nature portfolio

Peer Review File

Aberrant Intermediate Alveolar Epithelial Cells Promote Pathogenic Activation of Lung Fibroblasts in Preclinical Fibrosis Models

Corresponding Author: Dr Jeremy Katzen

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Hoffman and colleagues dissect the complex interactions between epithelial cells and mesenchymal cells in the fibrotic niche using two novel ex vivo models of cell-intrinsic AT2 dysfunction caused by surfactant protein C BRICHOS mutations at C121. Authors first characterize epithelial and mesenchymal cells in the mouse C121 model with single-cell sequencing, and generate hypotheses for epithelial-mesenchymal interactions using ligand-receptor analysis. Authors define and characterize a subset of Cd44 expressing cells that are enriched for transcriptional features reminiscent of “aberrant basaloid” cells in human IPF. Using Cd44 for epithelial sorting, authors performed organoid co-cultures with fibroblasts from normal and fibrotic mice and find that key phenotypic alterations (in both the epithelial and fibroblast compartments) tend to occur when Cd44+ AT2 cells from C121 mice are cultured with fibrotic mesenchyme. The most prominent examples of this synergy seems to relate to TGFbeta signaling and canonical transcriptional targets (Tgfb1, Itgb6, Ctgf, Cdkn1a, Col1a1), which is understandable given the feed-forward characteristics of this particular signaling pathway. Authors also show that C121 AT2 cells lose sensitivity to signaling through Fgfr2, primarily through downregulation of the receptor in the epithelium, and to a lesser extent due to loss of mesenchymal Fgf7 expression. Authors develop a novel IPSC-derived model derived from a patient with a C121 mutation and show cell-intrinsic gain of transcriptional markers of aberrant basaloid cells, which can be recapitulated in wildtype cells with the combination of loss of Wnt/Fgf signaling, and gain of TGFbeta signaling.

I applaud the authors for modeling the fibrotic niche and performing the combinatorial experiments necessary to dissect the complex interactions between cell-intrinsic and -extrinsic signals. A strength of the manuscript is the multiple modalities used to interrogate these interactions, including in vivo mouse model, rigorously isogenic human iPSC-derived AT2 cells; epithelial organoids and co-culture organoids; and conditioned media experiments.

This manuscript has important and special value to the community because of an interesting paradox at the center of the C121 mutant model: AT2 cells express C121 mutants, which triggers the UPR, which triggers loss of AT2 identity (which authors show previously and recapitulate here), whereupon cells express less SPC, thereby removing the stimulus for UPR activation. One might therefore expect this model to be one primarily of acute lung injury and resolution, yet fibrosis persists and worsens with successive doses of tamoxifen. This suggests that the fibrosis in this model must be caused by the establishment of a local niche that amplifies the effect of initial injury (Fig 4d).

The impact of this work would be further strengthened by authors illustrating this paradox more clearly, especially in the multiple-tamoxifen model. One way to do this might be to distinguish the phenotypes/fates of AT2 cells that themselves underwent recombination and expressed the mutant SPC, compared to those AT2 cells which did not undergo recombination but might nonetheless be in a hostile niche (analogous to experiments in Fig 4). Do unrecombined AT2 cells ever reach the Cd44-high state that authors say is similar to the human aberrant basaloid cell? Or must those cells themselves have experienced the stress that came from the genetic lesion?

The development of Cd44 sorting of intermediate cells describes a tool with potentially broad applications. The manuscript would be strengthened by additional characterization of Cd44+ sorted cells, both for its utility to the community and for the interpretation of mixed organoid experiments in Fig 4. Authors show by transcriptional analysis (RNAseq and qPCR) that the

Cd44 gating strategy successfully enriches for the “aberrant intermediate” cluster, but may also capture a substantial number of non-aberrant intermediate cells. Some data presented suggests that the Cd44 gating might be on the less-stringent side: Fig 3D shows that this gating strategy captures ~40% of the Cd200+ Cd104- AT2 cells, but single cell sequencing shown in Fig 3A suggests that the aberrant intermediate cluster accounts for a relatively small minority (probably much less than 40%) of AT2 and AT2-derived cells. Likewise the gating captures 10% of wildtype AT2 cells (Fig 3C) despite absence of aberrant intermediates in normal mice (Fig 1C). Some measure of the heterogeneity of the cells in this sorting strategy would help (e.g., cytospin staining of Cd44+ sorted cells for aberrant intermediate markers, or single-cell sequencing).

Additional comments:

Fig 1C. re-use of the blue/orange color scheme to represent mesenchyme/epithelium and also WT and C121G in the same subpanel makes this figure harder to parse than it needs to be.

Fig 2D, E, and related supplemental figures to NICHES analysis. What do the numbers in heatmaps/UMAPs for each signaling archetypes represent?

Fig 2 and 3. NICHES analysis would be strengthened by showing that key sender/receiver populations are co-localized, such as Krt8/Cd44 double-positive cells located near fibrotic fibroblasts. This has particular relevance to the TGFbeta signaling highlighted in the manuscript which might be expected to act at close distances.

Fig 3B. Why do authors think there is such an abundance of Cd44- Krt8+ cells in non-fibrotic regions, in fact at a similar density to fibrotic regions? Does authors’ model of microenvironmental amplification of epithelial fibrotic phenotypes include an element of stochasticity or “critical mass”?

Fig 4 and Supp Fig 9: What is the cell type composition of the Pdgfra+ mesenchyme from C121G mice? There has been a suggestion that Cthrc1-high fibrotic fibroblasts are Pdgfra negative and might reside in lower left corner of the MCAM/Pdgfra plot which was gated out (see PMID 32317643). This might explain why Cthrc1 and Col1a1 induction in the gated cells was relatively subtle compared to other studies even with unfractionated lung. I wonder if results in Fig 4 F-G would be even more prominent if this gate contained a higher proportion of fibrotic fibroblasts.

Fig 4B. Does culture of C121 AT2s with normal mesenchyme change expression of aberrant intermediate marker genes including Cd44, or perhaps cause gain of markers characteristic of Cd44- cells (from Fig 3)? That would suggest that the epithelial phenotypes can be substantially blunted by modulating the niche, even when the cells bear genetic lesions causing ongoing cell-intrinsic stress, which would have implications for IPF patients with shortened telomeres, SFTPC mutations, etc.

Fig 7: Do C121Y iAT2s reflect some of the transcriptional changes described for mouse C121G cells: TGFbeta module gene expression including ITGB6 and TGFB1 or FGFR2 downregulation? This would help further clarify whether those changes from mouse were cell-intrinsic or, as suggested, require the input of responsive mesenchyme. Likewise, is the gain of transitional markers by C121 iAT2s sensitive to Alk5 inhibition?

Reviewer #2

(Remarks to the Author)
In this very interesting article, Hoffman et al. performed a thorough analysis of elaborate preclinical models of lung fibrosis, focusing on the rise of aberrant intermediate alveolar cells and their interactions with mesenchymal cell populations. Using advanced techniques such as scRNA-seq analysis and organoid models, they provide compelling insights. To summarize (as the article is quite dense), they demonstrate that such aberrant intermediate alveolar cells are also observed in a genetic model of lung fibrosis (an Sftpc mutant), potentially driven by impaired epithelial–fibroblast interactions involving FGF and TGF-beta pathways. Their functional studies included elegant co-culture organoid systems, using sorted mouse cells and human mutated or corrected iAT2 cells (derived from iPSCs). Overall, these findings are highly relevant to the pathophysiology of lung fibrosis.
Since the study is already comprehensive, corroborated by multiple approaches, and presents convincing evidence, I have only a few comments.

Major Comments:

Figures 4 to 7: The analysis relies heavily on transcriptomic data. To strengthen their findings, it would be valuable for the authors to characterize the epithelial cells of their organoids at the protein level. Immunofluorescence (IF) could be used to confirm that these cells are indeed AT2 cells, with a more or less aberrant phenotype. This validation would be particularly critical for the data presented in Figures 4 and 8, especially regarding the expression of the aberrant phenotype.

Material and methods:
In the "Data and Code Availability" section, only data availability seems to be mentioned. For better reproducbilility ans to help other scientists dealing with scRNAseq analysis (including gene score calculation), the authors should make available all their R script used for analysis (zenodo or Github…)

Although the authors performed parametric analyses on populations with a limited number of replicates (n=3, see Figures 4 to 8), they should at least present their results as the median alone or with SD, rather than as the mean ± SEM (as stated in the "Statistics" section).

Reviewer #3

(Remarks to the Author)
This is a well written and comprehensive manuscript by Hoffman and colleagues. The authors use this groups Sftpc C121G mouse model to identify, isolate and study the role of aberrant-intermediate alveolar epithelial cells and their cross talk with fibroblasts during fibrosis. This presents a is a unique opportunity to study the aberrant intermediate AT2 population during ex vivo experiments, in vivo with a progressive fibrotic disease that replicates features of IPF patients and in patient derived iAT2 cells. There are 2 major concerns. First is the viability of using CD44 as a robust marker to identify this subpopulation of aberrant intermediate cells. Second is the interpretation of the proliferative capacity of the CD44+ cells from the Sftpc C121G mice or human iAT2s. Overall these and the other concerns should be addressable and strengthen the overall significance of this manuscript.

Major comments:
1. In Figure 2, the authors identifying ligan-receptor signaling pairs including Pdgfb-Pdgfrb. What role do they think pericytes (cluster 4) are playing in this process? Are they also becoming pro-fibrotic?
2. There has been considerable effort by this group to identify a unique cell surface marker to identify the aberrant intermediate cells. The use of CD44 is chosen and followed throughout the manuscript. The flow staining in Figure 3C is not entirely convincing of a unique population but identifies a small shoulder of CD200+ cells that have increased CD44 expression. Based on the data in a, it would be anticipated to be a much broader signal also visible in AT2, URP and Transitional cells as well as the aberrant intermediate population that the authors focus on. The major concern with this comment is that this would be difficult to identify if shown as histogram or even as a dot plot and to put this forward a novel marker that does not robustly pull a unique population by flow antibody staining will make the data difficult to reproduce by other labs.
3. Figure 3d states that ~10% of WT AT2 cells are CD44 hi. However, this is not reproduced in the immunostaining (Figure 3b) which also does not look very convincing for the identification of CD44+/KRT8+ cells that are accounting for ~40% of the AT2 population.
4. Frequencies are shown for Figure 3d. What are the total number of cells that are identified for the CD44hi population in WT and C121G mice?
5. Data in Figure 3 shows the identification of a small number of CD44hi cells in wild type mice. What role do the authors think these cells are playing at homeostasis (what does sequence in Figure 3e suggest?) Can the WT CD44hi population be included in the subsequent in vitro studies (Figure 4, Figure 5) to see if these cells in the context of no fibrosis signal are also sufficient to drive changes in the fibroblast phenotype?
6. The authors conclude from data presented in Supplemental Figure 10 that C121G CD44+ AT2 cells have a reduced progenitor capacity because G121G “fibrotic associated fibroblasts” increased AT2 progenitor capacity in WT AT2 cells but not in C121G AT2 cells. How do the authors put this data into the context of in vivo fibrosis where there are increased number and pro-fibrotic phenotype of fibroblasts and increased numbers of CD44+ aberrant intermediate AT2 cells in the fibrotic Sftpc C121G lungs (Figure 1, 2, 3). The authors conclude that proliferation may be dependent on the capacity of AT2 cells to respond to external cell-cell signaling – but again this doesn’t fit into an in vivo context or the NICHES data.
7. The FGF7 concentration data presented in Figure 5 is intriguing. However, I wonder if the authors should think about reversing how this data is presented. Not as a gradient from low to high FGF7 but from high ligand (as expressed in WT fibroblasts) and high receptor (AT2) to the lower expression in the Sftpc C121G mice (d,e, f). Then the story becomes what happens in WT mice and now what is not happening in the Sftpc C121G mice that could be contributing to a dysfunctional signaling Fgf7-Fgfr2 signaling loop and then correlates nicely with the phenotypic changes shown in g.
8. For data in Figure 5, there is no 0 ng/ml concentration shown which is an important baseline control.
9. For data in Figure 6, 8, Supplemental Figure 14 what are the basal/baseline concentrations of active TGF-beta being produced by the iAT2s in culture and does this vary with CD44 expression?
10. Do the patient-specific iPSC BRICHOSE SFTPC mutant cells (Figure 7) also have increased expression of TGF-beta (transcript, protein secretion)?
11. Figure 7b. The conclusion is that the mutant C121Y AT2 cells have a reduced proliferative/self-renewal capacity. However, based on the data presented it looks like they just have a delayed capacity. They have no expansion between p0 and p1 but the slope of the line is the same as the WT cells. When you compare p9 of the WT to p10 of the C121Y mutant they look pretty similar in numbers. The difference can be attributed to that initial delay passage delay. Thus, the conclusions drawn in the manuscript might not be accurate overall as the cells seem to have a similar growth curve once they begin proliferating.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this revised manuscript, the Hoffman and colleagues have added and/or clarified several areas and have addressed my comments to my satisfaction. Specifically, authors use a neat trick, detection of the retained neomycin expression cassette, to show that the majority of CD44-low and essentially all CD44-high AT2 cells have undergone SFTPC-C121G recombination. I appreciate that CD44 sorting is not dichotomous and that it represents an enrichment rather than a purification of the aberrant intermediate population of interest, and agree with the authors that the data should be interpreted with this in mind.

In summary, I again applaud the authors for their extensive work, which will have significant impact on the understanding of epithelial-mesenchymal interactions in the development of pulmonary fibrosis.

Reviewer #2

(Remarks to the Author)
The revised manuscript shows significant improvement, and my initial concerns have been adequately addressed and clarified.

Reviewer #3

(Remarks to the Author)
The authors have done significant work and have satisfactory addressed the comments raised during the initial review.

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Dear Reviewers,

We thank you for your time and thoughtful comments, and we appreciate the enthusiasm for our initial submission. In response to your feedback, we have significantly revised our manuscript and added new data and new analysis as outlined below. We think our manuscript has been strengthened greatly based upon these additional experiments.

Review #1

In this manuscript, Hoffman and colleagues dissect the complex interactions between epithelial cells and mesenchymal cells in the fibrotic niche using two novel ex vivo models of cell-intrinsic AT2 dysfunction caused by surfactant protein C BRICHOS mutations at C121. Authors first characterize epithelial and mesenchymal cells in the mouse C121 model with single-cell sequencing, and generate hypotheses for epithelial-mesenchymal interactions using ligand-receptor analysis. Authors define and characterize a subset of Cd44 expressing cells that are enriched for transcriptional features reminiscent of “aberrant basaloid” cells in human IPF. Using Cd44 for epithelial sorting, authors performed organoid co-cultures with fibroblasts from normal and fibrotic mice and find that key phenotypic alterations (in both the epithelial and fibroblast compartments) tend to occur when Cd44+ AT2 cells from C121 mice are cultured with fibrotic mesenchyme. The most prominent examples of this synergy seems to relate to TGFbeta signaling and canonical transcriptional targets (Tgfb1, Itgb6, Ctgf, Cdkn1a, Col1a1), which is understandable given the feed-forward characteristics of this particular signaling pathway. Authors also show that C121 AT2 cells lose sensitivity to signaling through Fgfr2, primarily through downregulation of the receptor in the epithelium, and to a lesser extent due to loss of mesenchymal Fgf7 expression. Authors develop a novel IPSC-derived model derived from a patient with a C121 mutation and show cell-intrinsic gain of transcriptional markers of aberrant basaloid cells, which can be recapitulated in wildtype cells with the combination of loss of Wnt/Fgf signaling, and gain of TGFbeta signaling.

I applaud the authors for modeling the fibrotic niche and performing the combinatorial experiments necessary to dissect the complex interactions between cell-intrinsic and -extrinsic signals. A strength of the manuscript is the multiple modalities used to interrogate these interactions, including in vivo mouse model, rigorously isogenic human iPSC-derived AT2 cells; epithelial organoids and co-culture organoids; and conditioned media experiments.

Major comment #1: This manuscript has important and special value to the community because of an interesting paradox at the center of the C121 mutant model: AT2 cells express C121 mutants, which triggers the UPR, which triggers loss of AT2 identity (which authors show previously and recapitulate here), whereupon cells express less SPC, thereby removing the stimulus for UPR activation...This suggests that the fibrosis in this model must be caused by the establishment of a local niche that amplifies the effect of initial injury (Fig 4d). The impact of this work would be further strengthened by authors illustrating this paradox more clearly, especially in the multiple-tamoxifen model. *One way to do this might be to distinguish the phenotypes/fates of*

AT2 cells that themselves underwent recombination and expressed the mutant SPC, compared to those AT2 cells which did not undergo recombination but might nonetheless be in a hostile niche (analogous to experiments in Fig 4). Do unrecombined AT2 cells ever reach the Cd44-high state that authors say is similar to the human aberrant basaloid cell? Or must those cells themselves have experienced the stress that came from the genetic lesion?

Response: We also find this paradox to be a focus of fascination and interest within the in vivo model. While our iAT2 model shows that expression of the mutation and induction of UPR in the iAT2s is sufficient to stimulate an aberrant cell alteration, in our in vivo model this remains an unknow. Based on the genetics of the model (using a Rosa26-Cre-ERT2) we do not have the ability to lineage trace AT2s to answer whether mutation expressing (lineage traced) cells are the only ones that enter this aberrant state. Given the only available AT2 lineage trace mouse uses the Sftpc-Cre-ERT2, and the need for biallelic Sftpc mutation in our model, we have accepted this barrier. However, we have developed a method to quantify excision of a neomycin cassette from the mutated *SFTPC* allele (PMID: 30721158 and 36473455) and had previously shown that at 7 weeks in the model we see 82.2% (SEM +/- 6.0%) recombination in the AT2 cell pool. Here, we sorted CD44-low and high AT2s from the model and found 78.1% (SEM +/- 3.2%) of the CD44-low and 95.9% (SEM +/- 1.0%) of the CD44-high underwent recombination. These data are now in **Supplemental Figure 6E**. Our interpretation of these data is that they affirm that un-recombined cells are not likely to enter the aberrant intermediate (CD44-high) cell state. Further, we note that roughly 10 % of AT2s from the *Sftpc*^{C121G} mouse cluster with the WT AT2s, and we hypothesize that these most likely represent the un-recombined CD44-low AT2s. An additional interpretation of these data is that the intrinsic stress from expressing the mutant SP-C is insufficient by itself for AT2s to enter the aberrant profibrotic cell state, since even within the CD44-low pool the majority of AT2s have undergone recombination to express the mutation. This conforms with our conceptual model where AT2 stress is the first step but requires signals from the alveolar niche to further propagate the cell transition to the most aberrant cell state.

Major comment #2: The development of Cd44 sorting of intermediate cells describes a tool with potentially broad applications. The manuscript would be strengthened by additional characterization of Cd44+ sorted cells….Some data presented suggests that the Cd44 gating might be on the less-stringent side: Fig 3D shows that this gating strategy captures ~40% of the Cd200+ Cd104- AT2 cells, but single cell sequencing shown in Fig 3A suggests that the aberrant intermediate cluster accounts for a relatively small minority (probably much less than 40%) of AT2 and AT2-derived cells. Likewise, the gating captures 10% of wildtype AT2 cells (Fig 3C) despite absence of aberrant intermediates in normal mice (Fig 1C). *Some measure of the heterogeneity of the cells in this sorting strategy would help (e.g., cytospin staining of Cd44+ sorted cells for aberrant intermediate markers, or single-cell sequencing).*

Response: Regarding the heterogeneity of the CD44-high C121G AT2s, we agree with the reviewer (also see response to **Reviewer 3's Major Comment 2**) that our scRNAseq data shows 19.3% of C121G AT2s cluster in the aberrant intermediate cell state and that our sorting strategy enriching for aberrant intermediate cells used a gating protocol that sorted 35.8% CD44-high

C121G AT2s. This same gating strategy isolated 8.1% CD44-high WT AT2s. In the C121G model, we feel that this strategy highly enriched for the aberrant intermediate cell state, but we agree with both Reviewer 1 and 3, that CD44 as a surface marker does not appear to be a dichotomous population in the C121G, and we think that reflects that the aberrant cell state is the termination of a continuum from homeostatic to aberrant. We debated whether to be more stringent with our gating, but given other publications noting a roughly 8% CD44-high AT2 population in the quiescent WT mouse lung, we felt our gating (using FMO) was best aligned with these papers. To address the question of heterogeneity, we made cytospins of the C121G CD44-high sorted cells and stained them for CD44 and Krt8. We found that 90.6% of these cells were CD44+/Krt8+ (and 94.9% were CD44+/Krt8+ or CD44+/Krt8-) (**Supplemental Figure 6F**). We feel this does reflect a degree of heterogeneity (with 5.1% of CD44+ cells being Krt8-) with our strategy, but also one that allows us to highly enrich for the aberrant intermediate state based on our transcriptional and functional assessment of the CD44-high vs low C121G AT2s.

Regarding the question of the CD44-high WT AT2s (also see **Reviewer 3's Major Comment 5**), we feel that in the quiescent lung, CD44 does not mark an aberrant intermediate cell. Supporting this, cytospins of the WT AT2s showed that only 27.8% of the CD44-high WT AT2s co-stained for Krt8, and these double positive cells were a very rare population (2.2% of AT2s). Our RNAseq analysis of the WT CD44-high and low AT2s support that we are not enriching for a profibrotic aberrant intermediate cell state (**Figure 3f-g,k** and **Supplemental Figure 7)**. We functionally assessed for this via organoid co-culture. In both mixed organoids with WT lung fibroblasts and fibrotic lung fibroblasts we did not find increased CFE in the CD44-high WT AT2s (**Supplemental Figure 11)**. This does not support the hypothesis that in the quiescent lung this CD44-high AT2 cell state has enhanced progenitor function. We further found that neither fibrotic (C121G) or WT lung fibroblasts in co-culture with the CD44-high WT AT2s had an altered gene signature indicative of fibrotic progression. We found that this supported our RNAseq analysis that the WT CD44-high AT2s are not enriched in profibrotic genes.

Minor Comment #1: Fig 1C. re-use of the blue/orange color scheme to represent mesenchyme/epithelium and also WT and C121G in the same subpanel makes this figure harder to parse than it needs to be.

Response: Colors have been adjusted.

Minor Comment #2: Fig 2D, E, and related supplemental figures to NICHES analysis. What do the numbers in heatmaps/UMAPs for each signaling archetypes represent?

Response: The numbers correspond to the order of number of cells within a cluster (0 being most cells within that cluster and 11 being least cells in the case of Epi-Mes). We have made this apparent in the text and the figure legend.

Minor Comment #3: Fig 2 and 3. *NICHES analysis would be strengthened by showing that key sender/receiver populations are co-localized, such as Krt8/Cd44 double-positive cells located near fibrotic fibroblasts.* This has particular relevance to the TGFbeta signaling highlighted in the manuscript which might be expected to act at close distances.

Response: We agree with the reviewer that the signaling implicated by the NICHES analysis and then modeled via organoid culture and the conditioned media experiments would suggest a proximity of KRT8/CD44 double-positive AT2s to fibrotic fibroblasts. While our initial submission showed (and quantified) that double positive cells were located within regions of collagen deposition (Figure 3B), we have now included immunofluorescent staining for smooth muscle actin (SMA), CD44, and KRT8 (**Figure 4A**). As we have previously shown in the C121G fibrosis model (PMID: 36473455), in regions of dense lung fibrosis, there are accumulations of parenchymal SMA+ cells, indicative of fibrotic fibroblasts (a protein marker used by others in the field to suggest a fibrotic phenotype, PMID: 39401861 and 39910313). Our staining identifies regions of SMA+ cells that have KRT8/CD44 double-positive cells interdigitating with the SMA+ positive cells. While this proximity does not imply proof of direct niche signaling, we feel it supports the premise of our transcriptomic NICHES data and then the relevance of our subsequent co-culture organoid and conditioned media experiments.

Minor Comment #4: Fig 3B. Why do authors think there is such an abundance of Cd44- Krt8+ cells in non-fibrotic regions, in fact at a similar density to fibrotic regions? Does authors' model of microenvironmental amplification of epithelial fibrotic phenotypes include an element of stochasticity or "critical mass"?

Response: We have previously shown that in the setting of AT2s expressing the misfolded C121G SP-C they decrease expression of AT2 marker genes and express *Krt8* and other markers of the "transitional" cell state. We have shown that the C121G expressing AT2s do this in a cell autonomous fashion without preceding lung injury (PMID: 36252035). Based on this, we expect to see KRT8+ AT2 cells in regions of seemingly normal appearing alveolated lung without histological injury or fibrosis. We have now included text to address the presence of KRT8+ AT2s in the non-fibrotic lungs (referencing to our prior work). Based on the abundance of KRT8+ cells in both regions of normal lung and in regions of fibrosis we are hesitant to put forth that a "critical mass" of KRT8+ cells leads to the progression to CD44+/KRT8+ cell state. Rather, we agree with the premise of this comment that there is likely microenvironmental signaling that contributes to a C121G AT2 that has entered a transitional cell state (KRT8+) progressing into a profibrotic CD44+/KRT8+ aberrant epithelial cells. We feel this concept aligns with the recent spatial transcriptomic findings (PMID 39901013) from "early" PF lungs that show abnormal molecular signatures in regions of histologically normal-appearing lungs without the aberrant cells states identified in late-stage fibrotic lung regions. Our ex vivo alveolosphere modeling and iAT2s would suggest that this microenvironmental signaling includes both the loss of homeostatic signals involved in AT2 maintenance (FGF) and also gain of aberrant signaling (TGFB signaling). Our construction of the model, where we "knock-in" the mutation through weekly tamoxifen into a subset of AT2s over time, produces a temporally heterogenous AT2 population. We could envision that the regions of fibrotic remodeling with CD44+/KRT8+ AT2s reflect a more advanced

lesion, with earlier monocyte recruitment or fibroblast activation generating the aberrant signals to produce CD44+/KRT8+ AT2s.

Minor Comment #5: Fig 4 and Supp Fig 9: *What is the cell type composition of the Pdgfra+ mesenchyme from C121G mice?* There has been a suggestion that Cthrc1-high fibrotic fibroblasts are Pdgfra negative and might reside in lower left corner of the MCAM/Pdgfra plot which was gated out (see PMID 32317643). This might explain why Cthrc1 and Col1a1 induction in the gated cells was relatively subtle compared to other studies even with unfractionated lung. *I wonder if results in Fig 4 F-G would be even more prominent if this gate contained a higher proportion of fibrotic fibroblasts.*

Response: We agree that our sorting strategy using Pdgfra+ for fibroblasts from the fibrotic C121G mouse lung likely contained the heterogeneous populations we see in the scRNAseq dataset, including alveolar, transitional/inflammatory, and fibrotic fibroblasts. This strategy may, if anything, decrease the fibrotic fibroblast fraction as the reviewer noted. However, we felt compelled to stick with this gating strategy for a couple of reasons. First, we were concerned that a gating strategy that sorted the pdgfra- or pdgfra-low population would include peribronchial fibroblasts and pericytes. Given our model does not have a lineage marker (like the Col-GFP that facilitated the sorting strategy in PMID 32317643 or pdgfra-ERT2cre) we had concerns about utilizing the CD9+ strategy to sort the Cthrc1+ subpopulation from pdgfra-lo peribronchial fibroblasts and pericytes. Second, given that we were comparing our mixed-organoid modeling findings to pdgfra+ fibroblasts from the WT mouse lungs, which also contain known heterogeneous populations, we felt that sub-fractionating out specific pdgfra- (or pdgfra-low) populations from the C121G lungs made a general comparison of “normal/homeostatic lung” and “fibrotic lung” fibroblasts in the organoid models difficult to interpret (and biased towards, if anything, a larger effect size). Third, the fibrogenic receptor-ligand signaling archetypes we see computationally between the CD44-high aberrant intermediate epithelial cells and the fibroblasts populations are not limited to just the most fibrotic fraction of cells, but rather should drive a fibrotic response, as we saw with the WT fibroblasts in co-culture, in “normal” populations as well. We agree that considering recent publications, this did require further addressing and we note in the discussion that with the increasing understanding of the heterogeneous populations of fibroblasts in the fibrosing lung, future mix-match organoid experiments with alveolar, adventitial, transitional/inflammatory, and fibrotic fibroblasts would be prudent.

Minor Comment #6: Fig 4B. *Does culture of C121 AT2s with normal mesenchyme change expression of aberrant intermediate marker genes including Cd44, or perhaps cause gain of markers characteristic of Cd44- cells (from Fig 3)?* That would suggest that the epithelial phenotypes can be substantially blunted by modulating the niche, even when the cells bear genetic lesions causing ongoing cell-intrinsic stress, which would have implications for IPF patients with shortened telomeres, SFTPC mutations, etc.

Response: We have reconfigured **Figure 4** and associated **supplemental figure 10** and added additional gene expression analysis to highlight the data answering this important comment. We

find that the culture of CD44 hi C121G AT2s with normal lung mesenchyme decreases expression of marker genes that define the aberrant intermediate cell state, including TGFB signaling genes, Fn1 and Cd44, suggesting modulating the niche can blunt the aberrant phenotype (despite the genetic lesion). However, we do not see the CD44 hi AT2s become enriched in quintessential AT2 marker genes when cultured with normal lung mesenchyme. We note, that the CD44 lo C121G AT2s are also not “normal” AT2s based on gene expression, so perhaps it should not be surprising that the normal lung mesenchyme fails to revert the C121G AT2s to a WT AT2 cell state. In addition to the additional data and figure reconfiguration to highlight these findings, we have added language in the text.

Minor Comment #7: Fig 7*: Do C121Y iAT2s reflect some of the transcriptional changes described for mouse C121G cells: TGFbeta module gene expression including ITGB6 and TGFB1 or FGFR2 downregulation?* This would help further clarify whether those changes from mouse were cell-intrinsic or, as suggested, require the input of responsive mesenchyme. *Likewise, is the gain of transitional markers by C121 iAT2s sensitive to Alk5 inhibition?*

Response: We have now included TGFB module score from Strunz et al. Nat. Communication 2020 in **Figure 7**. We see that expression of the module score is increased in the C121Y iAT2s compared to the corrected controls (**Figure 7K**). Additionally, by qPCR we see an increase in *TGFB1* and *CDKN1A* transcript (**Figure 7I**). We also see a decrease in *FGFR2* by qPCR (**Figure 7M**). In total, we think this analysis more strongly shows overlaps between the in vivo murine model and the human SP-C mutant iAT2s.

To further explore this, we cultured the mutant iAT2s with an ALK5 inhibitor based on the reviewer’s suggestion. We found that ALK5 inhibition decreased expression of the ABI marker genes *KRT7* and *KRT17* in mutant iAT2s (**Figure 8A,B**). However, it did not increase the expression of *FGFR2*. From this, we conclude that inhibition of TGFB signaling alone is insufficient to fully revert the mutant iAT2s to a state capable of responding to homeostatic niche signals. This finding motivated the experiments in Figure 8 in which we tested the combined effect of loss of homeostatic niche signals (CK) and gain of TGFB stimulation.

Review #2

In this very interesting article, Hoffman et al. performed a thorough analysis of elaborate preclinical models of lung fibrosis, focusing on the rise of aberrant intermediate alveolar cells and their interactions with mesenchymal cell populations. Using advanced techniques such as scRNA-seq analysis and organoid models, they provide compelling insights. To summarize (as the article is quite dense), they demonstrate that such aberrant intermediate alveolar cells are also observed in a genetic model of lung fibrosis (an Sftpc mutant), potentially driven by impaired epithelial–fibroblast interactions involving FGF and TGF-beta pathways. Their functional studies included elegant co-culture organoid systems, using sorted mouse cells and human mutated or corrected iAT2 cells (derived from iPSCs). Overall, these findings are highly relevant to the pathophysiology of lung fibrosis.

Since the study is already comprehensive, corroborated by multiple approaches, and presents convincing evidence, I have only a few comments.

Major Comment #1: Figures 4 to 7: The analysis relies heavily on transcriptomic data. To strengthen their findings, it would be valuable for the authors to characterize the epithelial cells of their organoids at the protein level. Immunofluorescence (IF) could be used to confirm that these cells are indeed AT2 cells, with a more or less aberrant phenotype. This validation would be particularly critical for the data presented in Figures 4 and 8, especially regarding the expression of the aberrant phenotype.

Response: We agree with the reviewer that much of the data presented in the ex vivo and in vitro culture system relies on gene expression. To address this, validate key findings, and to confirm that AT2 cells were indeed the input into our modeling, are we have now included IFC staining of AT2s from the AT2-only organoid culture for SP-C in Figure 5, which demonstrates the punctate SP-C protein pattern consistent with post-ER processing in *Sftpc*^{WT} AT2s compared to a reticular ER staining pattern with perinuclear aggregates in the CD44^{Hi} and CD44^{Lo} *Sftpc*^{C121G} AT2s. This pattern aligned with what we have observed in the in vivo model (PMID 36473455) and with the mutant iAT2 modeling IFC in Figure 7. Additionally, we observed an increase in IFC staining for KRT8 in the CD44^{Hi} and CD44^{Lo} *Sftpc*^{C121G} AT2s suggested that the *Sftpc*^{C121G} AT2s maintain key features of their aberrant phenotype in the ex vivo model. In figure 6, we analyzed the supernatant from the AT2s in culture for active TGFB1 via ELISA to validate the key secretome finding from that culture system, the expression of the profibrotic mediator, at a protein level. This we feel is the most functionally relevant finding to affirm the CD44^{Hi} *Sftpc*^{C121G} AT2s maintain the aberrant phenotype at a protein level in the ex vivo modeling. In the iAT2 modeling (Figure 8) we have now generated IF for KRT17 in the CK-DCI and the DCI-TGF-B conditions (the extremes of the phenotype) to validate the key reprogramming gene at a protein level. We struggled with IF validation for Figure 4. The key gene expression differences between the homeostatic and aberrant AT2s in that model are secreted proteins (TGFB1/2, CTGF) and FN1 (matrix protein), none of which are amenable to IF. The CD44hi AT2s in mixed organoid culture do not have enrichment in CLDN4 or KRT8 compared to WT. We attempted IF for CDKN1A (P21), which was differentially expressed, but were not convinced there was a clear signal in the protein to the point where we felt comfortable putting these data into the manuscript. Based on the cytospin analysis in Supplemental Figure 6) and AT2-only organoid IFC for SP-C and KRT8, we are very comfortable stating that AT2s were the input into these organoid culture, however, our thorough attempts to show “aberrant” versus normal AT2s in the mix-organoid culture system is something we were unable to accomplish despite analysis of multiple additional mixed organoid culture experiments.

Minor Comment #1 Material and methods In the "Data and Code Availability" section, only data availability seems to be mentioned. For better reproducibility and to help other scientists dealing with scRNAseq analysis (including gene score calculation), the authors should make available all their R script used for analysis (zenodo or Github…)

Response: We have made the code we developed for this project available on the Katzen Lab Github (https://github.com/KatzenLab).

Minor Comment #2 Although the authors performed parametric analyses on populations with a limited number of replicates (n=3, see Figures 4 to 8), they should at least present their results as the median alone or with SD, rather than as the mean ± SEM (as stated in the "Statistics" section).

Response: We appreciate this feedback and have adjusted the figures and the methods to reflect the proper presentation.

Review #3

This is a well written and comprehensive manuscript by Hoffman and colleagues. The authors use this groups Sftpc C121G mouse model to identify, isolate and study the role of aberrant-intermediate alveolar epithelial cells and their cross talk with fibroblasts during fibrosis. This presents a is a unique opportunity to study the aberrant intermediate AT2 population during ex vivo experiments, in vivo with a progressive fibrotic disease that replicates features of IPF patients and in patient derived iAT2 cells. There are 2 major concerns. First is the viability of using CD44 as a robust marker to identify this subpopulation of aberrant intermediate cells. Second is the interpretation of the proliferative capacity of the CD44+ cells from the Sftpc C121G mice or human iAT2s. Overall these and the other concerns should be addressable and strengthen the overall significance of this manuscript.

Major Comment #1: In Figure 2, the authors identifying ligan-receptor signaling pairs including Pdgfb-Pdgfrb. *What role do they think pericytes (cluster 4) are playing in this process? Are they also becoming pro-fibrotic?*

Response: Given this manuscript's focus on the emergence of fibrogenic epithelial to mesenchymal (and reciprocal) signaling that develop in the C121G model, we did not analyze for epithelial to epithelial signaling archetypes or mesenchymal to mesenchymal signaling. It is perhaps in the latter that we would see a clearer fibrotic role for the pericyte in this model. What we can say from our analysis is that in the mesenchymal to epithelial NICHES analysis, the pericytes ligand-receptor pairs cluster distinctly (cluster 6) from the other mesenchymal to epithelial interactions. The receiving populations in this cluster are not dominated by a single distal lung epithelial population, making it difficult to generate a hypothesis on distinct pathogenic signaling archetypes coming from the pericytes to a specific epithelial cell type/state. The primary ligands coming from the pericytes are *Angpt1*, *Vtn*, and *Hbegf*. Vitronectin is of specific interest, and perhaps reflects a pathogenic role of the pericyte in signaling to the epithelium given it is a ligand for itgav/b6. As noted by the reviewer, cluster 4 in the epithelial to mesenchymal NICHES analysis did resolve distinct ligand-receptor pairs from the epithelium to pericytes and smooth

muscle cells. However, there is not a clearly dominant sending epithelial population in this cluster. The highest expressed ligands coming from the epithelium in this cluster include the genes for collagen 4 and pdgfa and pdgfb, which makes us wonder whether these reflect a cluster dominated by AT1-pericyte interactions. We recognize that future unpacking of more of these potential signals between compartments is needed (and ex vivo assay development to interrogate putative interactions). We raise this in our discussion as a limitation of our approach and an area of future studies.

Major Comment #2: There has been considerable effort by this group to identify a unique cell surface marker to identify the aberrant intermediate cells. The use of CD44 is chosen and followed throughout the manuscript. The flow staining in Figure 3C is not entirely convincing of a unique population but identifies a small shoulder of CD200+ cells that have increased CD44 expression. Based on the data in a, it would be anticipated to be a much broader signal also visible in AT2, URP and Transitional cells as well as the aberrant intermediate population that the authors focus on. *The major concern with this comment is that this would be difficult to identify if shown as histogram or even as a dot plot and to put this forward a novel marker that does not robustly pull a unique population by flow antibody staining will make the data difficult to reproduce by other labs.*

Response: Review #1 raised a similar comment regarding heterogeneity of the CD44hi sorted population in our model and a concern that we sort 35% CD44hi cells in the mutant mice, while we see 20-25% cells cluster as CD44hi aberrant intermediate in the scRNAseq dataset. We have been careful in our description of our sorting strategy in the text (and have added additional language to make this clear) to note that we are enriching for an aberrant cell-state, which we feel exists as the extreme on a spectrum, to interrogate its biology. We agree that CD44 as a surface marker does not appear to be a dichotomous population, and we think that reflects that the aberrant cell state is the termination of a continuum from homeostatic to aberrant in the C121G model. We had debated whether to be more stringent with the CD44 gating to only sort 20-25% CD44+ AT2s from the mutation mice, but given other publications noting a CD44hi AT2 population in the quiescent lung (roughly 5-8% in the lungs), we chose a similar gating strategy (using an isotype control) since a right shifted gate would have shown less CD44hi AT2s in the quiescent lung. We feel the ability of our group to reproduce the sorting data published by another group (PMID 38759645) supports the robust nature of this sorting strategy. We would hypothesize that if we had been more stringent, we may have seen an even more robust phenotype in our ex vivo assays. We also note that using an AT2 lineage trace in the bleomycin model, we used the same sorting strategy to identify an aberrant population found a similar percentage of CD44-hi AT2s (32.6%). Further, as noted in the response below, we have used staining of our sorted AT2s to affirm our strategy at a protein level and the percentages we note align with our sorting.

Major Comment #3: Figure 3d states that ~10% of WT AT2 cells are CD44 hi. However, this is not reproduced in the immunostaining (Figure 3b) which also does not look very convincing for the identification of CD44+/KRT8+ cells that are accounting for ~40% of the AT2 population.

Response: To address this concern on the percent of CD44-hi WT AT2s more rigorously, we sorted AT2s from WT mice and stained cytospins to provide additional clarity on the percent of CD44+ AT2s in the WT lung (and affirm our sorting strategy in the C121G lungs) (**Supplemental Figure 6f**). We found that 5-9% of WT AT2s were CD44+ in the cytospins, which we felt affirmed our sorting strategy quantification. We further used this strategy to confirm our sorting strategy for the C121G AT2s, noting rare CD44+ cells in the CD44 lo sorted AT2s and 94.9% CD44+ AT2s in the CD44 hi AT2 sort. We further used this strategy to show that almost all the CD44+ AT2s were also KRT8+ in the C121G lungs, and that in the CD44 lo fraction there were 34.0% KRT8+ cells. Overall, we felt this additional experimentation supported the quantifications we provided in Figure 3b and d).

Major Comment #4: Frequencies are shown for Figure 3d. What are the total number of cells that are identified for the CD44hi population in WT and C121G mice?

Response: We have now included this data as a graph in **Supplemental Figure 6c and d**

Major Comment #5. Data in Figure 3 shows the identification of a small number of CD44hi cells in wild type mice. What role do the authors think these cells are playing at homeostasis (what does sequence in Figure 3e suggest?) Can the WT CD44hi population be included in the subsequent in vitro studies (Figure 4, Figure 5) to see if these cells in the context of no fibrosis signal are also sufficient to drive changes in the fibroblast phenotype?

Response: A recent publication found that CD44-high WT AT2s represent a population of AT2s primed for epithelial regeneration after lung injury with increased colony forming efficiency in organoid culture (PMID 38759645). Although we found, similar to this manuscript, that there was differential expression of cell proliferation genes between CD44-high and CD44-lo WT AT2s (**Supplemental Figure 7**), we did not find that CD44-high WT AT2s had enhanced organoid formation (**Supplmenetal Figure 11a-c**). We are thus unable to support their conclusion that this is a “primed” AT2 cell state for lung injury repair in the homeostatic lung. We did find that there was a differential expression of “inflammatory response genes” similar to this recent paper (**Supplemental Figure 7**). So, while outside the scope of this manuscript, we can support their finding that this is perhaps a cell population primed for differential response to lung infectious insults.

Regarding the role of this population in the homeostatic lung and response to fibrotic insults, as noted in our response to **Review 1 Major Comment 2**, in mixed organoid cultures with both fibrotic C121G lung and WT lung fibroblasts, CD44-high WT AT2s did not provoke a fibrotic activation of the mixed culture fibroblasts (**Supplmenetal Figure 11d**). Supporting that these cells do not represent a small population of fibrogenic aberrant intermediate epithelial cells in the normal lung, we now show in **Figure 3** heatmaps for the Murine Aberrant Basaloid gene signature and the Human Krt5-/Krt17+ gene signature with C121G and WT CD44 hi/lo AT2s. These show that compared to the C121G CD44-high and CD44-lo AT2s, the CD44-high WT AT2s are not shifted to this cell state. With this said, CD44-high WT AT2s have higher expression of certain

transitional cell genes compared to CD44-lo WT AT2s (**Supplemental Figure 7**), and perhaps represent an AT2 population primed to respond to fibrotic lung injury (though not born out in our mixed organoid culture). Current genetic model approaches for lineage tracing AT2s and CD44+ cells limit testing this hypothesis, but this can be an area of future focus.

Major Comment #6. The authors conclude from data presented in Supplemental Figure 10 that C121G CD44+ AT2 cells have a reduced progenitor capacity because G121G "fibrotic associated fibroblasts" increased AT2 progenitor capacity in WT AT2 cells but not in C121G AT2 cells. *How do the authors put this data into the context of in vivo fibrosis where there are increased number and pro-fibrotic phenotype of fibroblasts and increased numbers of CD44+ aberrant intermediate AT2 cells in the fibrotic Sftpc C121G lungs (Figure 1, 2, 3). The authors conclude that proliferation may be dependent on the capacity of AT2 cells to respond to external cell-cell signaling – but again this doesn't fit into an in vivo context or the NICHES data*.

Response: In vivo, we do not believe that the increased numbers of CD44 hi AT2s in the C121G lung reflect proliferation of a basal CD44 hi AT2 population, but rather differentiation of the mutant expressing AT2s into this aberrant cell state. The near equal quantification of AT2s sorted from the WT and C121G lung (response to comment #4, **Supplemental Figure 6c**) suggests that there is not an expansion of AT2s in the C121G model. Our trajectory inference analysis on AT2 progression into the CD44 hi state suggests that the CD44 hi AT2s in the C121G lung emerge via differentiation, and we feel this conforms with other group's conception of this aberrant cell state.

Regarding our interpretation of the mixed organoid co-culture modeling CFE, we were surprised to see increased CFE with WT AT2s co-cultured with fibrotic lung fibroblasts. As noted by reviewer 1, this likely reflects the heterogeneity (fibrotic, transitional/inflammatory, alveolar, etc) of the fibrotic lung fibroblasts (as seen in our scRNAseq analysis). We were seeking to note that CD44Hi C121G AT2s lacked this response to fibrotic lung fibroblasts and that this suggested that "the effect of fibroblast signaling on AT2 proliferation may be dependent on AT2 cell state and capacity of AT2s to respond to external cell-cell signaling." We feel this conforms with the NICHES analysis showing that in vivo the CD44 hi C121G AT2s lose quintessential fibroblast to AT2 signaling archetypes known to promote AT2 maintenance. However, to not over interpret the co-culture data we have revised the text to temper our interpretation, and as noted in response to Reviewer #1 above, future studies to disentangle the response of the different AT2 cell states to the various lung fibroblast populations that arise during fibrogenesis would be required to more completely understand these organoid proliferation responses.

Major Comment #7. The FGF7 concentration data presented in Figure 5 is intriguing. However, I wonder if the authors should think about reversing how this data is presented. Not as a gradient from low to high FGF7 but from high ligand (as expressed in WT fibroblasts) and high receptor (AT2) to the lower expression in the Sftpc C121G mice (d,e, f). Then the story becomes what happens in WT mice and now what is not happening in the Sftpc C121G mice that could be

contributing to a dysfunctional signaling Fgf7-Fgfr2 signaling loop and then correlates nicely with the phenotypic changes shown in g.

Response: We made this change and agree it helps to highlight the central finding of the figure.

Major Comment # 8. For data in Figure 5, there is no 0 ng/ml concentration shown which is an important baseline control.

Response: We twice sorted AT2s from WT and C121G mice (n=3 per experiment) and cultured AT2s in our basal media in the absence of FGF7 and both times found no alveolosphere growth in any of the three AT2 cell states (WT, C121G CD44 lo and C121G CD44 hi). We had thought that perhaps we would see growth in the C121G CD44 lo AT2s given their alveolosphere CFE in FGF 4ng/ml, however, this was not the case. We have included representative images of the FGF7 0ng/ml v 10ng/ml (as our "positive control" for CFE with FGF7 in **Supplemental Figure 12**) and commented on this in the text.

Major Comment #9. For data in Figure 6, 8, Supplemental Figure 14 what are the basal/baseline concentrations of active TGF-beta being produced by the iAT2s in culture and does this vary with CD44 expression?

Response: We performed an ELISA for active TGFB1 from the supernatant in Figure 6 (AT2 only organoid culture) and this is now shown in **Figure 6c**. We found that there was increased TGFB1 in the supernatant from the C121G CD44-high AT2s compared to WT AT2s and C121G CD44-lo AT2s. Given that the AT2 to AT1 cell culture conditions have serum in the media, we found the ELISA results to be uninterpretable given a very high background in the assay. Regarding the iAT2 model, see response below.

Major Comment #10. Do the patient-specific iPSC BRICHOSE SFTPC mutant cells (Figure 7) also have increased expression of TGF-beta?

Response: As noted to Reviewer #1 (Minor Comment #7), we have now included TGFB module scores in **Figure 7** from Strunz et al. Nat. Communication 2020. We see that expression of the module score is increased in the C121G iAT2s compared to the corrected controls (**Figure 7K**). Additionally, by qPCR we see an increase in *TGFB1* and *CDKN1A* transcript (**Figure 7I**). We also see a decrease in *FGFR2* by qPCR (**Figure 7M**). To further interrogate the relationship between TGFB signaling and the mutant iAT2 phenotype, we cultured the mutant iAT2s with an ALK5 inhibitor and found that ALK5 inhibition decreased the expression of ABI marker genes *KRT7* and *KRT17* in mutant iAT2s (**Figure 8A,B**). However, we did not see an increase in *FGFR2* expression. As noted above, our conclusion is that inhibition of TGFB signaling alone is insufficient to fully revert the mutant iAT2s to a state capable of responding to homeostatic niche signals.

Major Comment #11. Figure 7b. The conclusion is that the mutant C121Y AT2 cells have a reduced proliferative/self-renewal capacity. However, based on the data presented it looks like they just have a delayed capacity. They have no expansion between p0 and p1 but the slope of the line is the same as the WT cells. When you compare p9 of the WT to p10 of the C121Y mutant they look pretty similar in numbers. The difference can be attributed to that initial delay passage delay. Thus, the conclusions drawn in the manuscript might not be accurate overall as the cells seem to have a similar growth curve once they begin proliferating.

Response: We agree with this interpretation of the data as initially presented. We have now included the growth kinetic data from two iPSC differentiations into iAT2s and show the early passage (p0-p4) data on Figure 7b to highlight that during initial culture passages, mutant iAT2s demonstrate a significantly lower yield of $SFTPC^{tdT+}$ expressing cells, suggesting an early passage diminished self-renewal capacity.

Dear Reviewers,

We thank you for your time. In response to your feedback, our manuscript has been strengthened greatly. We appreciate your comments below noting enthusiasm for the findings in the revised manuscript without additional experimentation or edits requested.

Reviewer #1 (Remarks to the Author):

In this revised manuscript, the Hoffman and colleagues have added and/or clarified several areas and have addressed my comments to my satisfaction. Specifically, authors use a neat trick, detection of the retained neomycin expression cassette, to show that the majority of CD44-low and essentially all CD44-high AT2 cells have undergone SFTPC-C121G recombination. I appreciate that CD44 sorting is not dichotomous and that it represents an enrichment rather than a purification of the aberrant intermediate population of interest, and agree with the authors that the data should be interpreted with this in mind.

In summary, I again applaud the authors for their extensive work, which will have significant impact on the understanding of epithelial-mesenchymal interactions in the development of pulmonary fibrosis.

We thank the Reviewer for the positive feedback.

Reviewer #2 (Remarks to the Author):

The revised manuscript shows significant improvement, and my initial concerns have been adequately addressed and clarified.

We thank the Reviewer for the positive feedback.

Reviewer #3 (Remarks to the Author):

The authors have done significant work and have satisfactory addressed the comments raised during the initial review.

We thank the Reviewer for the positive feedback.