Three-axis classification of mouse lung mesenchymal cells reveals two populations of myofibroblasts

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DOI: 10.1242/dev.200081

Editor: Samantha Morris

Review timeline
Original submission: 5 August 2021
Editorial decision: 6 September 2021
First revision received: 10 January 2022
Editorial decision: 2 February 2022
Second revision received: 8 February 2022
Accepted: 21 February 2022

Original submission

First decision letter

MS ID#: DEVELOP/2021/200081

MS TITLE: Three-axis classification of mouse lung mesenchymal cells reveals two populations of myofibroblasts

AUTHORS: Odemaris Narvaez del Pilar and Jichao Chen

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. We encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the study by Del Pilar and Chen, the authors present a series of analyses derived from a scRNA-seq based time course of mouse lung development. The authors focus on the development of the lung mesoderm and its derivative cell populations that include fibroblasts and smooth muscle. The authors use a classification system that relies on a three-axis justification. The three axes are epithelial, vascular, and interstitial. They suggest that using this classification system would aid in the identification and validation of putative mesenchymal cells observed in the scRNA-seq space. The authors evaluate this axis classification system by analyzing each axis from the scRNA in subsequent figures. The authors also uncover a potentially novel conclusion, that the developing alveolar ducts and alveoli have two unique populations of myofibroblasts; ductal and alveolar, respectively.

What is the advance made in this paper and how relevant is it to the field?
First, this paper is an addition to a series of scRNA-time course studies focused on the lung mesenchyme, published (or in pre-print stage) in the past year (Zepp et al, 2021, Liu X et al, 2021). The authors do put forth the hypothesis that "…mesenchymal cells could be classified based on the structures they supported…", but the authors do not present experiments or perturbations that would add validity to the concept that the fibroblasts they classify "support" any structure. The concept that lung mesenchyme develops along these three axes is not new; clonogenic lineage tracing published in 2014 revealed this mode of mesenchymal development (Kumar, 2014) and was similarly resolved with additional lineage tracing this past year (Zepp, 2021). Further this classification system, does not add clarity to fibroblast heterogeneity as there are instances of ambiguity or discrepancies between lineage tracing and scRNA-seq (detailed below). Based on the scRNAseq, the authors do identify and attempt to validate the presence of two types of lung myofibroblasts. These data are new and potentially insightful to the field. However, the authors do not perform experiments that test functional differences between these two myofibroblasts, doing so would qualify this study as making a conceptual advance.

Do the data reported in the paper justify the conclusions? The title would suggest that the main takeaway from this paper is a three-axis classification of lung mesenchyme. There are several instances of ambiguities with this classification system, the data presented are descriptive. Hypothesis-driven experiments are needed to support this model.

Overall, in its current form, this paper is largely descriptive and further analyses and functional experiments are needed.

Comments for the author

Major points:
1) One of the main takeaways that the authors put forward is that lung mesenchymal cells can be classified based on a three-axis system: epithelial vascular, and interstitial. This classification system seems to be based entirely on single cell RNA-sequencing UMAP plots. Do these axes hold-up if the values underlying UMAP computation change? The nuances in the lineage tracing and histological data produced by the authors do not adhere to the three-axis rules set forth. For example, the line between vascular and epithelial axes blurs when it comes to assessing VSM and ASM in scRNA space. This is further complicated with the Pdgfrb-Lineage tracing, where the authors show Pdgfrb+ cell interdigitated between the ASM (Fig. S7B). Do these in vivo data suggest that Pdgfrb+ cells, that we are told are part of the vascular axis, are also part of the epithelial axis? The authors show that these cells may express Pi16 and Meox2, markers of the epithelial axis. How would the authors reconcile the discrepancies in their lineage tracing data with the scRNA-based 3-axis rules?
Further the authors need to present quantifiable data regarding the spatial relationship of these mesenchymal cells with the axis they support.
2) In line with the point above, one major implication of the 3-axis classification rule that the authors refer to extensively throughout the manuscript is that cells within these specific axes, exert some function that is important for that axis. For example, Pdgfrb+ cells would primarily be

interacting with the vascular axis. While this is a logical hypothesis, the authors do not present any new functional data to support the axis-based rules.
3) A confusing aspect of the classification system is what to make of the interstitial axis? The authors should refine the terms epithelial and vascular axes, to airway and macrovascular. But why are alveolar myofibroblasts not in the interstitial axis? In its current form, the epithelial axis designation only applies to the ASM in the adult lung (P70).
4) All the morphological assessments of these mesenchymal cell types are descriptive with only a few representative images shown. The authors should provide a morphometric quantification (cell volume, process number, process length, branch points, etc.) of these mesenchymal cells as these data would be very illustrative of the vast differences the authors claim to observe in their distinct axes. A comprehensive morphometric assessment of the mesenchymal cells would elevate this paper and offer something new to the field.
5) One new piece of data to come from this study is the possible identification of ductal and alveolar myofibroblasts. While this is interesting the authors present no functional data and the analysis in its current form is difficult to interpret. For example, the CDH4/HHIP staining appears to stain many cells outside of the outlined 'alveolar duct'(Fig.4C). The authors present a more convincing Cdh4-CreER lineage tracing in Fig S5. The data shown in Fig 4E
(lacking morphometric analyses) needs to show a macroscopic view and quantification showing the specificity of targeting only the ductal myofibroblasts and NOT the alveolar myofibroblasts. If this lineage tracing tool is useful in selectively isolating this novel cell-type, more functional analysis would elevate this paper and be an important addition to the field.

Minor Points:
1) The authors should amend the language used to describe the isolation of fibroblasts for the scRNA-seq. The mesenchymal cell-types appear to be sub selected from whole lung scRNA data published by this group before. The referring language should remove "gating" as this implies a negative selection by bead or FACS based purification.
2) The PDGFRA antibody is unclear. What does perinuclear staining of Pdgfra indicate? Does this have something to do with the receptivity to Pdgf ligands and can the authors test this? The authors should add quantification of this.
3) The authors do provide some lineage tracing based quantification in the supplemental tables. These quantitative data should be presented in the main figures to aid the assessment of rigor in these experiments. Further quantification of the new Cdh4-CreER line should also be added.

Reviewer 2

Advance summary and potential significance to field

This work rigorously examined the gene expression, morphology, localization and to a more limited extent, lineage of different mesenchymal cell types identified in the developing and mature mouse lung. The findings led to the organization of lung mesenchymal cells into three axis, each with proximal versus distal diversity of cell types. The results also highlighted a myofibroblast sub-type that is specified in embryonic lung and persists into adult stages. This manuscript is eloquently written. While a bulk of the single cell RNAseq data confirm published findings, as acknowledged by the authors, the careful spatial localization of cell types provided a much needed big-picture view of the lung mesenchymal cell types.

Comments for the author

The following criticisms should be addressed.
1. The localization of the ductal myofibroblasts need to be better demonstrated: by illustrating in a 3D demonstration of the alveolar duct in the context of AT1/AT2 cells, as well is wider angle view of gene expression deeper into the lung along the more proximal airway. Figure 4D the colocation of tdT and HHIP in the alveolar duct is minimal.
2. The "shift" of clusters described in Figure 3 of this study "reflecting changes in gene expression and/or cell composition" needs to be elaborated with list of differential genes that can be validated to ensure that the shift is not from analyses artifact.

3. Lgr6-CreEr labeling of the “persistent” myofibroblasts recapitulate what had been observed by Lee et al., 2017. To be specific, both current study and Lee and Colleagues observed alveolar and peri-airway labeling of Lgr6 expressing cells. However, this study did not provide data showing whether Lgr6-CreEr labels ASM or myofibroblast, or both cell types in the peri-airway region.
4. The authors use Pdgfrb-CreER to label pericyte, and Pdgfra-CreER to label myofibroblasts, in order to demonstrate their morphology. But they also show that these cre lines each label multiple cell types.

Reviewer 3

Advance summary and potential significance to field

This manuscript identifies heterogeneity of mesenchymal cell populations in three tissue compartments, vascular, epithelial, and interstitial, of mouse lung based on a comprehensive cell sorting scRNA seq analysis of mesenchymal cells. Mesenchymal cells were isolated by FACS by elimination of other specific cell types. Single cell seq analysis identified 24 cell clusters that could be arranged into the three major lung compartments. These data are strongly supported by detailed immunostaining that categorizes mesenchymal cells based on their location relative to nearby landmark cells. This paper further advances the field by identifying new mesenchymal-specific Cre drivers. The data sets in this study will be very useful to the field of lung biology. I would encourage these data sets to be submitted to the Lunggens data base to allow easy accessibility.

Comments for the author

The validation of the scRNA seq data, by necessity, is descriptive but nevertheless useful. My main critique of the validation studies is that they lack quantitation, so we don’t know if these are cherry picked examples that fit the author’s models or robust observations. I would therefore strongly recommend quantifying, at least the lineage trace data, to show the fraction of labeled cells that persist or are lost over time or that fill a particular cellular niche. For example, vascular associated cells could be quantified relative to the diameter of the vessel that they are associated with; on page 8 “Intriguingly, these elongated proximal interstitial cells were wedged between and basal to ASM cells”: is this a rare event or is it observed for most proximal interstitial cells?; it would help to determine if there is/is not any association of MEOX+ cells with AT2 cells, as this has been reported in the literature. A nearest neighbor analysis of MEOX+ cells for both the proximal and distal compartments would be very useful. .

Specific comments:
Page 5 “to identify the transition from VSM cells to pericytes” implies a lineage relationship. This should be rephrased as a transition zone indicating an anatomic location and not a lineage relationship.
Figure 2B, not clear what the righthand boxes and lower left box are showing. It this tdT and brightfield overlay and ACTA2/brightfield overlay? In general, the figure legends should be more detailed.
Figure 3B, the evidence for perinuclear localization of PDGFRa is not clearly defined in the figure legend or text. Presumably the bottom 2 panels show this, but it is very difficult for the reader to interpret. Separate DAPI and GFP images might help to show co-localization, Figure 7D. The observation of cleaved caspase 3 at this stage of lung development is somewhat controversial in the literature. The immunostaining for cleaved caspase 3 should include co-localization with Dapi and quantitation at a few relevant time points. This observation should be discussed relative to the literature.

Minor comment: ROSA should be all caps.

First revision

Author response to reviewers' comments

We thank the reviewers for their insightful suggestions and positive remarks to improve our manuscript. Reviewers' suggestions have been addressed accordingly.

REVIEWER 1:

The authors do put forth the hypothesis that "…mesenchymal cells could be classified based on the structures they supported…", but the authors do not present experiments or perturbations that would add validity to the concept that the fibroblasts they classify "support" any structure.

The sentence is reworded as "mesenchymal cells could be classified based on their neighboring structures…". Regarding functional experiments, given the new knowledge from this study, we feel that mesenchymal cells used in existing organoid systems require better characterization and possibly require co-culturing with the corresponding epithelial and endothelial cells; this, as well as in vivo experiments, is deemed beyond the scope of this study. We also note that we find "quick" cell ablation experiments in vivo difficult to interpret given the loss of both physical environment and all chemical signals associated with the ablated cells.

The concept that lung mesenchyme develops along these three axes is not new; clonogenic lineage tracing published in 2014 revealed this mode of mesenchymal development (Kumar, 2014) and was similarly resolved with additional lineage tracing this past year (Zepp, 2021).

Our original submission cited Kumar 2014 in Discussion "…consistent with their distinct developmental origins – as evidenced by the respective radial versus distal recruitment of VSM and ASM cells (Greif et al., 2012; Kumar et al., 2014)…". The Kumar study focuses on early embryonic stages and does not address 4 out of 7 mesenchymal cell types in this study: ductal and alveolar myofibroblasts, and proximal and distal interstitial cells.

The Zepp 2021 study reaches different conclusions. (1) They refer to a population dubbed AMPs (Axin2+ Myofibrogenic Progenitor); these cells have been described extensively in the field and are known as pericytes, not AMPs. (2) They also refer to a population named MANCs, mesenchymal alveolar niche cells; our data shows that these are not located in the alveolar region. Instead, these "MANCs" are the proximal interstitial cells that co-express genes such as *Twist2, Il33, Pdgfra, Pdgfrb,* and *Meox2*. There is currently no evidence that these proximal interstitial cells form the alveolar type 2 stem cell niche.

Based on the scRNAseq, the authors do identify and attempt to validate the presence of two types of lung myofibroblasts. These data are new and potentially insightful to the field. However, the authors do not perform experiments that test functional differences between these two myofibroblasts, doing so would qualify this study as making a conceptual advance. There are several instances of ambiguities with this classification system, the data presented are descriptive. Hypothesis-driven experiments are needed to support this model. Overall, in its current form, this paper is largely descriptive and further analyses and functional experiments are needed.

Our three-axis classification system including identification of the two myofibroblast populations serves as a conceptual framework for future functional experiments. For example, our newly characterized markers CDH4, HHIP, and MEOX2 will help clarify the type of mesenchymal cells used in existing organoid cultures, which would be the first step in a definitive functional experiment. In vivo functional experiments for the ductal myofibroblasts would take years and are deemed beyond the scope of this study. In Discussion, we highlighted our contributions and future directions: "In this study, we introduce a three-axis classification system for lung mesenchymal cells that integrates single-cell transcriptomic, spatiotemporal, morphological, and lineage information." "This classification provides a framework to define the heterogeneous lung mesenchymal cells in vivo and in cultured organoids, and predicts their functions and signaling interactions with nearby cell lineages, and can be extended to other organs."

Below, we will address the said apparent ambiguities.

Major points:

One of the main takeaways that the authors put forward is that lung mesenchymal cells can be classified based on a three-axis system: epithelial, vascular, and interstitial. This classification system seems to be based entirely on single cell RNA-sequencing UMAP plots. Do these axes hold-up if the values underlying UMAP computation change?

The 3-axis model is also supported by Monocle (Fig. 1D) and validated by extensive immunostaining and lineage marking experiments. We used the default Seurat UMAP parameters and the entire code was provided as supplemental data to ensure reproducibility.

The nuances in the lineage tracing and histological data produced by the authors do not adhere to the three-axis rules set forth. For example, the line between vascular and epithelial axes blurs when it comes to assessing VSM and ASM in scRNA space.

As their names imply, airway SM and vascular SM share many contractile genes and are thus related in the scRNA space, which measures transcriptional similarity. This is not too different from clustering of proliferative cells of multiple axes. “The proliferative clusters (11, 14, and 21) included cells from each of the three axes as cell cycle genes dominated over cell type markers. This dominance of a single biological characteristic in cell clustering also contributed to the proximity of the VSM and ASM clusters.” That is why we additionally used immunostaining to map ASM and VSM spatially.

This is further complicated with the Pdgfrb-Lineage tracing, where the authors show Pdgfrb+ cell interdigitated between the ASM (Fig. S7B). Do these in vivo data suggest that Pdgfrb+ cells, that we are told are part of the vascular axis, are also part of the epithelial axis? The authors show that these cells may express Pi16 and Meox2, markers of the epithelial axis. How would the authors reconcile the discrepancies in their lineage tracing data with the scRNA- based 3-axis rules?

As stated in Introduction, “…the field has relied on components of signaling pathways of known importance in mesenchymal biology, such as Pdgf…”; “Due to the dynamic nature of signaling pathways and their deployment in multiple concurrent processes, mesenchymal cell types tagged by candidate signaling molecules might not align with those classically defined by molecular and cellular criteria.” The Pdgfrb data is a case in point in that “scRNA-seq predicted (it) to be active in both pericytes and proximal interstitial cells”. Therefore, to distinguish various Pdgfrb cells, we also relied on cell morphology and spatial information including proximal VS distal distributions and proximity to vessels. “This battery of molecular tools shed light on lineage-tracing experiments.” PI16 and MEOX2 are markers of the interstitial, not epithelial, axis.

Further the authors need to present quantifiable data regarding the spatial relationship of these mesenchymal cells with the axis they support.

We now include nearest neighbor analyses of MEOX2+ proximal interstitial cells to airways and macrovessels, and MEOX2+ distal interstitial cells to AT1, AT2, and DAPI cells (Fig. 5B, 6A).
one major implication of the 3-axis classification rule that the authors refer to extensively throughout the manuscript is that cells within these specific axes, exert some function that is important for that axis. For example, Pdgfrb+ cells would primarily be interacting with the vascular axis. While this is a logical hypothesis, the authors do not present any new functional data to support the axis-based rules.

We now included nearest neighbor analyses for lung mesenchymal subpopulations as stated above; functional experiments are deemed beyond the scope of this study. As mentioned earlier in the rebuttal, in Discussion, we highlighted our contributions and future directions: “In this study, we introduce a three-axis classification system for lung mesenchymal cells that integrates single-cell transcriptomic, spatiotemporal, morphological, and lineage information.” “This classification provides a framework to define the heterogeneous lung mesenchymal cells in vivo and in cultured organoids, and predicts their functions and signaling interactions with nearby cell

lineages, and can be extended to other organs."

A confusing aspect of the classification system is what to make of the interstitial axis? The authors should refine the terms epithelial and vascular axes, to airway and macrovascular. But why are alveolar myofibroblasts not in the interstitial axis? In its current form, the epithelial axis designation only applies to the ASM in the adult lung (P70).

The interstitial axis is transcriptionally distinct with proximal and distal cells and shares a new marker MEOX2. "Interstitial" is a fitting name for proximal MEOX2+ cells as they are "between the epithelial and endothelial trees within the proximal bronchovascular bundles". "Assignment of distal MEOX2+ cells to the interstitial axis was further supported by their discrete localization from PDGFRA+ and PDGFRB+ cells in the embryonic lung before the distal interstitial space became unrecognizable due to postnatal expansion of the alveolar airspace (Fig. 5F)."

We used the term "epithelial axis" because airway smooth muscle cells envelop the airways, the novel ductal myofibroblast surround alveolar ducts, and alveolar myofibroblasts surround distal alveoli. None of these cells nor cells of the vascular axis express MEOX2. In the adult lung (P70), the epithelial axis designation applies to both ASM and ductal myofibroblasts as ductal myofibroblasts persist after alveologenesis whereas alveolar myofibroblasts undergo developmental apoptosis.

All the morphological assessments of these mesenchymal cell types are descriptive with only a few representative images shown. The authors should provide a morphometric quantification (cell volume, process number, process length, branch points, etc.) of these mesenchymal cells as these data would be very illustrative of the vast differences the authors claim to observe in their distinct axes. A comprehensive morphometric assessment of the mesenchymal cells would elevate this paper and offer something new to the field.

We have now included measurements of cell perimeter, processes, and termini per process. "Comparison of cell morphology distinguished various mesenchymal cell types: pericytes were most complex with a larger perimeter, more processes and termini; compared to ductal myofibroblasts, alveolar myofibroblasts were smaller but with more processes, consistent with the geometry of alveolar ducts versus alveoli that they surrounded (Fig. 6F)."

One new piece of data to come from this study is the possible identification of ductal and alveolar myofibroblasts. While this is interesting, the authors present no functional data and the analysis in its current form is difficult to interpret. For example, the CDH4/HHIP staining appears to stain many cells outside of the outlined 'alveolar duct'(Fig.4C). The authors present a more convincing Cdh4-CreER lineage tracing in Fig S5. The data shown in Fig 4E (lacking morphometric analyses) needs to show a macroscopic view and quantification showing the specificity of targeting only the ductal myofibroblasts and NOT the alveolar myofibroblasts. If this lineage tracing tool is useful in selectively isolating this novel cell-type, more functional analysis would elevate this paper and be an important addition to the field.

We have addressed the functional data comments earlier in this rebuttal, and have further characterized the ductal myofibroblasts. Additional CDH4/HHIP staining outside the outlined alveolar duct is because "Arising from branch stalks as the airways did, alveolar ducts had wider airspace than the surrounding alveoli and could be best identified as they extended toward the lateral edge, instead of the lobe surface where tissue geometry made tubes less recognizable as they were shorter and interrupted by branching". To strengthen this point, we have updated Fig. 4A and added a new Fig. S3D to provide a macroscopic view of these tubular alveolar ducts surrounded by CDH4 ductal myofibroblasts, distinct from PDGFRA+ alveolar myofibroblasts. A new Fig. 4D provides another macroscopic view of multiple alveolar ducts containing *Cdh4*^{CreER} lineage-labeled cells. This driver is however inefficient "(~4%; 734 HHIP+ cells)". As mentioned, we have included measurements of cell morphology.

Minor points:
The authors should amend the language used to describe the isolation of fibroblasts for the scRNA-seq. The mesenchymal cell-types appear to be sub selected from whole lung scRNA data

published by this group before. The referring language should remove “gating” as this implies a negative selection by bead or FACS based purification.

Cells are not from whole lung, but FACS gating. We sorted for epithelial, endothelial and immune cells; the remaining, triple-negative cells were also collected as lung mesenchymal cells. Having the four purified cell lineages allows us to mitigate the dominance and thus sampling bias of immune and endothelial cells. “The resulting four purified cell lineages were remixed in equal proportions for cost efficiency and sequenced using 10x Genomics. We previously showed that this cell isolation strategy allowed balanced, sufficient sampling of all major cell types in both developing and mature mouse lungs (Cain et al., 2020; Vila Ellis et al., 2020)”.

The PDGFRA antibody is unclear. What does perinuclear staining of Pdgfra indicate? Does this have something to do with the receptivity to Pdgf ligands and can the authors test this? The authors should add quantification of this.

Indeed, Pennock et al. (PMID 27325673) suggest that ligand-bound, activated PDGFRA is endocytosed and accumulates in the perinuclear region of cultured cells; image quantification was difficult and not tempted there. The text now reads “PDGFRA staining was … concentrated in a perinuclear compartment in the neonatal lung, possibly indicating receptor activation and endocytosis (Pennock et al., 2016)”. We have further reported cells with perinuclear PDGFRA staining as having high GFP from two *Pdgfra* knock-in alleles. Functional tests of ligand receptivity require robust in vivo readouts of signaling activation and/or reliable cultured myofibroblasts that are currently unavailable.

The authors do provide some lineage tracing based quantification in the supplemental tables. These quantitative data should be presented in the main figures to aid the assessment of rigor in these experiments. Further quantification of the new Cdh4-CreER line should also be added.

Fig. 7F has been updated to include quantification. Cdh4-CreER line is inefficient “(~4%; 734 HHIP+ cells)”, and was used to illustrate cell morphology and location of ductal myofibroblasts.

REVIEWER 2:

This manuscript is eloquently written. While a bulk of the single cell RNAseq data confirm published findings, as acknowledged by the authors, the careful spatial localization of cell types provided a much needed big-picture view of the lung mesenchymal cell types.

We thank the reviewer for the encouraging comments and thoughtful suggestions to improve the manuscript.

The localization of the ductal myofibroblasts need to be better demonstrated: by illustrating in a 3D demonstration of the alveolar duct in the context of AT1/AT2 cells, as well is wider angle view of gene expression deeper into the lung along the more proximal airway. Figure 4D the colocation of tdT and HHIP in the alveolar duct is minimal.

We have updated and added figures to show multiple macroscopic views of ductal myofibroblasts surrounding the tubular alveolar ducts (Fig. 4A, 4D, S3D). As requested, Fig. S3D has AQP5 counterstaining for AT1 cell membrane to show alveolar ducts. tdT accumulates to the nucleus whereas HHIP is perinuclear (Fig. 4C), reducing colocalization on section views. We have used open and filled arrowheads to highlight representative tdT-expressing ASM cells and ductal myofibroblasts in the *Lgr6*$^{GFP:CreER}$ images (new Fig. 4F).

The “shift” of clusters described in Figure 3 of this study “reflecting changes in gene expression and/or cell composition” needs to be elaborated with list of differential genes that can be validated to ensure that the shift is not from analyses artifact.

The “shift” in the Fig. 3A UMAPs occurs to myofibroblasts but not airway smooth muscle cells. We have included differentially expressed genes in volcano plots (Fig. S3F) and Table S3. Lgr6-CreEr labeling of the “persistent” myofibroblasts recapitulate what had been observed by

Lee et al., 2017. To be specific, both current study and Lee and Colleagues observed alveolar and peri-airway labeling of Lgr6 expressing cells. However, this study did not provide data showing whether Lgr6-CreEr labels ASM or myofibroblast, or both cell types in the peri-airway region.

We have used open and filled arrowheads to highlight representative tdT-expressing ASM cells and ductal myofibroblasts in the *Lgr6*$^{GFP:CreER}$ images (new Fig. 4F). "Similarly, an *Lgr6*$^{GFP:CreER}$ driver (Snippert et al., 2010), as predicted by scRNA-seq (Fig. 3A), labeled ACTA2+ contractile cells around airways and alveolar ducts – the latter of which were marked by HHIP – but not vessels; neonatal lineage-labeled cells also persisted in the mature lung (Fig. 4F). This labeling pattern was consistent with a prior report (Lee et al., 2017)."

The authors use Pdgfrb-CreER to label pericyte, and Pdgfra-CreER to label myofibroblasts, to demonstrate their morphology. But they also show that these cre lines each label multiple cell types.

Indeed, such non-exclusiveness of popular drivers is a challenge in lung mesenchymal cell research. We validated the relevant cell types using spatiotemporal information and other markers. For example, *Pdgfrb*^{CreER} additionally labels proximal interstitial cells that are within bronchovascular bundles and express MEOX2. At neonatal stages, *Pdgfra*^{CreER} inefficiently labels distal interstitial cells that do not have perinuclear PDGFRA but express MEOX2.

REVIEWER 3:

This paper further advances the field by identifying new mesenchymal-specific Cre drivers. The data sets in this study will be very useful to the field of lung biology. I would encourage these data sets to be submitted to the Lunggens data base to allow easy accessibility.

We thank the reviewer for supporting this kind of research and positive remarks. "Raw data were deposited at GEO under accession number GSE180822". We will reach out to Lunggens about data deposition there.

My main critique of the validation studies is that they lack quantitation, so we don't know if these are cherry picked examples that fit the author's models or robust observations. I would therefore strongly recommend quantifying, at least the lineage trace data, to show the fraction of labeled cells that persist or are lost over time or that fill a particular cellular niche.

Quantification of the lineage tracing data was included in Table S5 and is now also shown in Fig. 7E. We have also quantified cell morphology and localization (Fig. 2B, 5B, 6A, 6F).

For example, vascular associated cells could be quantified relative to the diameter of the vessel that they are associated with.

We have now quantified the diameters of proximal, transition zone, and capillary vessels in Fig. 2B.

on page 8 "Intriguingly, these elongated proximal interstitial cells were wedged between and basal to ASM cells": is this a rare event or is it observed for most proximal interstitial cells? It would help to determine if there is/is not any association of MEOX+ cells with AT2 cells, as this has been reported in the literature. A nearest neighbor analysis of MEOX+ cells for both the proximal and distal compartments would be very useful.

Following the reviewer's recommendation, we performed nearest neighbor analyses of proximal (Fig. 6A) and distal (Fig. 5B) interstitial cells.

Specific comments:
Page 5 “to identify the transition from VSM cells to pericytes” implies a lineage relationship. This should be rephrased as a transition zone indicating an anatomic location and not a lineage relationship.

Thank you for the suggestion. It now reads “to identify the transition zone from VSM cells to pericytes”.

Figure 2B, not clear what the righthand boxes and lower left box are showing. Is this tdT and brightfield overlay and ACTA2/brightfield overlay? In general, the figure legends should be more detailed.

We have enlarged the numbers (1, 2, and 3) for the highlighted boxes. The legend now reads “Imaris normal shading view is shown for ACTA2 and tdT images”. This image rendering gives better 3D perception, and is noted in other figure legends when needed.

Figure 3B, the evidence for perinuclear localization of PDGFRa is not clearly defined in the figure legend or text. Presumably the bottom 2 panels show this, but it is very difficult for the reader to interpret. Separate DAPI and GFP images might help to show co-localization,

Thank you for the suggestion. We have included single channel images.

Figure 7D. The observation of cleaved caspase 3 at this stage of lung development is somewhat controversial in the literature. The immunostaining for cleaved caspase 3 should include co-localization with Dapi and quantitation at a few relevant time points. This observation should be discussed relative to the literature.

Examples of condensed chromatin and quantifications of apoptosis over time are included as Fig. 7E. The Discussion reads “The distinct developmental fates of ductal and alveolar myofibroblasts could explain the remaining Fgf18-lineage cells, which are expected to include both myofibroblasts (Fig. 1B) (Hagan et al., 2020), and also highlight the heterogeneity within secondary crest myofibroblasts that include cells around embryonic branch stalks and thus future alveolar ducts (Li et al., 2015; Zepp et al., 2021). Future studied are needed to understand the differential regulation, fate, and function of the two myofibroblast populations.”

Minor comment: ROSA should be all caps.

We added in methods “The official locus name for the *Rosa* reporters is *ROSA*.”

Second decision letter

MS ID#: DEVELOP/2021/200081

MS TITLE: Three-axis classification of mouse lung mesenchymal cells reveals two populations of myofibroblasts

AUTHORS: Odemaris Narvaez del Pilar, Maria Jose Gacha Garay, and Jichao Chen

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is

so. In this case, to address some of the concerns raised by the first reviewer, I suggest a change in article type to a 'Techniques and Resources' article.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

In the revised manuscript entitled “Three-axis classification of mouse lung mesenchymal cells reveals two populations of myofibroblasts,” del Pilar and colleagues address some of the critiques raised during the initial review. As requested, the authors now provide a morphometric analysis of the pericytes, myofibroblasts, and interstitial fibroblasts (see comment below for clarification). They have also added ‘nearest neighbor’ distance measurements of Meox2+ cells (see comment below). These data are useful additions to this study.

In the first review, this reviewer commented that experiments be performed to support the “three-axis classification system” and/or validate the apparently different (and potentially novel!) myofibroblasts. These were central points to this study as highlighted in the title. To these points, the authors did not address this reviewer’s concerns. Specifically, the authors did not test the validity or utility of the 3-axis classification (comments below) and remarked that functional experiments related to the ductal myofibroblast are “deemed beyond the scope of this study”. Specific comments about the utility of the "classification system" and/or suggestions for further consideration regarding data reporting and the writing are listed below.

Comments for the author

Major concerns referring to this as a classification system with applicability to other organs.
1) The authors “hypothesized that mesenchymal cells could be classified based on their neighboring structures…”. How did the authors test if this hypothesis is true? What were the rules assigned to this classification system?
How would a different lab with a unique lineage tracing tool use these rules to ‘classify’ their stromal cell? The authors discuss their classification system as “its root in the cylindrical coordinate system with an axial height for proximal-distal location, a radial distance for the layered wrapping…and an azimuth for the cylindrical symmetry”. Such distances and measures are not presented in this study. The terms used by the authors, such as “neighboring”, “nearby” and “in-between” are not defined values.
2) Based on the title, the authors indicate that the classification system revealed two populations of myofibroblasts. In the absence of the classification system, would the authors not have identified the two myofibroblasts? Is this statement still valid if the authors only rely on the clustering of the scRNA?
Several lung development atlases have been generated, published, and data deposited. Does the classification system and/or clustering “reveal” these “cells” in other scRNA datasets?
3) The “nearest neighbor” measurements suggest that a mesenchymal cell is spatially associated with the authors’ “axis” definition. However, by only examining a single “axis” marked by expression of Meox2, the authors do not address the specificity of their classification system. These data would be more useful if the authors include an additional "axis"-defined cell type marker.
This reviewer would suggest another nuclear marker such as Ebf1 for the Pdgfrb cells(described in Liu et al., 2021, PMID:34151224) for the “vascular” axis as a comparison.

Minor concerns to address in the manuscript.
4) This reviewer’s previous comment about the term “gating” used by the authors needs clarification. The authors prep epithelial, endothelial, and mesenchymal cells using a FACS based strategy (‘gating’). Based on the methods this reviewer’s understanding is that these cells were

mixed and then loaded onto a 10x chromium system. The UMAP and other data presented in this paper were derived from a mesenchymal cell 'subset' derived from this whole-lung scRNA (figure S1). The authors should clarify whether 'clusters' were used for subsetting or gene expression for Col3a1? As written, "computationally identified the mesenchymal cells as positive for a matrix gene Col3a1.." It is advised to sub-select 'clusters' for downstream analysis. This can be addressed in the methods.

5) In the text, there are several statements the authors write that question the validity of other studies. This reviewer would suggest removing these statements as they serve no purpose for the interpretation of the authors'
analyses and are based on limited data and no experiments.

a. Page 4, bottom: Sentence begins with "These two groups…Col13a1 and Col14a1…." Remove the subordinate "although" clause.

b. Page 5, top: Sentence includes "…used marker Plin2 was non-specific raising questions.." Remove the "raising questions" clause.

c. Page 9, bottom: Sentence includes "…the latter of which has been called lipofibroblasts…albeit…". Remove the "albeit" clause.

6) The authors present Meox2 and Cdh4 as new markers for subsets of fibroblasts. Do the authors think that these markers have a functional role in their respective fibroblast lineages? A short addition to the discussion would be welcomed.

7) The authors need to include the time point(s) used for the cell morphology analyses presented in Figure 6F in the legend or the plots. The authors refer to "Fig 2-5" but this is unclear. For example there are two timepoints in figure 2 for Pdgfrb-lineage, 6-week and P3, but the authors only plot a single column. Please clarify.

Reviewer 3

Advance summary and potential significance to field

This manuscript identifies heterogeneity of mesenchymal cell populations in three tissue compartments, vascular, epithelial, and interstitial, of mouse lung based on a comprehensive cell sorting scRNA seq analysis of mesenchymal cells. Mesenchymal cells were isolated by FACS by elimination of other specific cell types. Single cell seq analysis identified 24 cell clusters that could be arranged into the three major lung compartments. These data are strongly supported by detailed immunostaining that categorizes mesenchymal cells based on their location relative to nearby landmark cells. This paper further advances the field by identifying new mesenchymal-specific Cre drivers. The data sets in this study will be very useful to the field of lung biology.

Comments for the author

The authors have done a good job in addressing most suggestions of the reviewers. In particular, they have placed image quantification along with the image data. However, these quantitative data need to be explained in the results and discussion to support the conclusions. Additionally, quantitative measures in Figures 2B, 5B, 6A,F, 7E need statistical analysis (in most cases ANOVA with multiple comparisons) marked on the figures with lines or asterisks and described in the legend. These data need to be interpreted in the results to highlight significant differences or relevant non-significant differences.

Dot plot data should also have supporting statistics and relevant interpretation in the results. For example, in fig 2A is increased Gap43 and Gucy1a1 in pericytes significant compared to other cell types? In figure 4A, is Tagln expression significantly higher in ASM compared to other cell types, and how is this interpreted.

I agree with the authors that functional studies are beyond the scope of this work.

Minor comments.

The authors should consider updating the figures with the correct accepted gene nomenclature for mice. ROSA not Rosa.
Also note that transgenes are not italicized but knockin alleles are in italics.

Second revision

Author response to reviewers' comments

We thank the reviewers for their positive remarks and additional suggestions to make our study more useful to the field. We have provided our point-to-point response below.

REVIEWER 1

Major concerns referring to this as a classification system with applicability to other organs.

1) The authors "hypothesized that mesenchymal cells could be classified based on their neighboring structures…". How did the authors test if this hypothesis is true? What were the rules assigned to this classification system? How would a different lab with a unique lineage tracing tool use these rules to 'classify' their stromal cell? The authors discuss their classification system as "its root in the cylindrical coordinate system with an axial height for proximal-distal location, a radial distance for the layered wrapping…and an azimuth for the cylindrical symmetry". Such distances and measures are not presented in this study. The terms used by the authors, such as "neighboring", "nearby" and "in- between" are not defined values.

We used the word "hypothesized" to introduce our reasoning to conceptualize mesenchymal cell classification. The sentence now reads "As reasoned in the introduction, mesenchymal cells could be conceptually classified based on their neighboring structures, most notably the vascular and epithelial trees."

Similarly, "the cylindrical coordinate system" in Discussion is a mathematical concept that provides a new perspective to biology, and unlike the XYZ coordinates, is "a natural way to characterize biology because tubes are fundamental building blocks". We have presented that the proximal (airway) and distal (alveolar) mesenchymal cell populations within each axis, radial organization around epithelial and vascular tubes (most strikingly in Fig. 5F), cross-sectional and longitudinal views of tubes to illustrate the cylindrical symmetry (azimuth).

We propose our 3-axis system as a new conceptual framework in lung mesenchymal cell research. "This tube-centered axial system is conceptually applicable to the mesenchyme in other organs, ranging from smooth muscle cells and pericytes surrounding the omnipresent vascular network, peristaltic muscles along the digestive tract, the hierarchical insulation and organization of axons within nerve bundles by myelination, endoneurium, and perineurium." Recognizing such analogy does not require precise measurements.

2) Based on the title, the authors indicate that the classification system revealed two populations of myofibroblasts. In the absence of the classification system, would the authors not have identified the two myofibroblasts? Is this statement still valid if the authors only rely on the clustering of the scRNA? Several lung development atlases have been generated, published, and data deposited. Does the classification system and/or clustering "reveal" these "cells" in other scRNA datasets?

ScRNA is such robust technology that existing datasets are largely consistent; the challenge lies in the spatial location of cell clusters and hence interpretation of the data. Our classification system is useful as "A notable prediction of the axial system that we have validated experimentally is the presence of ductal myofibroblasts associated with alveolar ducts, an epithelial structure connecting proximal airways with distal alveoli". The axial system also allows us to predict that the interstitial cells "belonged to a third axis and named it the interstitial axis to refer to the

space between the epithelial and endothelial trees", and show that they indeed share a common marker MEOX2.

3) The "nearest neighbor" measurements suggest that a mesenchymal cell is spatially associated with the authors' "axis" definition. However, by only examining a single "axis" marked by expression of Meox2, the authors do not address the specificity of their classification system. These data would be more useful if the authors include an additional "axis"-defined cell type marker. This reviewer would suggest another nuclear marker such as Ebf1 for the Pdgfrb cells(described in Liu et al., 2021, PMID:34151224) for the "vascular" axis as a comparison.

A collection of reasoning and data (not limited to nearest neighbor measurements) support our 3-axis model. The reasoning for the proximal-distal 3 axes is below **(1) vascular axis:** "It was self-evident to assign vascular smooth muscle cells and pericytes to the vascular tree, which we named the vascular axis because the two mesenchymal cell types situated along a proximal-distal axis." **(2) epithelial axis:** "Applying the same concept, we assigned to the epithelial axis the proximal ASM cells (cluster 10) and the distal alveolar myofibroblasts (clusters 6 and majority of 1), both of which constrain and shape the epithelium (Kim and Vu, 2006), and predicted that the other transcriptionally-related clusters (5, 8, 12, and 13) were associated with the epithelium in-between, namely the alveolar ducts – for which we provided evidence later in this study." **(3) interstitial axis:** "recognizing that the Twist2-expressing cells were shown in a mouse phenotyping database (Koscielny et al., 2014) to localize between the epithelial and endothelial trees within the proximal bronchovascular bundles and that the ratio of Twist2- expressing and Wnt2-expressing cells was what one would expect for proximal and distal compartments, we predicted that they belonged to a third axis and named it the interstitial axis to refer to the space between the epithelial and endothelial trees."

"Supporting this three-axis classification, Monocle trajectory analysis coerced the associated mesenchymal cells into three paths that terminated in Pdgfrb+ pericytes, Actc1+ ASM cells, and Wnt2+ fibroblasts – corresponding to the vascular, epithelial, and interstitial axes, respectively". Subsequently, "we focused on each axis individually to define constituent cell populations, map in 3D their proximal- distal distributions, and categorize cell morphology."

Complementary to the nearest neighbor analysis, a visually striking image in Fig. 5F shows that "Assignment of distal MEOX2+ cells to the interstitial axis was further supported by their discrete localization from PDGFRA+ and PDGFRB+ cells in the embryonic lung before the distal interstitial space became unrecognizable due to postnatal expansion of the alveolar airspace".

The recommended marker Ebf1 is abundant in B cells and thus not used. The referenced paper Liu et al. 2021 is cited.
Minor concerns to address in the manuscript.

4) This reviewer's previous comment about the term "gating" used by the authors needs clarification. The authors prep epithelial, endothelial, and mesenchymal cells using a FACS based strategy ('gating'). Based on the methods, this reviewer's understanding is that these cells were mixed and then loaded onto a 10x chromium system. The UMAP and other data presented in this paper were derived from a mesenchymal cell 'subset' derived from this whole-lung scRNA (figure S1). The authors should clarify whether 'clusters' were used for subsetting or gene expression for Col3a1? As written, "computationally identified the mesenchymal cells as positive for a matrix gene Col3a1.." It is advised to sub-select 'clusters' for downstream analysis. This can be addressed in the methods.

We have added to the methods "Clusters positive for Col3a1 but negative for Nkx2-1, Cdh5, and Ptprc were subsetted as mesenchymal cells." The main text is also updated as "computationally identified the mesenchymal cell clusters as positive for a matrix gene Col3a1 and negative for other cell lineage markers including Nkx2-1 (epithelial), Cdh5 (endothelial), and Ptprc (immune) (Fig. S1B)."

5) In the text, there are several statements the authors write that question the validity of other studies. This reviewer would suggest removing these statements as they serve no purpose for the interpretation of the authors' analyses and are based on limited data and no experiments.

a. Page 4, bottom: Sentence begins with "These two groups…Col13a1 and Col14a1…." Remove the subordinate "although" clause.

This clause is supported by scRNA-seq analysis of all collagen genes. It now reads "These two groups were recently named after additional markers as Col13a1 and Col14a1 matrix fibroblasts (Xie et al., 2018); we noted that most mesenchymal cells as well as endothelial and epithelial cells produced matrices (Fig. S1C)."

b. Page 5, top: Sentence includes "…used marker Plin2 was non-specific, raising questions.." Remove the "raising questions" clause.

This clause is removed.

c. Page 9, bottom: Sentence includes "…the latter of which has been called lipofibroblasts…albeit…". Remove the "albeit" clause.

This clause is removed.

6) The authors present Meox2 and Cdh4 as new markers for subsets of fibroblasts. Do the authors think that these markers have a functional role in their respective fibroblast lineages? A short addition to the discussion would be welcomed.

Thank you for the suggestion. We have added "Future studies are needed to probe if new markers such as MEOX2 and CDH4 regulate the transcriptional program and cell sorting of specific mesenchymal cell populations".

7) The authors need to include the time point(s) used for the cell morphology analyses presented in Figure 6F in the legend or the plots. The authors refer to "Fig 2-5" but this is unclear. For example there are two timepoints in figure 2 for Pdgfrb-lineage, 6-week and P3, but the authors only plot a single column. Please clarify.

Thank you for the suggestion. The legend now reads "Schematic and quantification of cell morphology of color-coded cell types in Fig. 2C (P3 pericytes), 3D (P7 alveolar myofibroblasts), 4E (P21 ductal myofibroblasts), and 5E (6-wk distal interstitial cells)."

REVIEWER 3

The authors have done a good job in addressing most suggestions of the reviewers. In particular, they have placed image quantification along with the image data. However, these quantitative data need to be explained in the results and discussion to support the conclusions.

The following text is added.

"To visualize the vascular axis, we used a PdgfrbCreER driver (Cuervo et al., 2017) and wholemount immunostaining to identify the transition zone from VSM cells to pericytes based on the gradual decrease in vessel diameter with the transition zone being intermediate between proximal macro- vessels and distal capillaries as well as ACTA2 (also known as SMA) staining (Fig. 2B)."

"MEOX2+ cells were in the vicinity of alveolar type 2 (AT2) cells, but not significantly closer than alveolar type 1 (AT1) cell or non-epithelial cell nuclei (Fig. 5B, S6B)."

"Intriguingly, these elongated proximal interstitial cells were wedged between and basal to ASM cells (Fig. S7B, 6D), within 10 um to the airway basement membrane, while those closer to macro-vessels could be further away in the adventitia (Fig. 6A)."

"Comparison of cell morphology distinguished various mesenchymal cell types: pericytes were most complex with a larger perimeter, more processes and termini; compared to ductal

myofibroblasts, alveolar myofibroblasts were smaller but with more processes, consistent with the geometry of alveolar ducts versus alveoli that they surrounded (Fig. 6F)."

"Notably, both Myh11-CreER and PdgfraGFPCreER labeled cells, which overlapped for alveolar myofibroblasts, were found to express cleaved-Caspase 3 – consistent with apoptosis that began after P12, as also supported by chromatin condensation (Fig. 7D, 7E). We also noted that a smaller number of PDGFRB+ pericytes as well as non-PDGFRA/B cells were positive for cleaved-Caspase 3, implying additional cell trimming in neonatal lungs (Fig. 7E)."

Additionally, quantitative measures in Figures 2B, 5B, 6A,F, 7E need statistical analysis (in most cases ANOVA with multiple comparisons) marked on the figures with lines or asterisks and described in the legend. These data need to be interpreted in the results to highlight significant differences or relevant non-significant differences.

Ordinary ANOVA with Tukey or Dunnett test is included in Figures and legends, and described in the main text as above.

Dot plot data should also have supporting statistics and relevant interpretation in the results. For example, in fig 2A is increased Gap43 and Gucy1a1 in pericytes significant compared to other cell types? In figure 4A, is Tagln expression significantly higher in ASM compared to other cell types, and how is this interpreted.

Statistics for each dot plot is included in additional spreadsheets in Table S2-4. "mature pericytes mostly had cells from P13, P20, and P70 and expressed significantly more Gap43 and Gucy1a1 (Fig. 2A, Table S2) – suggesting that pericytes, unlike VSM cells, mature after birth." "readily identified an Actc1+ ASM cluster that was largely unchanged on the UMAPs over time with the highest levels of contractile genes Acta2/Tagln/Myh11, possibly reflecting their higher mechanical load for airway constriction"

I agree with the authors that functional studies are beyond the scope of this work.

Thank you for the support.

Minor comments.

The authors should consider updating the figures with the correct accepted gene nomenclature for mice. ROSA not Rosa.

Also note that transgenes are not italicized but knockin alleles are in italics.

ROSA is used in figures. Cdh5-CreER and Myh11-CreER are no longer italicized.

Third decision letter

MS ID#: DEVELOP/2021/200081

MS TITLE: Three-axis classification of mouse lung mesenchymal cells reveals two populations of myofibroblasts

AUTHORS: Odemaris Narvaez del Pilar, Maria Jose Gacha Garay, and Jichao Chen
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. I have carefully considered your request to not publish as a T&R article, and I agree with you that the work meets the standard for publication as a Research Article.