveolarization until ~P36-P39, we reasoned that this additional recovery time following cessation of hyperoxia would allow for spontaneous recovery if this injury was transient. Additionally, shown below are unpublished flexiVent data in which mice were treated for 10 days with 75% O₂ and recovered until analysis at 10 weeks of age. These results are entirely consistent with the flexiVent data we have included in the manuscript, and the persistence of lung physiologic changes in adult mice suggest the presence of permanent underlying structural changes. We did not conduct morphometry/MLI studies at later timepoints, but we have no reason to suspect a different outcome given the clear results from lung physiology.

Author response image 6.

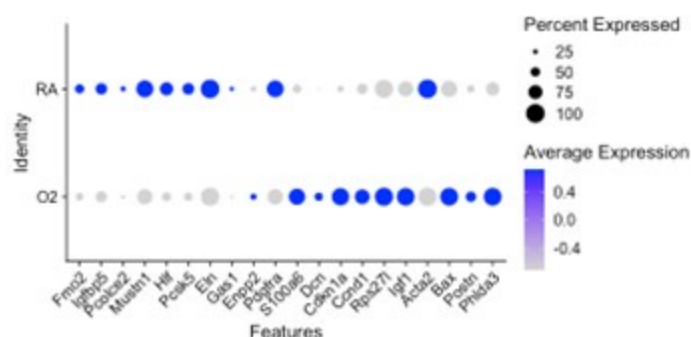


(4) Thibeault *et al* published a single-cell analysis of neonatal hyperoxia in 2021, with seemingly contrasting findings. How does this dataset compare in context?

Our data is complimentary to the single-cell analysis published by Thebaud *et al.* We included a re-analysis of their mesenchymal data in Supplementary Figure 2 which shows they also observed a relative decrease in myofibroblast clusters at the P7 and P14 timepoints following hyperoxia treatment. Figure 4 of their paper highlights the top differentially expressed genes between RA and O2 in Col13a1 FB and myofibroblasts, and we observe nearly identical findings in our data set within each of these clusters. Below we have created dotplots of P7 wildtype samples for the same selected genes shown in Figure 4G of the Thebaud *et al.* paper. It is important to note that their clustering pooled all myofibroblasts into one cluster, while our data is divided into alveolar myofibroblasts and ductal myofibroblasts. The other difference is their data set includes all timepoints P3, P7 and P14 pooled for display, while the plot we selected for simplicity here is only P7 cells. From these data we can see that the general trends are identical to those observed by Thebaud *et al.*, and the differences in genes such as *Acta2* can be accounted for by different changes observed in the different myofibroblast clusters – which is identical to what is shown in the violin plots above – namely that *Acta2* is reduced in hyperoxia in alveolar myofibroblasts while increased in the ductal myofibroblasts.

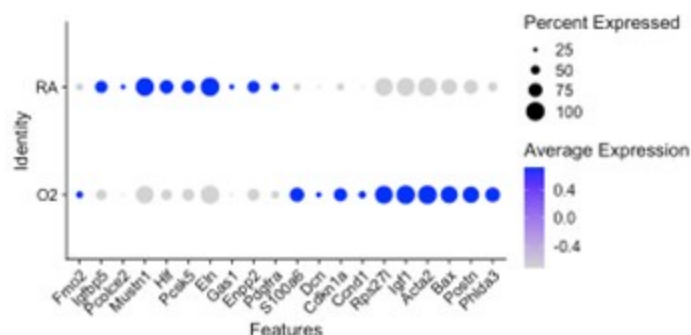
Author response image 7.

Alveolar myoFB



Author response image 8.

Ductal myoFB



One difference between our two datasets is the relative contribution of myofibroblast and Col13a1 fibroblasts to the entire mesenchymal population of cells. Over 50% of all mesenchymal cells in our preps consist of myofibroblasts, while most of their mesenchymal cells are Col13a1 fibroblasts. These differences are likely accounted for by differences in tissue digestion and cell preparation protocols. However, despite these differences, their data

show the same trends of decreased myofibroblasts and a relative expansion in Col13a1 fibroblasts.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

(1) Figure 1, for the hyperoxia model, it is informative to have the analysis done at P40, while most of the previous studies using this model focus on outcomes shortly after the end of the hyperoxia regimen. The authors state "we did not see evidence of fibrosis, scarring, or inflammation." It will be helpful to include data supporting this conclusion, especially ACTA2, CTHRC1, and CD45 staining.

We did not conduct trichrome staining or hydroxyproline assays to quantify the absence of fibrotic changes because there were no gross histologic changes consistent with scarring or fibrosis by H&E staining. We have amended the text to say "we did not see evidence of fibrosis or scarring" since we did not publish any changes to characterize the immune cell compartment.

(2) Figure 3, single cell analysis, naming of the clusters is confusing. Is "alveolar myofibroblasts" the same as "secondary crest myofibroblasts"? Is "Col13a1 FB" the same as "alveolar fibroblasts" and "Col14a1 FB" the same as "adventitial fibroblasts"? The loss of myofibroblasts is intriguing because, by staining, there is an increase of ACTA2+ cells. Are ACTA2+ cells not myofibroblasts in scRNAseq data?

As mentioned in responses above, we used Jichou Chen's nomenclature of "alveolar myofibroblasts" and "ductal myofibroblasts", but we agree that the former cluster is most consistent with "secondary crest myofibroblasts". To distinguish the two remaining clusters of fibroblasts we used the same nomenclature as found in Thebaud et al's single cell data set- "Col13a1 FB and "Col14a1 FB". The Col13a1 FB cluster is most consistent with "alveolar fibroblasts" and contains high expression of several genes used to define "lipofibroblasts", though it is unclear whether the latter may represent a subcluster within the Col13a1 FB cluster.

As shown above, *Acta2* is expressed broadly within the lung mesenchyme with highest levels found in myofibroblasts and smooth muscle cells.

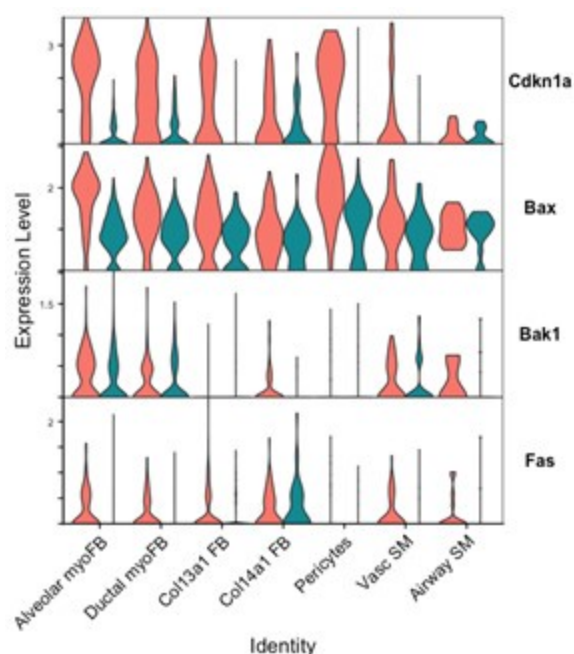
(3) Phosphorylated SMAD2/3 staining (e.g. Cell Signaling antibody) in the two models will be informative to show where TGF signaling activity is altered.

We have not been successful in using SMAD2/3 staining to infer changes in TGFβ signaling at the resolution needed to address this question. Other groups have shown qPCR and western blot data for SMAD2/3 signaling from whole lung extracts, but these approaches lack cell type and specificity and do not address spatial changes. We attempted to incorporate pSMAD2/3 staining into our flow cytometry experiments, but the staining protocol did not work in our hands.

(4) Is cell death increased in the multiple models that showed simplification?

While our EdU experiments address proliferation, we were unable to perform PDGFRα and TUNEL/caspase co-staining by histology to address apoptosis/cell death in our different models. Shown here is data from P7 wildtype mice in which *Cdkn1a* (promoting arrest of cell cycle), and pro-apoptotic genes *Bax*, *Bak1*, and *Fas* are all upregulated in hyperoxia in several mesenchymal cell populations including myofibroblasts.

Author response image 9.



(5) Wording: "These data suggest that *avb6* does not play a role in TGF β activation during normal development or neonatal hyperoxia, while *av*-integrins in the lung mesenchyme are required for normal development and play a protective role in response to hyperoxia." The first half of the sentence is missing a reference to the epithelium.

Text now reads "These data suggest that epithelial *avb6* does not play a role..."

Reviewer #2 (Recommendations For The Authors):

The reviewer greatly appreciates the work presented here, especially the hard task of addressing combined signaling pathway input into key mesenchymal cell types during an essential expansion of alveolar surface area in postnatal lung and its effect upon disturbance.

The issues of concern are mentioned in the public review and are expanded upon below:

(1) Expanded characterization of PDGFR α + expressing cells in the scRNA dataset is needed (see public review). Also included should be some of the key myofibroblast genes (*elastin*, *Acta2*, etc.) and their changes in the relevant cell populations. It would be important to show (at least at the transcriptional level) that myofibroblast differentiation is impaired if the author claims that the alveolarization defect is due to functional myofibroblast impairment. Furthermore, *Ect2* expression and changes with treatments should be shown for the different cell populations (relevant to Figure 9).

See responses above

(2) The authors stated that they did not find evidence of fibrosis, scarring, and inflammation, but did not provide data to support this statement. Given the importance of at least the inflammation component in BPD, the absence of inflammation needs to be

shown, especially in the model using the TGFBR2-CKO mouse, where at least their data show a trend to increased CD45 cell numbers (Figure 2), and upregulated inflammatory upstream regulators (IL10, IFN α , IKBKB, CEBPB upregulated) in the IPA (Figure 3). BAL and/or tissue by flow or IHC have been used to assess different immune cell populations. In terms of evaluation of vascular impairment, the single-cell data set contains endothelial cells, vascular smooth muscle, and pericytes, which allows interrogation following the two different types of injury (hyperoxia CKO TGFBR2) used for the scRNA-seq experiments).

A full characterization of the immune cell or vascular/endothelial cell compartment within our models is beyond the scope of this current study as we were focusing on the shared changes observed within the lung mesenchyme. None of these compartments exist in isolation, so of course there are likely to be correlative and/or causative changes observed in each of the different models which we studied. We did consider further phenotypic analysis of the immune cells by flow cytometry within our different models, but deferred these experiments for future studies. As mentioned earlier we have omitted the reference to “no inflammation”.

(3) The authors should report several litters per experiment and experimental group, mortality in the groups, and if present, visualize using e.g. Kaplan-Meier curves. The switch of the mothers during treatment, the early postnatal injections and treatments, and variability in outcome measures between different litters have to be anticipated. Therefore at least 2 litters, but preferably 3 litters per experiment should be examined, to show reproducibility.

All experiments were conducted with at least 2-3 contemporaneous litters in each treatment group as this was necessary to have enough animals per treatment condition/group to achieve statistical significance. This was essential as all experiments were conducted on the C57BL/6 background where litter sizes are typically 6-8 pups in our colony. We did not encounter any maternal mortality related to hyperoxia exposure while rotating between hyperoxia and normoxia every 48 hrs. Loss of pups in our experiments was mostly due to cannibalism either immediately after birth or from neglect due to failure of cross-fostering.

(4) The reviewer is concerned about using PBS as a control for experiments involving antibody treatment, in this case, 1D11. The use of an isotype IgG would be the most appropriate and convincing control. In this case, an isotype-matched murine IgG1 control (13C4) has already been generated and is commercially available. While the reviewer does not suggest repeating all experiments, at least one small experiment showing that control IgG does not alter the lung phenotype with hyperoxia when compared with 1D11 would be important.

We appreciate the reviewer’s suggestion and will consider an isotype antibody comparison in future studies. While not directly comparing 1D11 to isotype, we can share data in which we compared PBS to a different antibody. In this experiment, we attempted to use antibody blockade during the first 10 days of life while mice were undergoing hyperoxia treatment to target a specific component of the TGF β pathway. We observed no difference in outcomes either in RA or O₂ when comparing PBS to xxx antibody. We cannot share the antibody identity due to intellectual property reasons, however additional studies confirmed that this antibody likely had no impact due to poor in vivo blocking activity.

Names of transgenic lines used through manuscript:

Please use the correct name, as per JAX would be either Gli1^{tm3}(cre/ERT2)Alj/J or Gli1-CreERT2.

Please use the correct name, as per JAX would be either Pdgfratm1.1(cre/ERT2)Blh/J or Pdgfra-CreERT2.

PDGFRA-CRE would be JAX# 013148.

The transgenic lines have been noted in the methods, and we have edited the text of the manuscript to reflect the correct names of these lines. For the supplementary figure 4 which compares Gli1-CreERT2 to Pdgfra-CreERT2, we left our prior nomenclature intact because it better reflects that each of these lines are haploinsufficient at their targeted loci, and that the controls are cre-negative littermates.

We did not use the PDGFRA-CRE line (JAX# 013148).

Reviewer #3 (Recommendations For The Authors):

- More transparency about the single-cell analysis is required: 1) how are cell types and clusters defined? 2) what strategy was used for ambient RNA? 3) how do the controls compare with recently published mouse developmental datasets? 4) how does this model compare with the single-cell dataset published by Thibeault et al in 2021 (neonatal hyperoxia x 14 days with multiple time points used)?

See responses above.

- Tissue level validation of these findings is essential by RNA ISH or IF. While validation that the same process is at play in human tissue would be ideal, if this is not available, the conclusions must be tempered in the discussion.

See responses above.

- Is this more mild neonatal injury reversible in mice? As noted above, more characterization of this model (and placing it in the context of other more widely published models would be helpful).

See responses above.

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