

Directed Differentiation with Mechanical Simulation Enhances Alveolar-like Features in Human Pluripotent Stem Cell-Derived Lung Epithelium

Corresponding Author: Dr Amy Wong

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)

Brief summary of the manuscript:

Song et al. report the differentiation of human pluripotent stem cells into fetal lung progenitor (FLP) and distal bipotent (DBT) organoid lineages that give rise to alveolar-type epithelial cells. By combining iPSC-derived FLPs with human fetal lung fibroblasts (HFLFs) and applying cyclic mechanical cues within a microfluidic air–liquid interface (ALI) system, the authors generate epithelial sheets containing ABCA3⁺ and HTI-56⁺ cells.

Overall impression of the work:

The study is technically competent and addresses an important question in human lung differentiation. However, the principal novelty appears confined to the microfluidic co-culture experiments (Figure 6), and further data are required to substantiate reproducibility, mechanistic insight, and biological significance. Moreover, the manuscript does not clearly resolve the ongoing debate regarding whether bipotent progenitor cells (BPCs) are indispensable intermediates during human lung development (He et al., *Cell*, 2022).

If the following comments are addressed, the work could make a meaningful contribution to the field.

Major Comments:

1. Evidence for the Existence and Necessity of BPCs in Human Lung Development

The authors emphasise the induction of AT1/AT2 bipotent progenitor cells (BPCs) from human iPSCs as a central rationale. Although the BPC concept was first proposed in mouse fetal lung development (Desai TJ et al., *Nature*, 2014; Frank DB et al., *PNAS*, 2019), recent single-cell multi-omics analyses of human fetal lungs (He et al., *Cell* 2022; Fig. 3) suggest a distinct trajectory in which early/late tip cells differentiate into fetal AT2 or AT1 cells via late-stalk intermediates, bypassing a discrete BPC stage. Given these findings, it remains uncertain whether BPCs are essential in humans. As direct access to fetal lung tissue is limited, the authors should reanalyze the dataset from He et al. dataset to verify the presence of SFTPC⁺ PDPN⁺ BPC-like populations during human lung development and provide clear evidence supporting their developmental necessity and relevance to the current human iPSCs-system.

2. Evaluation of AT1/AT2 Maturity in the iPSC Differentiation System

Functional maturation is critical in iPSC-derived alveolar differentiation. Although the manuscript claims the generation of “functionally mature alveolar cells,” the evidence—limited to ABCA3 and HTI-56 expression and electron microscopy images—is not sufficient.

Please (i) justify why these markers can reliably indicate functional maturity, and (ii) provide qualitative and quantitative analyses demonstrating that the observed lamellar body-like structures correspond to mature AT2 cells.

3. Robustness of Data Supporting the Novelty Claimed in Figure 6

The microfluidic co-culture of FLPs and HFLFs under cyclic mechanical strain represents the principal innovative aspect of this manuscript. However, as noted in the limitation section, no experimental data are presented to validate the chosen cyclic-stretch condition (5 % strain at 0.5 Hz). Since the originality of the manuscript relies largely on this result (Figure 6), please supply evidence that this condition is optimal for promoting alveolar epithelial maturation, ideally through a systematic parameter assessment and an appropriate functional endpoint assay.

4. Evidence for the Universality of the “No-Sorting” FLP Differentiation Approach

The authors report > 85 % efficiency in generating NKX2.1⁺ SOX2⁺ SOX9⁺ CD47⁺/CPM⁺ FLPs, enabling subsequent maturation without cell sorting. However, the protocol up to the FLP stage appears similar to prior reports, and this high efficiency may be clone-specific. It is essential to repeat the differentiation using more than three iPSC lines and confirm that the sorting-free approach reproducibly yields FLPs suitable for maturation.

5. Clarification of Cell Lines Used for the Data Presented in Each Figure

In the Method and Materials section, the authors reported the use of the human ESC line CA1 and the iPSC lines BU3, CFGE1 and CFGE2. To demonstrate the robustness of the protocol, the data should be presented separately for each hPSC line. To establish generalizability, please clarify what “N=3” represent in each experiment. Independent biological replicates, rather than technical replicates alone, are required. Additionally, in the comparison of the conditions for inducing AT1-like cells, did the authors observe consistent results across all hPSC lines?

Minor Comments:

1. Highlight 1: The points of “superiority” over existing methods should be clearly stated.

2. Incomplete citation of prior iPSC-lung differentiation studies

The manuscript should reference Gotoh et al., *Stem Cell Rep* 2014 and Yamamoto Y et al., *Nat Methods* 2017, particularly where it states (line 136) that “most iPSC-based systems neglect epithelial–mesenchymal crosstalk.”

3. Quantification of SOX2⁺, SOX9⁺, and double-positive cells (Figure 1C)

Please quantify the proportions of SOX2⁺-only, SOX9⁺-only, and SOX2⁺SOX9⁺ cells within the induced FLP population.

4. Supplementary Fig 3b

NKX2.1 should be localised to the nucleus. Please confirm whether the images are correct.

5. Improved analysis of AT2 differentiation conditions (Figure 2B)

As ABCA3 alone is not sufficient to define AT2 identity (Sun YL et al., *Am J Respir Cell Mol Biol*, 2021), please quantify the relative frequencies of ABCA3⁺, SFTPC⁺, and double-positive cells under each condition.

6. Thickness of the “ultra-thin EC (UMM) membrane”

Please specify the thickness of the membrane.

7. Characterization of ABCA3⁺ HTI-56⁺ populations (Figure 6A)

A substantial subset of double-negative cells is present within the epithelial sheet. Please characterise these cells to clarify their identity and possible contribution.

8. Stability of marker expression in the microfluidic device

How long are AT1 markers, SFTPC and other AT2 markers maintained in the microfluidics device? This is crucial information when using this method for assays in the future.

9. Image resolution and subcellular localisation.

Please include inset magnifications in all immunofluorescence panels to demonstrate intracellular localization patterns of marker proteins.

Reviewer #2

(Remarks to the Author)

Summary

In this manuscript, the authors first presented a protocol for generating distal bud tip organoids containing immature alveolar type 1 (AT1) and AT2-like cells. They report that co-culture with fetal lung fibroblasts or exposure to fibroblast-conditioned media enhances differentiation efficiency toward fetal AT1 and AT2 cell fates. The study further demonstrates that alveolar maturation can be augmented by modulating Hippo-YAP1 signaling and by applying biophysical cues through cyclic stretch. Finally, the authors introduce a human iPSC-derived alveolar-on-chip system designed to mimic aspects of human fetal lung development. While the work is conceptually interesting, several critical issues must be addressed to strengthen the conclusions.

Major Points

1. A large portion of the conclusions rely on quantifying positive cells from microscopy images. However, most of the representative IF panels show nonspecific staining, very high background, overexposure, or inconsistent magnification between experimental conditions (e.g., Fig. 5A, 5D, 6A, in Supp Fig. 3b: Scale bars differ between channels for the same image). In addition, both organoid morphology and cell density vary considerably, which may compromise quantification accuracy. The authors should provide isotype controls, higher-quality images, and a clearer explanation of how quantifications were performed (e.g., whole organoid vs. single z-plane, number of organoids analyzed). Line 203: Authors report 50-60% AT1/AT2 bipotent cells, but Fig. 1E shows 34.8% BPCs. Clarify this discrepancy.

2. In Fig. 2C, the quantification of lamellar bodies or microvilli is unclear, were individual cells or whole organoids quantified? A more robust analysis, such as Western blotting for surfactant proteins, would be more convincing. In Fig. 2L,

an AT1 cell is claimed to be present, but no morphological criteria support this. Gene expression analysis (e.g., RT-qPCR or RNA-seq for AT1 markers) should be included to validate AT1 cell identity.

3. In Fig. 3A, the loading controls (Calnexin) are barely visible, and the final condition appears to be from a different gel. This experiment should be repeated for clarity and reliability. In Fig. 3D, the flow cytometry gating strategy needs adjustment and should include appropriate minus-fluorescence or isotype controls.

4. The Western blot in Fig. 4F shows a double band that is not addressed in the text. The authors should clarify whether this reflects isoforms, post-translational modifications, or a technical artifact.

5. Because the authors state that fibroblasts influence epithelial development via growth factor secretion, they should distinguish direct cellular interaction from paracrine signaling. Including direct co-cultures, conditioned media, and transwell cultures (preventing direct contact) would help dissect whether the observed effects arise from direct cell–cell contact or secreted factors.

Minor Points

1. At the beginning of the Results, the authors state that both an embryonic stem cell line and an iPSC line were used; however, it is unclear which data correspond to which line. If both lines were used, data for both should be shown.

2. Lines 67–68: The sentence describing the co-culture setup is unclear and should be rephrased.

3. Line 165: A comma is missing (“To do this, ...”).

4. Supplementary Fig. 1b: The y-axis label should specify which population the percentage refers to (e.g., all live cells or a specific gate).

5. Line 170: The authors state that FOXA2/SOX17 co-expression was assessed by flow cytometry, but Methods (lines 468–469) and Supplementary Fig. 1a/b show IF staining. Please reconcile this.

6. Supplementary Fig. 2: Rename P1–P4; this terminology is commonly used for cell passages and can be misleading.

7. Supplementary Fig. 2a–c: Indicate the developmental stage in each panel, as done for S2b.

8. Supplementary Fig. 1e: FACS plots for FGF2/SHH+ cells should also be included.

9. Flow cytometry percentage displays are inconsistent throughout the figures. The placement of legends (side vs. below vs. above) should be unified.

10. Supplementary Fig. 1d: AVFE is only defined in the figure legend; it should also be introduced in the main text.

11. Fig. 1c: The small inset images in the corner are unclear; label them in the text or annotate with arrows.

12. Supplementary Fig. 3a: Gating terminology differs between control and DBT; use consistent labels. Plots should be labeled with negative and DBT samples.

13. Lines 177–178 and 200–202 refer to Supplementary Fig. 2c but assign it to different stages (Stage 2b vs. Stage 4). Clarify and label the figure accordingly.

14. Fig. 1D: This is not a TEM image.

15. Lines 214–215: Authors state that only some cells are ECAD+ and AQP5+, but the ECAD staining in Fig. 1F appears widespread. Clarify.

16. Line 221: Replace “mediums” with media.

17. Fig. 2B: “FC quantification” may be mistaken for “fold change.” Rephrase and ensure consistent labeling across flow cytometry figures.

18. Lines 236–237 refer to Fig. 2D for microvilli, but Fig. 2D shows lamellar body quantification; correct the reference.

19. Supplementary Fig. 6c is missing or unlabeled.

20. Line 257: Grammar correction needed (“The bipotent DBT marker ... was significantly decreased ...”).

21. Line 279: Introduce the abbreviation AL (adult lung) before use.

22. Line 413: *in vitro* should be italicized.

23. Line 609: Missing words, should read: “adherent differentiated cells were washed with PBS three times...”.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

Song et al. introduce an improved differentiation strategy that more efficiently generates distal lung epithelial cells and establishes scalable distal bud tip organoids capable of producing bipotent alveolar progenitors. Although pharmacologic maturation effects are limited, microenvironmental cues—including fibroblast co-culture, mechanical stretch, and air exposure—enhance alveolar type 1 cell fate maturation. Addressing the following concerns below will be required for consideration in Communications Biology.

Major points

1. Fetal lung development: Direct differentiation of PSCs into lung epithelial cells recapitulates aspects of fetal lung development; therefore, PSC-derived cells generally resemble fetal-stage cell types. The authors quantified AT1/AT2 bipotent cells and fetal AT1 and AT2 cells using HT2-280, PDPN, and AQP5. However, these markers are not characteristic indicators of AT1 or AT2 cell fate during the fetal stage. PDPN and AQP5 are expressed in developing airway epithelium, not in the bud tip epithelium, and HTI-280 is also not a valid marker for fetal AT2 in human fetal lungs 1,2. For example,

HTII-280+ cells within an organoid are not matched with ABCA3+ cells (figure 2E). Furthermore, the model, a bipotent lung progenitor capable of differentiating to both alveolar type 1 and 2 cells still remain controversial in human lung development². Thus, the validity of using PDPN, AQP5, HT2-280, and SOX9 to define fetal biopotential lung progenitor or fetal AT1/AT2 lineages in this manuscript is not well supported and raises concerns. It is highly recommended to consider human fetal lung development and additionally reliable cell type markers, such as SFTPC, CAV1, and TP63, when authors revisit, re-evaluate, and clarify the dataset presented in figure 1. A combined gene expression analysis using qRT-PCR and immunostaining will be useful.

- Bud tip progenitor: SOX9 and SOX2 dual positive
- A typical AT2 marker: SFTPC, the gold standard marker (proSPC, immature and mature AT2; mature SPC, mature AT2)
- Other AT2 markers: LAMP3, ABCA3, HOPX
- AT1 markers: AQP5, CAV1, PDPN (if the organoid does not contain basal cell-like cells)
- HTII-280: Adult AT2 cell marker; useful but not always clearly indicating AT2 cells in fetal lung

2. Scalable culture of differentiating lung epithelium including BPCs and fetal AT2: How much are the PSC-derived BPCs scalable in 3D? How many passages it can be propagated for scalable production of alveolar cells? The lung BPCs can be considered 'proliferative' but the authors need to provide the relevant data. Furthermore, this is directly linked to the stability of BPC state under in vitro condition.

3. Surfactant protein expression: The authors compared several alveolar differentiation conditions, CK+DCI, CKD, CK, and CSBK to investigate the ideal alveolar induction in this context. However, they are missing surfactant genes when judging the differentiation efficiency – for example, lack of SFTPC, SFTPB, and LAMP3 expression was suggested. ABCA3 is also an important gene associated with alveolar differentiation but the gold standard gene, SFTPC, at least must be included. Although detection of lamellar bodies by TEM analysis (figure 2) and one SFTPC staining image (in supplementary fig 5b) were provided, but a higher resolution of surfactant protein localization pattern will have to be evident.

4. Positive control (adult human lung) for evaluating AT1 fate differentiation: The authors used adult human lung (AL) as a control to compare AT1 marker expression levels in the differentiated organoids. However, the gene expression levels of AT1 markers upon AT1 differentiation seems not to be corresponded to those of whole adult lung. Did the authors use the purified cells when comparing the levels? The whole tissue lysates from adult and fetal lungs could not be optimal as a control. Therefore, it would be difficult to conclude that the upregulation of AT1 markers such as CAV1 and SPOCK2, as well as YAP1, TEAD1, and LATS1, is physiologically relevant to those in adult AT1 cells.

Additionally, HOPX is considered as an AT2 marker in human, in contrast to mouse lungs where it is commonly used as an AT1 marker. Therefore, the images on figure 3F could be strong evidence that the AT1 differentiation is yet fully achieved in this manuscript. As a minor, the quality of western blotting image in figure 3 also needs to be improved as well.

5. Heterogeneity on 2D stretched condition: Nuclear YAP localization needs to be properly suggested, which is the direct evidence of active YAP signaling. In line with this, the nuclear YAP translocation shown in figure 5A is very promising to show the active Yap signalling upon the stretching event. However, the percentage of cells and expression of the AT1 and AT2 markers seems highly heterogenous although they are plated on the same membrane. This could be because the authors used BPCs than fetal AT2-like cells. It would be informative if the authors address this issue.

Minor points

1. Immunostaining for ABCA3 in the supplementary fig. 3b is not convincing – it seems that the signal came from a necrotic core.

2. In the supplementary fig. 6b, the DAPI/PDPN and HT2-280/AQP5/Merged images presented for the AVOD organoid group appear inconsistent and do not seem to depict the same fields.

References

1. Nikolić, M.Z., Caritg, O., Jeng, Q., Johnson, J.-A., Sun, D., Howell, K.J., Brady, J.L., Laresgoiti, U., Allen, G., Butler, R., et al. (2017). Human embryonic lung epithelial tips are multipotent progenitors that can be expanded in vitro as long-term self-renewing organoids. *Elife* 6. <https://doi.org/10.7554/eLife.26575>.
2. Lim, K., Donovan, A.P.A., Tang, W., Sun, D., He, P., Pett, J.P., Teichmann, S.A., Marioni, J.C., Meyer, K.B., Brand, A.H., et al. (2023). Organoid modeling of human fetal lung alveolar development reveals mechanisms of cell fate patterning and neonatal respiratory disease. *Cell Stem Cell* 30, 20-37.e9. <https://doi.org/10.1016/j.stem.2022.11.013>.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I thank the authors for a thorough and substantial revision. The manuscript is considerably improved. The developmental frame has been aligned with current human data. Several claims have been appropriately tempered to match the evidence. Image quality and quantification have been strengthened, and the corresponding transcriptomic data now support the imaging-based conclusions. The great majority of our previous comments have been addressed well. One central issue remains unresolved, and it concerns the principal novelty of the manuscript (the microfluidic cyclic-stretch platform, Figure 6). Because this point is directly relevant to whether the study's core claim is experimentally supported, I recommend a further round of revision for the comment below. Minimal additional experiments are warranted.

Major comments:

1. The principal concern remains the biological validation of the cyclic stretch condition. The chosen cyclic-stretch condition is not justified as optimal for promoting alveolar maturation, because the authors state that no parameter validation was performed and defer it to future work. The new Supplementary Figure 6 confirms that the membrane reaches -5% strain under the applied pressure, but this is only an engineering calibration. It validates that the setpoint is reached, not that -5% is the biologically optimal magnitude for maturation.

We therefore request a minimal experiment to provide direct evidence that the maturation response is parameter-dependent around the chosen setpoint. Rather than a comprehensive optimization, it would be sufficient to compare the current condition with one lower and one higher strain (for example approximately 3%, 5%, and 8–10%), while maintaining the same frequency. Quantification of % ABCA3⁺ AT2, % HT1-56⁺/AGER⁺ AT1, % double-positive, and % double-negative cells would be helpful for readers to understand whether the reported maturation phenotype is specifically associated with the selected mechanical input rather than representing a stretch versus no-stretch effect.

2. If the authors interpret YAP activation as a mechanistic driver of the observed phenotype rather than merely an associated event, this interpretation should either be experimentally supported (for example by pharmacological inhibition of YAP/TEAD signaling) or more carefully qualified throughout the manuscript.

Minor comment:

1. The strongest superiority data (the NKX2.1/SOX2/SOX9 comparison across the four protocols) now appears only in the rebuttal letter, not in the manuscript. Readers cannot evaluate the superiority claim from the paper itself. Please restore this comparison (at least to the Supplementary Material) so that the head-to-head advantage in transcription factor-defined FLP identity is part of the published record, alongside the retained CD47/PDX1 comparison.

Reviewer #2

(Remarks to the Author)

The authors have adequately addressed all of my concerns and provided satisfactory revisions to the manuscript. I think the paper has improved substantially, and I have no further comments.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

A majority of the concerns raised from my side have been adequately addressed in the revised manuscript. The authors have improved the presentation of their distal tip-like lung organoid model and provided clearer evidence of SOX9, SOX2, and NKX2.1 expression following modification of previously reported culture conditions.

Nevertheless, I remain unconvinced by the evidence supporting the presence of AT1-like cells in these organoids and under the stretched 2D ALI condition. For example, the stretched cell morphologies observed in the 2D cultures remain highly heterogeneous based on their quantification by HT1-56 and ABCA3 staining, suggesting that the induction of the AT1-like state is not yet well controlled in vitro.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

General comment:

Most of our previous concerns have now been adequately addressed, although the requested strain-response experiment was not performed. I do not consider it necessary to insist on additional experiments at this stage. Instead, the manuscript

should be carefully revised to reflect what is directly supported by the current data. In particular, whether mechanical stretch itself promotes alveolar maturation remains unresolved, while the microfluidic culture system and the associated phenotypic changes represent positive findings of the study.

Major comments:

1. The manuscript still contains several causal and maturation-related claims that are stronger than supported by the current data. Because only one strain magnitude was tested and the effects of stretch and ALI exposure are not fully separated, the experiments do not establish that mechanical stretch itself promotes alveolar maturation. I therefore suggest the following revisions:

(a) The Title should avoid implying that mechanical stimulation itself enhances the generation of alveolar cells. For example, "Directed Differentiation and Microfluidic Culture Enhance Alveolar-like Features in Human Pluripotent Stem Cell-Derived Lung Epithelium" would more accurately reflect the findings.

(b) Highlight 4, the Abstract, and the Editorial Summary should similarly describe the observed changes under the combined microfluidic culture condition rather than attribute alveolar maturation directly to mechanical stimulation. For the Editorial Summary, for example, I suggest: "The combined microfluidic culture condition incorporating air-liquid interface exposure and cyclic stretch is associated with epithelial flattening, increased nuclear YAP localization, and more restricted expression of AT1- and AT2-associated markers."

(c) Several statements and section headings in the Results also imply causal effects of cyclic stretch on alveolar differentiation or maturation that are not established by the experimental design. Examples include "Biophysical Stimulation Promotes Further Alveolar Epithelial Differentiation," "Cyclic Stretch Activates Multiple Mechanotransduction Pathways to Drive Cytoskeletal Reorganization..." and the concluding statement that air exposure combined with cyclic stretch is "essential for enhancing" AT1 and AT2 maturation. These statements should be revised to distinguish directly observed changes from conclusions regarding alveolar maturation or lineage specification.

(d) Similar qualification is needed throughout the Discussion. Statements that "cyclic stretch promotes the emergence" of specific AT1/AT2-associated populations or that mechanical cues "drive the formation of mature AT1 and AT2 cells" are not established by the current experiments. In addition, the statement that the platform generates "bona fide AT1 and AT2 cells" appears stronger than warranted given the acknowledged lack of functional and developmental benchmarking. These statements should be revised to distinguish directly observed morphological and marker-expression changes from conclusions regarding alveolar maturation or lineage specification.

2. Although the rebuttal states that the limitation of the strain analysis has been explicitly acknowledged, this is not sufficiently clear in the manuscript. Please state that the study does not establish a strain-response relationship and that the specific contribution of mechanical stretch to the observed phenotype remains unresolved.

3. Please reconcile the description of ~4–12% strain as physiological (Lines 800–801) with the statement that $\geq 10\%$ represents pathological overdilation (Lines 806–807), as these ranges overlap.

Minor comment:

1. For the analyses in Figs. 5B-D, data from the Submerged Stretch condition are not shown, whereas this intermediate condition is included in Figs. 5F and 5G. Because comparison with Submerged Stretch could help distinguish changes associated with cyclic stretch from those arising after introduction of ALI, please clarify why this condition was not included in these analyses. If these data are available, their inclusion would help clarify the respective contributions of stretch and ALI.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Dear Reviewers and Editors,

We are very grateful to the reviewers for their constructive feedback and suggestions which we have carefully addressed in the revised manuscript and in our point-by-point response. We feel these changes have greatly improved our paper and hope our revisions and responses are satisfactory to the reviewers.

Reviewer #1:

Brief summary of the manuscript: Song et al. report the differentiation of human pluripotent stem cells into fetal lung progenitor (FLP) and distal bipotent (DBT) organoid lineages that give rise to alveolar-type epithelial cells. By combining iPSC-derived FLPs with human fetal lung fibroblasts (HFLFs) and applying cyclic mechanical cues within a microfluidic air–liquid interface (ALI) system, the authors generate epithelial sheets containing ABCA3⁺ and HTI-56⁺ cells.

Overall impression of the work: The study is technically competent and addresses an important question in human lung differentiation. However, the principal novelty appears confined to the microfluidic co-culture experiments (Figure 6), and further data are required to substantiate reproducibility, mechanistic insight, and biological significance. Moreover, the manuscript does not clearly resolve the ongoing debate regarding whether bipotent progenitor cells (BPCs) are indispensable intermediates during human lung development (He et al., *Cell*, 2022). If the following comments are addressed, the work could make a meaningful contribution to the field.

We thank the reviewer for the thoughtful and constructive feedback and for recognizing the technical rigor and relevance of our study. Regarding the comment on the ongoing debate about whether bipotent progenitor cells represent indispensable intermediates during human lung development (e.g., PMID: 36493756), we agree that this is an important and actively evolving area in developmental biology. However, resolving this question falls beyond the primary scope and objectives of the present study.

Our work is focused on optimizing the efficient generation of fetal lung progenitors and their subsequent differentiation into alveolar epithelial lineages within a bioengineered microenvironment. While our system gives rise to distal bipotent tip (DBT)-like populations with the capacity to generate alveolar cell types, it was not designed to definitively address lineage hierarchy or to functionally test the necessity of BPC intermediates during human lung development *in vivo*. Given the complexity of this question and the need for dedicated lineage-tracing and *in vivo* validation approaches, we believe that a conclusive resolution of the BPC debate would require a distinct experimental framework using primary fetal explants/organoids as the ones developed by Dr. Rawlin's group (PMID: 36493780).

We hope the reviewer agrees that, while not directly resolving this question, our study provides a robust and reproducible platform for generating lung progenitor populations and may serve as a useful system for future investigations into human lung lineage relationships, including the role of BPCs.

Major Comments:

1. Evidence for the Existence and Necessity of BPCs in Human Lung Development. The authors emphasise the induction of AT1/AT2 bipotent progenitor cells (BPCs) from human iPSCs as a central rationale. Although the BPC concept was first proposed in mouse fetal lung development (Desai TJ et al., *Nature*, 2014; Frank DB et al., *PNAS*, 2019), recent single-cell multi-omics analyses of human fetal lungs (He et al., *Cell* 2022; Fig. 3) suggest a distinct trajectory in which early/late tip cells differentiate into fetal AT2 or AT1 cells via late-stalk intermediates, bypassing a discrete BPC stage. Given these findings, it remains uncertain whether BPCs are essential in humans. As direct access to fetal lung tissue is limited, the authors should reanalyze the dataset from He et al. dataset to verify the presence of SFTPC⁺ PDPN⁺ BPC-like populations during human lung development and provide clear evidence supporting their developmental necessity and relevance to the current human iPSCs-system.

We thank the reviewer for this insightful and important comment regarding the existence and necessity of AT1/AT2 bipotent progenitor cells (BPCs) in human lung development, and for highlighting the work by He et al. (2022) (PMID: 36493756). We agree that the classical concept of a discrete AT1/AT2 bipotent progenitor population was established in mouse fetal lung development (PMID: 24499815; PMID: 30782824), and that recent single-cell multi-omics analyses of human fetal lung tissue suggest a different developmental trajectory. In particular, He et al., (2022) (PMID: 36493756) propose that early and late tip progenitors give rise to AT1 and AT2 lineages via late stalk intermediates, without evidence for a stable, transcriptionally distinct SFTPC⁺PDPN⁺ BPC population.

In response to the reviewer's suggestion, we carefully re-examined the findings reported in PMID: 36493756, focusing on the presence of cells co-expressing canonical AT1 (*AGER*, *AQP4*, *CAV1*, *HOPX*) and AT2 (*SFTPC*, *SFTPB*, *LAMP3*, *NAPSA*) markers. Consistent with their conclusions, these analyses indicate that while transient co-expression of lineage-associated markers may occur, a discrete and transcriptionally stable BPC cluster is not clearly resolved in human fetal lung datasets. Rather, the data support a model of progressive lineage specification through **late tip** and **late stalk** intermediates. Importantly, our study does not rely on or require the existence of a discrete, obligate BPC state. To avoid overinterpretation, we have revised the manuscript to remove or replace the term "BPC" with "late tip" and "late stalk" progenitors, aligning our terminology with current human developmental models (Line 196-199). In this context, we define distal progenitors functionally, based on their capacity to generate both AT1- and AT2-like cells under appropriate conditions, rather than as a transcriptionally fixed intermediate.

Our bulk RNA-seq analysis of stage 4 distal bud tip (DBT) organoids supports their identity as late-stage distal progenitors, showing enrichment of late tip and late stalk gene programs along with fetal AT1 and AT2 transcriptional signatures. These features closely correspond to the developmental window described in PMID: 36493756, during which progenitors retain multilineage differentiation potential, even in the absence of a discrete BPC population.

We hope this clarification addresses the reviewer's concern and better aligns our interpretation with current understanding in the field.

2. Evaluation of AT1/AT2 Maturity in the iPSC Differentiation System. Functional maturation is critical in iPSC-derived alveolar differentiation. Although the manuscript claims the generation of “functionally mature alveolar cells,” the evidence—limited to ABCA3 and HTI-56 expression and electron microscopy images—is not sufficient.

Please (i) justify why these markers can reliably indicate functional maturity, and (ii) provide qualitative and quantitative analyses demonstrating that the observed lamellar body-like structures correspond to mature AT2 cells.

We thank the reviewer for emphasizing the importance of functional maturation, which is indeed a critical benchmark for iPSC-derived alveolar differentiation systems.

(i) Rationale for ABCA3 and HTI-56 as indicators of advanced alveolar epithelial maturation - We would like to clarify that we do not intend to claim the generation of fully mature adult AT1 or AT2 cells and have ensured our revised manuscript does not imply generation of mature cells equivalent to those found in the adult lungs. Rather, our conclusion is that biomechanical stimulation promotes a more developmentally advanced, alveolar-like epithelial state compared to existing in vitro systems where we can identify cells that express exclusive markers of AT1 (ABCA3+) and AT2 (HTI-56+) cells (New Figure 6D). ABCA3 and HTI-56 were selected as markers because they are tightly associated with late-stage alveolar epithelial differentiation and functional specialization, rather than early lineage commitment based on previous studies by PMID: 16982851 and PMID: 9889249. To clarify, ABCA3 plays a critical functional role in surfactant biogenesis by transporting phospholipids into lamellar bodies PMID: 16982851. Loss-of-function mutations in ABCA3 lead to severe neonatal respiratory failure (PMID: 32532878), underscoring its essential role in functional AT2 cell biology. Importantly, ABCA3 expression emerges only at later stages of human lung development (approximately GW20–23), coinciding with the onset of surfactant production capacity (PMID: 16982851). Meanwhile, HTI-56 marks differentiated AT1 cells and is associated with epithelial flattening and barrier formation, key structural features of the gas exchange surface that arise during late gestation.

We agree with the reviewer that marker expression alone does not establish full functional maturity. Accordingly, we have revised the manuscript to clarify that ABCA3, and HTI-56 expression should be interpreted as indicators of advanced differentiation toward functional AT2- and AT1-like states, rather than definitive evidence of fully mature alveolar epithelial function.

(ii) Evaluation of lamellar body–like structures - We appreciate the reviewer's request for more rigorous qualitative and quantitative characterization of lamellar bodies. In our current dataset, transmission electron microscopy (TEM) analysis of Stage 4 DBT organoids and Stage 5 organoids reveals membrane-bound, electron-dense, concentric lamellar inclusions (New Figure 2K), which are morphologically consistent with early lamellar body–like structures described in late fetal AT2 cells. These structures are absent in earlier stages, supporting their association with advanced differentiation.

However, we acknowledge that definitive identification of fully mature lamellar bodies would require either high-resolution TEM with systematic morphometric quantification or functional assays of surfactant synthesis and secretion. We have performed biochemical analysis to measure SFTPC protein levels by Western blot and show increased production of this surfactant in stage 4 and 5 organoids (New Supplementary Figure 4a). In alignment with other studies assessing differentiation efficiencies of hPSC, we feel this analysis is sufficient to suggest our protocol can generate AT2-like cells.

We have revised the manuscript to explicitly describe this technical limitation in the discussion (Line 528-531), clarified that the observed ultrastructural features provide supportive, but not conclusive, evidence of lamellar body formation, and avoid overinterpretation of these findings as proof of full AT2 functional maturity.

In summary, while we do not claim the generation of fully mature adult alveolar epithelial cells, the combination of 1) developmentally restricted marker expression (ABCA3+, HT1-56+); 2) emergence of lamellar body–like ultrastructures, and, 3) their specific induction under cyclic mechanical stimulation, supports the conclusion that our system achieves a level of alveolar epithelial maturation not previously reported in static organoid or Transwell-based cultures.

3. Robustness of Data Supporting the Novelty Claimed in Figure 6. The microfluidic co-culture of FLPs and HFLFs under cyclic mechanical strain represents the principal innovative aspect of this manuscript. However, as noted in the limitation section, no experimental data are presented to validate the chosen cyclic-stretch condition (5 % strain at 0.5 Hz). Since the originality of the manuscript relies largely on this result (Figure 6), please supply evidence that this condition is optimal for promoting alveolar epithelial maturation, ideally through a systematic parameter assessment and an appropriate functional endpoint assay.

We thank the reviewer for highlighting the importance of validating the cyclic mechanical stretch parameters, as this is indeed central to the novelty of our microfluidic co-culture platform.

First, we would like to clarify that experimental validation of the applied strain magnitude was performed and is presented in new Supplementary Figure 6. In this experiment, we systematically tuned the concentration of the BS3 crosslinker to modulate membrane stiffness and quantitatively calibrated membrane deformation under negative pressure. This enabled us to reproducibly achieve a 5% linear strain across the culture region, confirming that the applied mechanical input is well-defined and experimentally controlled. We have revised the main text to more clearly direct readers to this figure and explicitly state this validation (Line 368-370).

Second, the choice of 5% cyclic strain at 0.5 Hz was guided by well-established physiological and mechanobiological principles rather than being arbitrary. As noted in the PMID: 21873445 and PMID: 18805958, normal tidal breathing induces alveolar epithelial strain in the range of approximately 4–12% under non-pathological conditions. Strain below this range may be insufficient to activate mechanotransduction pathways, whereas excessive strain can induce stress or injury responses.

Consistent with this, prior studies have demonstrated that low-amplitude cyclic stretch (~5%) supports epithelial homeostasis, whereas higher levels ($\geq 10\%$) can impair alveolar epithelial function, including migration and tissue integrity (PMID: 18805958). These findings support the selection of 5% strain as a physiologically relevant and non-disruptive mechanical cue appropriate for promoting differentiation rather than stress responses.

Importantly, while we did not perform a full parameter sweep, our data demonstrate that this ALI Stretch condition is biologically effective from static culture to generate ABCA3⁺ and HT1-56⁺ cells (New Figure 6D). Notably, the observed phenotype is absent under identical biochemical conditions without cyclic stretch (Static condition), directly demonstrating that the applied mechanical input is a key driver of the reported maturation effects

With respect to the reviewer's request for systematic parameter optimization and functional endpoint assays, we fully agree that such analyses would be valuable. However, a comprehensive exploration of strain magnitude and frequency, in the context of long-term differentiation, would substantially expand the scope of the current study. Our primary goal here was to establish a proof-of-concept that physiologically relevant cyclic mechanical cues can promote advanced alveolar epithelial characteristics in a human iPSC-derived system. Future studies will build on this platform to systematically vary mechanical parameters and incorporate direct functional readouts, such as surfactant synthesis and secretion. We have revised the Discussion to more clearly position the current work as an initial mechanobiological validation and explicitly outline this as a future direction (Line 532-538).

4. Evidence for the Universality of the “No-Sorting” FLP Differentiation Approach. The authors report > 85 % efficiency in generating NKX2.1⁺ SOX2⁺ SOX9⁺ CD47⁺/CPM⁺ FLPs, enabling subsequent maturation without cell sorting. However, the protocol up to the FLP stage appears similar to prior reports, and this high efficiency may be clone-specific. It is essential to repeat the differentiation using more than three iPSC lines and confirm that the sorting-free approach reproducibly yields FLPs suitable for maturation.

We thank the reviewer for raising this important point regarding the robustness and reproducibility of the sorting-free FLP differentiation approach. We have performed this differentiation protocol in 3 independent hPSC lines (1 human embryonic stem cell line, CA1, and 1 human iPSC lines BU3 (courtesy of Dr. Darrell Kotton, Boston University) and 1 human cystic fibrosis gene edited iPSC line CFGE1, each with at least three independent differentiation replicates, we feel this is sufficient to address concerns of reproducibility. We agree that additional lines will strengthen robustness of differentiation, but addition of another few lines does not preclude the robustness of differentiation from the three independent lines we used in our study. Across all lines tested, we consistently observed a high efficiency (>85%) generation of NKX2.1⁺ SOX2⁺ SOX9⁺ CD47⁺/CPM⁺ fetal lung progenitors (New Figure 1B-

D, New Supplementary Figure 2a) without the need for cell sorting, demonstrating that this outcome is not clone-specific but reproducible across distinct genetic backgrounds.

Crucially, fetal lung progenitor cells were fully competent for downstream differentiation, reproducibly giving rise to distal bud tip (DBT) organoids (New Figure 1E-J) and alveolar epithelial lineages (New Figure 2E-H) under subsequent culture conditions.

We have revised the Methods and Results sections to explicitly list all hPSC lines used in our study and report the number of biological replicates per line in the figure legends. Due to the high efficiency of generating fetal lung progenitor cells across all three independent lines and every replicate differentiation we opted to not rely on cell sorting to enrich for these cells in downstream differentiation.

5. Clarification of Cell Lines Used for the Data Presented in Each Figure. In the Method and Materials section, the authors reported the use of the human ESC line CA1 and the iPSC lines BU3, CFGE1 and CFGE2. To demonstrate the robustness of the protocol, the data should be presented separately for each hPSC line. To establish generalizability, please clarify what "N=3" represent in each experiment. Independent biological replicates, rather than technical replicates alone, are required. Additionally, in comparison to the conditions for inducing AT1-like cells, did the authors observe consistent results across all hPSC lines?

We thank the reviewer for this important request for clarification regarding cell line usage, biological replication, and generalizability of the differentiation outcomes.

As mentioned in our response above, the differentiation and maturation protocol was performed using three independent hPSC lines. Each cell line was subjected to multiple independent differentiation experiments (≥ 3 differentiation sets per line), ensuring that the observed results reflect reproducibility both within and across genetic backgrounds.

We acknowledge that the original manuscript did not clearly distinguish between biological replicates and cell line usage. To clarify, "N" refers to the number of independent cell lines and have clarified this in the manuscript on all the figure legends.

In addition, we have clarified in the figures (where possible) the data points for all the lines to show differentiation efficiency and inter-line variability.

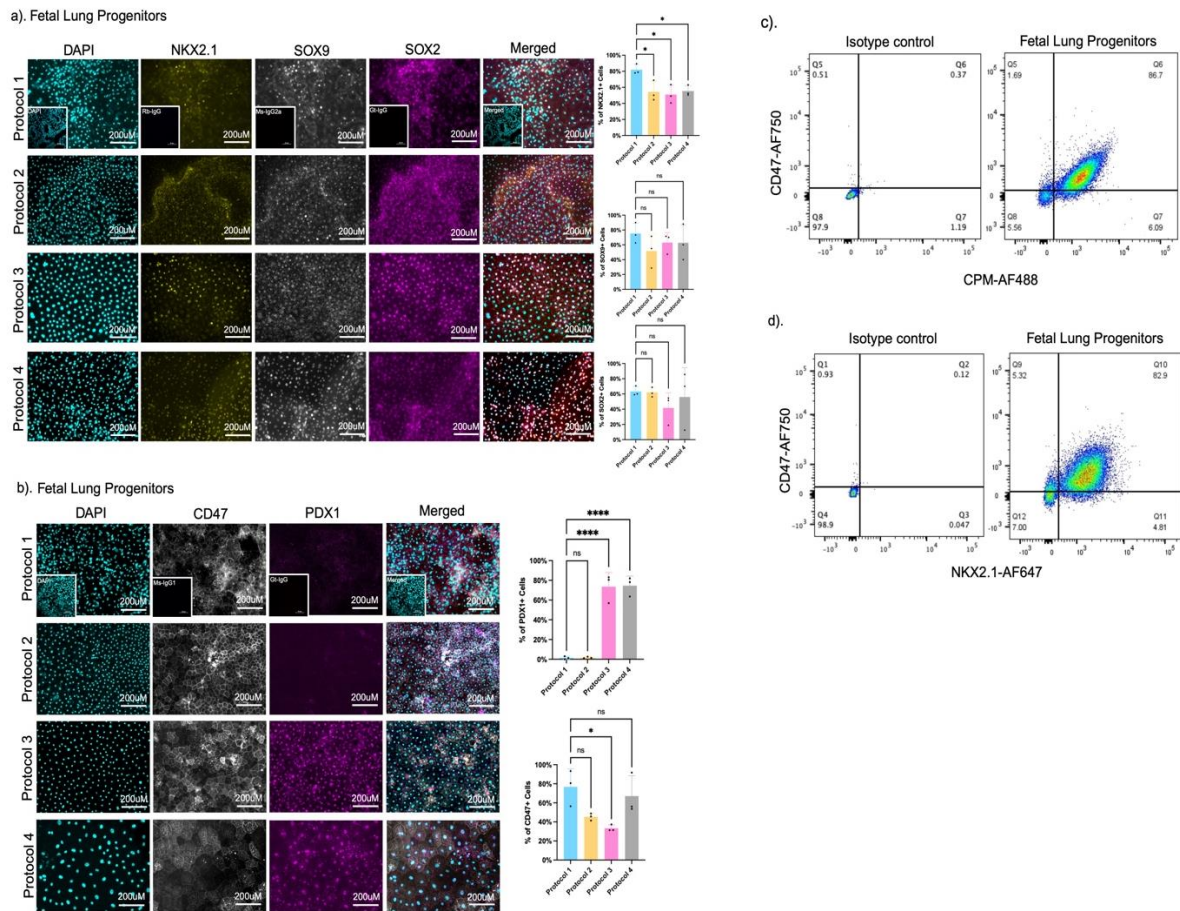
Regarding AT1-like cell induction, we confirm that consistent and reproducible results were observed across all hPSC lines tested. Specifically, HT1-56+ AT1-like cells were robustly induced under the same experimental conditions across all lines (New Figure 6D) and quantitative analysis (New Figure 6E) show differentiation outcomes of all the independent hPSC lines.

Minor Comments:

1. The points of "superiority" over existing methods should be clearly stated.

The points of superiority of our method relative to existing differentiation protocols is important and while our original version included direct comparison to 3 other protocols to generate fetal lung progenitors defined by percentage of NKX2.1, SOX2, and SOX9 positive cells (Panel a), we have opted to remove this data from the new version of this manuscript mainly to simplify the results. Instead, we are including the data here in the point-by-point response to show our method "Protocol 1" (blue bar) yielded the highest percentage of

NKX2.1+ (magenta), SOX2+(yellow), and SOX9+ (white) cells (See figure below) compared to published protocols (Protocol 2, yellow bar, Bluhmki et al., 2021; Protocol 3, pink bar, Jacob et al., 2017; Protocol 4, grey bar, Kanagaki et al., 2021).



Moreover, using our Protocol, we do not observe PDX1+ cells (indicative of pancreatic endoderm lineage) compared to Protocols 3 and 4 (Panel b). The percentage of NKX2.1+CD47+ or CPM+CD47+ cells is consistently above 85% (Panel c,d).

Collectively, this indicates more efficient specification of fetal lung progenitors at a critical developmental stage that we believe will enable more efficient differentiation towards distal lung phenotypes. The high purity of fetal lung progenitors enables seamless progression into distal organoid and alveolar differentiation systems without sorting, preserving cell yield and simplifying experimental workflows. This is particularly advantageous for applications requiring large-scale or reproducible tissue generation.

2. Incomplete citation of prior iPSC-lung differentiation studies - The manuscript should reference Gotoh et al., *Stem Cell Rep* 2014 and Yamamoto Y et al., *Nat Methods* 2017, particularly where it states (line 136) that “most iPSC-based systems neglect epithelial–mesenchymal crosstalk.”

We thank the reviewer for pointing out the omission of these important prior studies. We agree that the work by Gotoh et al. (2014) and Yamamoto et al. (2017) represents foundational

contributions to iPSC-based lung differentiation, including early demonstrations of epithelial specification and co-culture approaches. We have now revised the manuscript to include these citations in the relevant section on line 101-103.

3. Quantification of SOX2+, SOX9+, and double-positive cells (Figure 1C). Please quantify the proportions of SOX2+-only, SOX9+-only, and SOX2+SOX9+ cells within the induced FLP population.

This is a great suggestion, and we have now included these quantifications in the new Figure 1C.

4. Supplementary Fig 3b - NKX2.1 should be localised to the nucleus. Please confirm whether the images are correct.

We thank the reviewer for pointing out the concern regarding NKX2.1 staining and have performed new staining of the DBT organoids for NKX2.1 in new Supplementary Figure 3b which show clearer nuclear localization of this protein.

5. Improved analysis of AT2 differentiation conditions (Figure 2B). As ABCA3 alone is not sufficient to define AT2 identity (Sun YL et al., *Am J Respir Cell Mol Biol*, 2021), please quantify the relative frequencies of ABCA3+, SFTPC+, and double-positive cells under each condition.

We thank the reviewer for this important comment and agree that ABCA3 expression alone is not sufficient to define AT2 identity. First, to address this, we have expanded our analysis to directly quantify AT2-associated marker expression across conditions. Since it is very difficult to quantify the cells that express ABCA3 and SFTPC in the organoids cultured in CKD media based solely on immunostaining (New Figure 2H), we used flow cytometric analysis to quantify the percentage of ABCA3+ and AGER+/PDPN+ cells (New Supplementary Figure 4b, 4c) and quantified the level of SFTPC in these organoids by Western blot analysis (New Supplementary Figure 4a).

Second, we performed bulk RNA-sequencing on the organoids and using Bisque deconvolution (BisqueRNA), which is a computational method used to estimate cell type composition from bulk RNA-seq data using reference single-cell RNA-seq (scRNA-seq) datasets. We used the scRNA-seq dataset from Peng He et al., 2022. This analysis shows comparative increase in genes associated with fetal AT2 and AT1 cells in primary fetal lungs (New Figure 2F, 2G). This analysis independently supports the emergence of a fetal AT2-like transcriptional program under the optimized differentiation conditions.

Together, the combined quantification by flow cytometry, Western blot and transcriptomic profiling provide a more comprehensive assessment of AT1- and AT2-like differentiation.

6. Thickness of the “ultra-thin EC (UMM) membrane” - Please specify the thickness of the membrane.

We have clarified that the thickness of the UMM membrane used in this study is 5.5 um to 6.5um, consistent with previous characterization (PMID: 41348548). This information has been explicitly added to the Methods section on line 733-736, along with the appropriate citation to guide readers to the original detailed description.

7. Characterization of ABCA3- HT1-56- populations (Figure 6A) - A substantial subset of double-negative cells is present within the epithelial sheet. Please characterize these cells to clarify their identity and possible contribution.

We thank the reviewer for this important comment regarding the ABCA3- HT1-56- double-negative population observed in New Figure 6D. Indeed, there is a small population of ABCA3- HT1-56- double-negative epithelial cells after ALI Stretch. The identity of these cells remains unclear. We hypothesize these may be other epithelial cell types that are not alveolar (ie. distal epithelial progenitors) that have not yet fully differentiated into AT1- or AT2-like states), or are intermediate or transitional states during AT1/AT2 differentiation, in which canonical marker expression is dynamic and not yet stabilized. This interpretation is consistent with recent single-cell analyses of human fetal lung development by Peng He et al., 2022, which demonstrate that alveolar differentiation proceeds through transitional epithelial states that may lack strong expression of mature lineage markers. Importantly, the presence of this population does not detract from our central finding that cyclic stretch promotes the emergence of exclusive ABCA3+ and HT1-56+ cells but rather reflects the expected cellular heterogeneity of differentiation systems and ongoing lineage progression. Nonetheless, future studies will need to further characterize the entire population of cells after ALI Stretch to better understand the efficiency of generate alveolar-like cells using our platform, in addition to the impact of modifying stretch frequency and magnitude. We have included this in the Discussion of the manuscript on Line 550-563.

8. Stability of marker expression in the microfluidic device - How long are AT1 markers, SFTPC and other AT2 markers maintained in the microfluidics device? This is crucial information when using this method for assays in the future.

We thank the reviewer for raising this important point regarding the stability of alveolar epithelial marker expression within the microfluidic system. We show alveolar marker expression after 8 days under cyclic stretch conditions (New Figure 6). We have not tested beyond 8 days in our cyclic stretch conditions, and we agree that it is important to determine the extent to which these cells maintain their phenotype beyond 8 days. However, we believe this is beyond the current scope of the study and is a great follow-up study in the future where we can mass produce these devices to do larger scale time-defined experimental tests. To our knowledge, there are currently limited studies that evaluate sustained alveolar epithelial differentiation under continuous cyclic stretch beyond approximately one week, highlighting the need for platforms such as ours to systematically address long-term stability. Most lung-on-chip studies that apply stretch and assess changes in gene expression are limited to short 5 days or less (PMID: 41200831 and DOI: <https://doi.org/10.1183/13993003.congress-2020.556>).

9. Image resolution and subcellular localisation. Please include inset magnifications in all immunofluorescence panels to demonstrate intracellular localization patterns of marker

proteins.

We thank the reviewer for this suggestion. We have added inset magnifications to the immunofluorescence panels and the figure legend in the revised manuscript to better show subcellular localization.

Reviewer #2:

Summary

In this manuscript, the authors first presented a protocol for generating distal bud tip organoids containing immature alveolar type 1 (AT1) and AT2-like cells. They report that co-culture with fetal lung fibroblasts or exposure to fibroblast-conditioned media enhances differentiation efficiency toward fetal AT1 and AT2 cell fates. The study further demonstrates that alveolar maturation can be augmented by modulating Hippo-YAP1 signaling and by applying biophysical cues through cyclic stretch. Finally, the authors introduce a human iPSC-derived alveolar-on-chip system designed to mimic aspects of human fetal lung development. While the work is conceptually interesting, several critical issues must be addressed to strengthen the conclusions.

Major Points

1. A large portion of the conclusions rely on quantifying positive cells from microscopy images. However, most of the representative IF panels show nonspecific staining, very high background, overexposure, or inconsistent magnification between experimental conditions (e.g., Fig. 5A, 5D, 6A, in Supp Fig. 3b: Scale bars differ between channels for the same image). In addition, both organoid morphology and cell density vary considerably, which may compromise quantification accuracy. The authors should provide isotype controls, higher-quality images, and a clearer explanation of how quantifications were performed (e.g., whole organoid vs. single z-plane, number of organoids analyzed). Line 203: Authors report 50-60% AT1/AT2 bipotent cells, but Fig. 1E shows 34.8% BPCs. Clarify this discrepancy. - include isotope, and fix the contrast.

We thank the reviewer for this careful and important critique. We agree that image quality, appropriate controls, and transparent quantification methods are essential, particularly given the reliance on microscopy-based analyses. We have therefore implemented the following revisions to improve rigor, consistency, and clarity:

1) Image quality, background, and presentation consistency - We acknowledge that several immunofluorescence panels in the original submission exhibited high background, overexposure, and inconsistent presentation. To address this, we have:

- Reacquired and/or reprocessed representative images using matched acquisition settings (laser power, gain, and exposure) across conditions within each experiment for new Figures 1J, 2H, 4D.
- Applied uniform contrast adjustments to avoid signal saturation and reduce background artifacts for new Figure 1J, 2H, 4D.

- Included higher-magnification inset (zoom-in) panels to better resolve marker localization within the epithelium for new Figures 1J, 2H, 3G, 4D, 5E, 6D.
- Ensured consistent magnification and scale bars across all conditions within each figure for all image panels.
- Corrected the issue in new Supplementary Figure. 3b, where scale bars previously differed between channels; all channels for a given field now share identical scaling.

2) Inclusion of appropriate negative controls - To address concerns regarding nonspecific staining, we have now included matched isotype controls (same species, isotype, and concentration) processed in parallel under identical staining and imaging conditions. These controls are presented in the small inlet of each figure correspondingly and referenced in the respective figure legends. Except for the new Figure 5E and 6D have shared isotype control at new Supplementary Figure 9a.

3) Quantification methodology and robustness - We agree that variability in organoid morphology and cell density can affect quantification if not rigorously controlled. Therefore, to clarify quantification was performed using standardized image analysis pipelines applied consistently across all samples where multiple organoids ($n > 3$) per conditions were randomly imaged for quantification for three hPSC lines ($N = 3$). Marker positive cells were counted by a student who was blinded and did not know the sample groups to prevent bias in the counts. We now explicitly describe these procedures in the Methods section, including sampling strategy and normalization approach.

In this revised manuscript, the trends observed from imaging-based quantification are supported by orthogonal transcriptomic analyses (bulk RNA-seq), which independently confirm the same differentiation patterns (New Figure 1G, 2E, 3C, 6A).

4) Clarification of the reported AT1/AT2 progenitor percentage - We thank the reviewer for identifying this discrepancy. In the revised manuscript, we have addressed this issue in two ways. First, we have removed ambiguous use of the term “bipotent progenitor cell (BPC)”, as we feel this term needs to be functionally validated. Instead, we have referred to cells that co-express genes canonically associated with AT1 or AT2 cells as “late stalk and late tip” cells based on the shared differentially expressed genes identified in He et al 2022 dataset. Second, the previously stated 50–60% value has been corrected, and all values are now aligned with the quantitative data presented in new Figure 1E.

2. In Fig. 2C, the quantification of lamellar bodies or microvilli is unclear, were individual cells or whole organoids quantified? A more robust analysis, such as Western blotting for surfactant proteins, would be more convincing. In Fig. 2L, an AT1 cell is claimed to be present, but no morphological criteria support this. Gene expression analysis (e.g., RT-qPCR or RNA-seq for AT1 markers) should be included to validate AT1 cell identity. - include in supplementary western blots.

We have clarified that lamellar body-like structures were quantified at the single-cell level, rather than at the whole organoid level. Specifically, quantification was performed as the

number of lamellar bodies per individual AT2-like cell cross-section, based on TEM images acquired at consistent magnification. Due to the sectional nature of TEM imaging, whole-organoid quantification is not feasible. We now explicitly describe as the unit of analysis (individual cells), the sampling strategy (fields and organoids analyzed), and the total number of cells/fields quantified in the Methods Section on line 837-846 and Figure legend for new Figure 2L.

With respect to microvilli, we acknowledge that the original wording was ambiguous. We did not perform quantitative analysis of microvilli. Instead, microvilli were used as a qualitative ultrastructural feature to show that Stage 4 DBT organoids did not show microvilli but microvilli were present on apical surfaces of AT2-like cells in Stage 5 organoids grown in CKD media. We have revised the text to clearly describe this as a qualitative observation and removed any implication of quantitative analysis.

Finally, to further strengthen the interpretation of AT2-like identity beyond morphology, we have now included Western blot analysis for SFTPC comparing Stage 4 DBT organoids and Stage 5 organoids grown in CKD media (New Supplementary Figure 4a). This provides an independent protein-level validation supporting acquisition of an AT2-associated program.

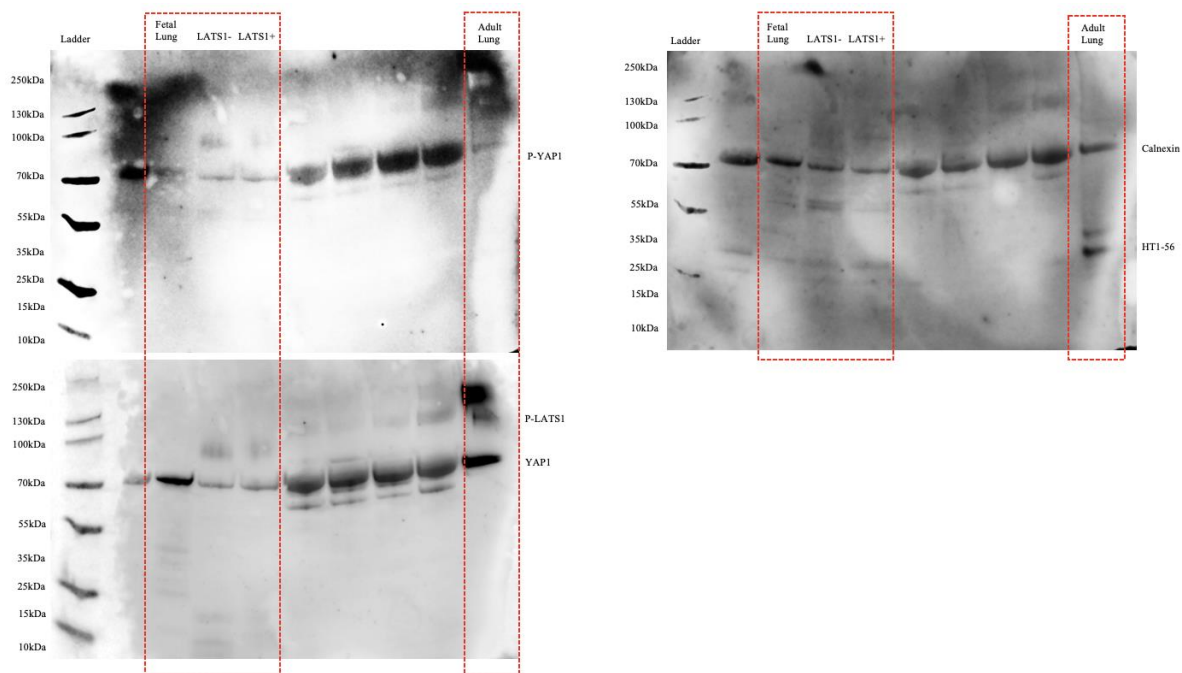
Validation of AT1-like cell identity - We acknowledge that AT1 cells under TEM imaging, show flattened, elongated cell morphology, thin cytoplasmic extensions, and spread cellular architecture, this alone is insufficient to imply these cells are indeed AT1. Accordingly, we have revised the manuscript to avoid overinterpretation and now refer to these cells as “AT1-like” and have included additional data such as bulk RNA sequencing which demonstrates upregulation of AT1-associated gene programs in the relevant differentiation conditions (New Figure 2F). This provides a more robust and orthogonal validation of AT1-like identity beyond morphology alone.

3. In Fig. 3A, the loading controls (Calnexin) are barely visible, and the final condition appears to be from a different gel. This experiment should be repeated for clarity and reliability. In Fig. 3D, the flow cytometry gating strategy needs adjustment and should include appropriate minus-fluorescence or isotype controls.

We thank the reviewer for this careful assessment and for highlighting issues related to data presentation and flow cytometry controls. We acknowledge that the Calnexin loading control was not clearly visible in the originally presented figure. In the revised manuscript, we have improved the presentation by including the calnexin loading control with optimized contrast/exposure to ensure clear visibility and confirm that all lanes shown in revised new Figure. 3E (old Figure. 3A) were run and transferred on the same gel/blot. Additionally, the samples were not loaded onto separate gels, instead the image was strictly cropped for figure assembly for presentation. To ensure full transparency, we have included the uncropped, original Western blot images (including Calnexin) below and will also include this as raw Source Data upon acceptance of our manuscript.

Note

Note, the bands not highlighted in the red box were for other samples for unrelated project.



We agree that rigorous gating for flow cytometry analysis requires appropriate controls. clarify, we now include full gating strategies and have added appropriate isotype controls for all the flow cytometry results, processed under identical conditions (New Supplementary figure 2b, 4b, 4c).

4. The Western blot in Fig. 4F shows a double band that is not addressed in the text. The authors should clarify whether this reflects isoforms, post-translational modifications, or a technical artifact.

We thank the reviewer for noting the presence of the double band in old Figure. 4F (New Figure 5B) and agree that this should be clarified. In the revised manuscript, we have addressed this directly. The observed double band is consistent with the expected migration pattern of phosphorylated YAP1 (Ser127) and likely reflects a combination of YAP1 isoforms and/or phosphorylation-dependent mobility shifts, which commonly produce closely spaced bands in the ~65–78 kDa range. This interpretation is supported by the manufacturer's validation data for the phospho-YAP1 (Ser127) antibody (Cell Signaling Technology, #4911), which indicate that multiple bands can be detected due to differences in isoform composition and post-translational modification status. Accordingly, we have revised both the main text (line 414-416) and the new Figure 5B legend to explicitly state that the double band likely represents biologically relevant YAP1 species, rather than a technical artifact. We have also included the antibody reference in the Methods section key resource table (P-YAP1, Cell Signaling Technology, #4911) to guide readers.

5. Because the authors state that fibroblasts influence epithelial development via growth factor secretion, they should distinguish direct cellular interaction from paracrine signaling. Including

direct co-cultures, conditioned media, and transwell cultures (preventing direct contact) would help dissect whether the observed effects arise from direct cell–cell contact or secreted factors. We thank the reviewer for this important suggestion and agree that distinguishing between direct cell–cell interactions and paracrine signaling is critical for interpreting the role of fibroblasts in epithelial maturation.

In our original experimental design, co-culture was performed under direct-contact conditions, permitting both physical interaction and exchange of soluble factors between human fetal lung fibroblasts and epithelial cells from DBT organoids cultured in CKD media. To directly address the reviewer’s concern, we have now performed complementary indirect co-culture experiments using a Transwell system, which prevents direct cell–cell contact while allowing diffusion of secreted factors (new Supplementary Figure. 5). Under these indirect conditions, we observed that paracrine signaling alone is insufficient to fully recapitulate the epithelial maturation phenotype observed in direct co-culture. Specifically, the robust induction of alveolar epithelial features seen in direct contact conditions was markedly reduced in the Transwell configuration. These results indicate that direct fibroblast–epithelial interactions are required for optimal maturation, and that the observed effects cannot be explained solely by soluble factors.

To further support this interpretation, we now include an analysis of predicted ligand–receptor interactions between stromal “sender” cells and epithelial “receiver” cells (new Supplementary Figure. 5c). This analysis identifies candidate paracrine signaling pathways consistent with known human fetal lung cell communication networks. However, when considered alongside the Transwell results, these data suggest that paracrine signaling likely contributes but is not sufficient on its own, implicating an additional role for contact-dependent mechanisms, such as extracellular matrix interactions or juxtacrine signaling. We have revised the Results section to explicitly state that fibroblast-mediated effects in our system reflect a combination of contact-dependent and paracrine cues, and we now outline future studies (e.g., conditioned media and ECM-based approaches) to further dissect these contributions (line 529-535 and line 561-567).

Minor Points

1. At the beginning of the Results, the authors state that both an embryonic stem cell line and an iPSC line were used; however, it is unclear which data correspond to which line. If both lines were used, data for both should be shown.

We thank the reviewer for requesting this clarification. We have clarified the specific hPSC line(s) used for each experiment in the figure legends (including whether data derive from CA1 (hESC), BU3 or CFGE1 (both hiPSC)). Where statistical analyses were involved, we have included datapoints for all three lines. It is important to note, that for all three lines, there was not large differences in the yield of cell types along the differentiation trajectory.

2. Lines 67–68: The sentence describing the co-culture setup is unclear and should be rephrased. We have now clarified the coculture setup on line 215-220. It is now read. “*To do this, DBT organoids were dissociated and co-cultured with MRC-5 in a semi-solid culture of Matrigel to*

Medium ratio of 75%:25%". In addition, we have modified the figure (New Figure 2A) demonstrating the coculture set-up for clarity.

3. Line 165: A comma is missing ("To do this, ...").

We fixed this typo.

4. Supplementary Fig. 1b: The y-axis label should specify which population the percentage refers to (e.g., all live cells or a specific gate).

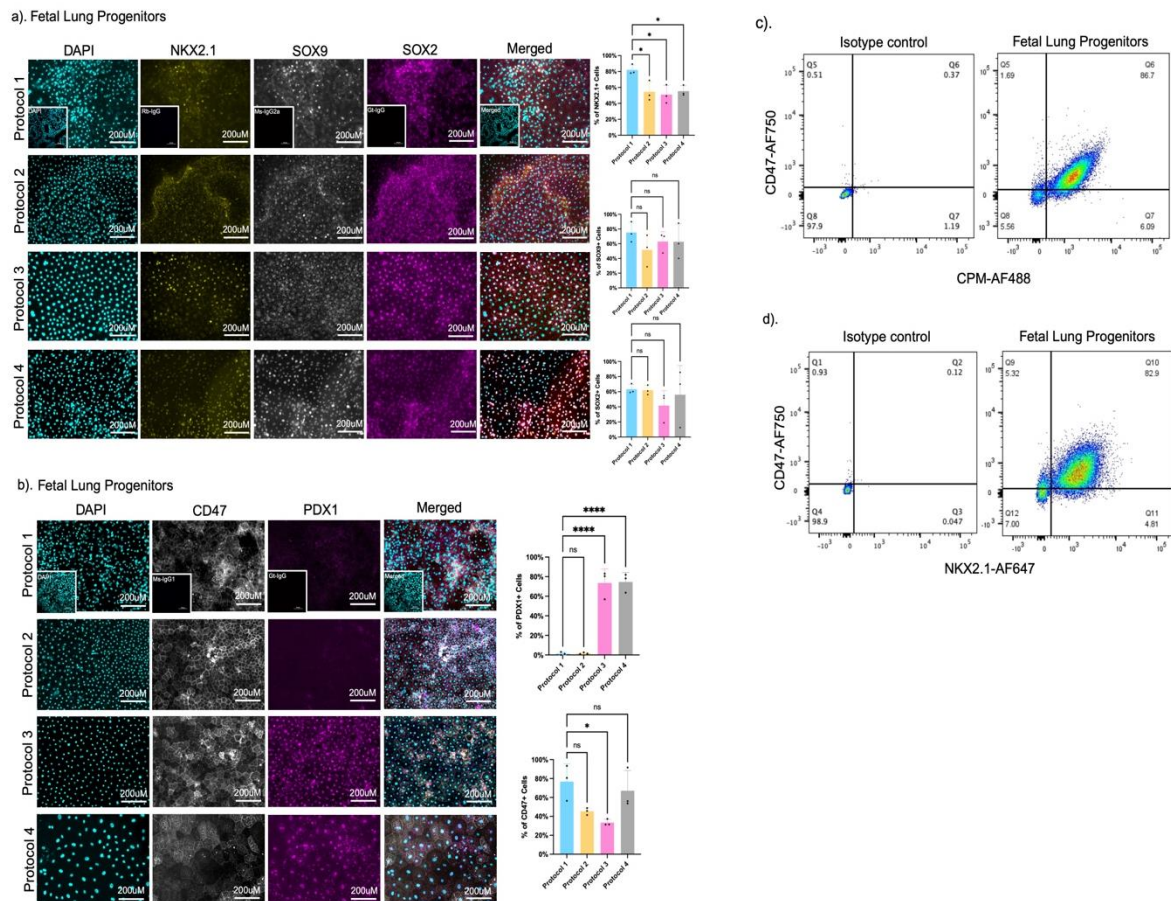
Supplementary Figure. 1b is a quantification of immunofluorescence staining from several fields of view. Therefore, this is the % positive cells per total DAPI (nuclei) staining. This is now clarified in the new Supplementary Figure 1b.

5. Line 170: The authors state that FOXA2/SOX17 co-expression was assessed by flow cytometry, but Methods (lines 468–469) and Supplementary Fig. 1a/b show IF staining. Please reconcile this.

We thank the reviewer for catching this error. We can confirm that FOXA2/SOX17 co-expression was assessed by immunofluorescence staining, not by flow cytometry. We have corrected this statement in the manuscript on line 150-152.

6. Supplementary Fig. 2: Rename P1–P4; this terminology is commonly used for cell passages and can be misleading.

We thank the reviewer for raising this concern and understand where these labels can be confusing. We have opted to remove this data from the new version of this manuscript mainly to simplify the results. Instead, we are including the data here in the point-by-point response to show our method "Protocol 1" (blue bar) yielded the highest percentage of NKX2.1+ (magenta), SOX2+(yellow), and SOX9+ (white) cells (See figure below) compared to published protocols (Protocol 2, yellow bar, Bluhmki et al., 2021; Protocol 3, pink bar, Jacob et al., 2017; Protocol 4, grey bar, Kanagaki et al., 2021).



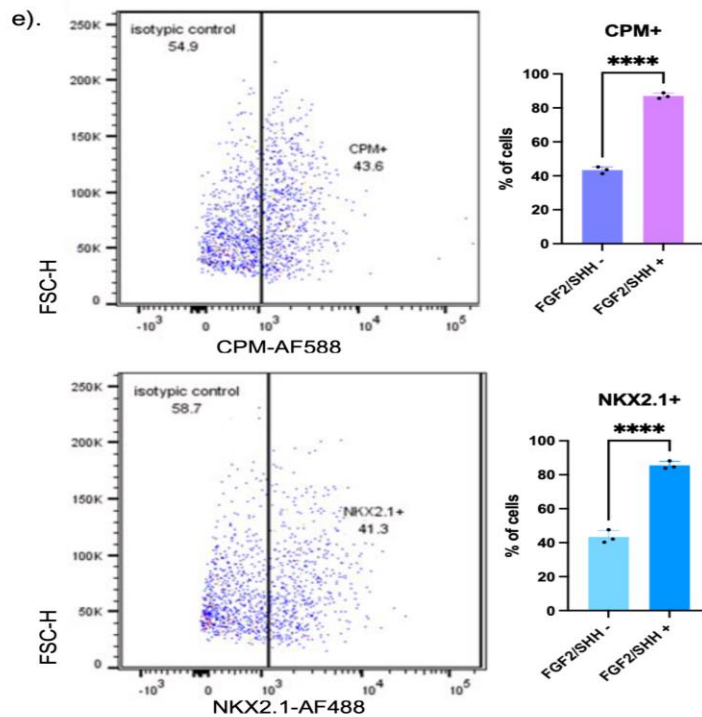
Moreover, using our Protocol, we do not observe PDX1+ cells (indicative of pancreatic endoderm lineage) compared to Protocols 3 and 4 (Panel b). The percentage of NKX2.1+CD47+ or CPM+CD47+ cells is consistently above 85% (Panel c,d).

Collectively, this indicates more efficient specification of fetal lung progenitors at a critical developmental stage that we believe will enable more efficient differentiation towards distal lung phenotypes. The high purity of fetal lung progenitors enables seamless progression into distal organoid and alveolar differentiation systems without sorting, preserving cell yield and simplifying experimental workflows. This is particularly advantageous for applications requiring large-scale or reproducible tissue generation.

7. Supplementary Fig. 2a–c: Indicate the developmental stage in each panel, as done for S2b. We have clarified the developmental stage “Fetal lung progenitor” in each panel in New Supplementary Figure 2.

8. Supplementary Fig. 1e: FACS plots for FGF2/SHH+ cells should also be included.

Here we show that without treatment of the DE cells with FGF2/SHH, the percentage of NKX2.1+ and CPM+ cells is significantly less than cells specified into AFVE with FGF2/SHH as expected. While FGF2/SHH-mediated AFVE specification enhances the efficiency of lung lineage induction in our system, it is important to note that several published differentiation protocols do not include this intermediate step and instead employ alternative patterning strategies PMID: 28965766, PMID: 33241896 and PMID: 34426605.



9. Flow cytometry percentage displays are inconsistent throughout the figures. The placement of legends (side vs. below vs. above) should be unified.

We thank the reviewer for noting the inconsistencies in flow cytometry annotations and legend placement. In the revised manuscript, we standardized the formatting across all flow cytometry panels: for single marker plots, the reported positive-cell percentage is now placed centrally within the gated region, while for dual marker plots, quadrant percentages are consistently placed at the four corners. In addition, we unified legend positioning so that all legends are placed consistently at the center of the x- and y-axis sides across figures. These changes improve readability and ensure consistent interpretation throughout the manuscript.

10. Supplementary Fig. 1d: AVFE is only defined in the figure legend; it should also be introduced in the main text.

We have defined AFVE in the main text on line 147.

11. Fig. 1c: The small inset images in the corner are unclear; label them in the text or annotate with arrows.

In the revised manuscript, Fig 1C is now New Fig 1J. All inset images are now labeled.

12. Supplementary Fig. 3a: Gating terminology differs between control and DBT; use consistent labels. Plots should be labeled with negative and DBT samples.

We have ensured the labels for each figure is labelled with negative (Isotype control) on the top panel and DBT Organoids on the bottom. The gating for the CD47+ and EpCAM+ was intentionally made more restricted in the DBT sample to ensure we are only capturing double positive cells.

13. Lines 177–178 and 200-202 refer to Supplementary Fig. 2c but assign it to different stages (Stage 2b vs. Stage 4). Clarify and label the figure accordingly.

We have carefully corrected all figure labels.

14. Fig. 1D: This is not a TEM image.

We have corrected all image labels in the revised manuscript to ensure brightfield, fluorescence, SEM, and TEM images are correctly ordered or described.

15. Lines 214–215: Authors state that only some cells are ECAD⁺ and AQP5⁺, but the ECAD staining in Fig. 1F appears widespread. Clarify.

We thank the reviewer for this mistake. We have elected to restrain new organoids with ECAD (E-cadherin) which is broadly expressed across epithelial cells, as shown in new Fig. 1J. We co-stained with AGER, a canonical AT1 cell marker, in the DBT organoids as shown in the new Figure. 1J, 1K. We observed a small number of ECAD⁺AGER⁺ cells within the DBT organoids.

16. Line 221: Replace “mediums” with media.

We have made this change throughout the manuscript.

17. Fig. 2B: “FC quantification” may be mistaken for “fold change.” Rephrase and ensure consistent labeling across flow cytometry figures.

We thank the reviewer for noting this potential ambiguity. In the revised manuscript, we have replaced “FC” with the full term “flow cytometry” where flow cytometry-related figures/legends are reported for clarity.

18. Lines 236–237 refer to Fig. 2D for microvilli, but Fig. 2D shows lamellar body quantification; correct the reference.

We thank the reviewer for catching this error. We have corrected the figure which is now new Figure 2M.

19. Supplementary Fig. 6c is missing or unlabeled.

We thank the reviewer for noticing this labeling issue. In this revised submission, we have carefully ensured all figures including in the supplementary figures are labelled.

20. Line 257: Grammar correction needed (“The bipotent DBT marker ... was significantly decreased ...”).

We corrected this grammar accordingly on line 315-317 which now states, “Immunostaining showed the presence of exclusive cell clusters of HOPX (AT1 marker, 39%, $p < 0.001$) and LAMP3 (AT2 marker, 17%) cells in LATS1i⁺ organoids, whereas there were significantly less like late tip and late stalk cells (based on co-expression of HOPX and LAMP3) ($p < 0.0001$).”

21. Line 279: Introduce the abbreviation AL (adult lung) before use.

We removed limited abbreviations in our revised manuscript for clarity and adult lung is no longer abbreviated.

22. Line 413: *in vitro* should be italicized.

We have made this correction in the revised manuscript.

23. Line 609: Missing words, should read: “adherent differentiated cells were washed with PBS three times...”.

We thank the reviewer for catching this omission. We have corrected the sentence on line 757-758.

Reviewers #3&4:

Song et al. introduce an improved differentiation strategy that more efficiently generates distal lung epithelial cells and establishes scalable distal bud tip organoids capable of producing bipotent alveolar progenitors. Although pharmacologic maturation effects are limited, microenvironmental cues—including fibroblast co-culture, mechanical stretch, and air exposure—enhance alveolar type 1 cell fate maturation. Addressing the following concerns below will be required for consideration in Communications Biology.

Major points

1. Fetal lung development: Direct differentiation of PSCs into lung epithelial cells recapitulates aspects of fetal lung development; therefore, PSC-derived cells generally resemble fetal-stage cell types. The authors quantified AT1/AT2 bipotent cells and fetal AT1 and AT2 cells using HT2-280, PDPN, and AQP5. However, these markers are not characteristic indicators of AT1 or AT2 cell fate during the fetal stage. PDPN and AQP5 are expressed in developing airway epithelium, not in the bud tip epithelium, and HTII-280 is also not a valid marker for fetal AT2 in human fetal lungs 1,2. For example, HTII-280+ cells within an organoid are not matched with ABCA3+ cells (Figure 2E). Furthermore, the model, a bipotent lung progenitor capable of differentiating to both alveolar type 1 and 2 cells still remain controversial in human lung development². Thus, the validity of using PDPN, AQP5, HT2-280, and SOX9 to define fetal biopotential lung progenitor or fetal AT1/AT2 lineages in this manuscript is not well supported and raises concerns. It is highly recommended to consider human fetal lung development and additionally reliable cell type markers, such as SFTPC, CAV1, and TP63, when authors revisit, re-evaluate, and clarify the dataset presented in Figure 1. A combined gene expression analysis using qRT-PCR and immunostaining will be useful.

- Bud tip progenitor: SOX9 and SOX2 dual positive
- A typical AT2 marker: SFTPC, the gold standard marker (proSPC, immature and mature AT2; mature SPC, mature AT2)
- Other AT2 markers: LAMP3, ABCA3, HOPX
- AT1 markers: AQP5, CAV1, PDPN (if the organoid does not contain basal cell-like cells)
- HTII-280: Adult AT2 cell marker; useful but not always clearly indicating AT2 cells in fetal lung

We thank the reviewers for this thoughtful and important critique. We agree that some PSC-derived lung epithelial differentiation systems predominantly reflect fetal-stage developmental programs, and that appropriate marker selection must be grounded in human fetal lung biology. We also agree that HTII-280, PDPN, and AQP5 alone are not sufficient to define fetal AT1 or AT2 identity, and that the concept of a discrete AT1/AT2 bipotent progenitor population remains debated in humans. In response, we have substantially revised both our interpretation and supporting data as follows:

1) Revision of developmental framework and removal of BPC overinterpretation - We have removed the implication of a discrete, obligatory AT1/AT2 “bipotent progenitor cell (BPC)” population. Instead, we now describe the relevant population within distal bud tip (DBT) organoids as late distal tip/stalk-like progenitors, consistent with human fetal lung developmental trajectories described by He et al. (2022). This aligns our interpretation with current human single-cell data.

2) Refinement of AT2 marker usage - We acknowledge the reviewer’s concern regarding HTII-280. While HTII-280 is first emerged at gestational week 11 and more widely spread at gestational week 17 or more, it has been used as a more mature AT2 marker (PMID: 20566753), it is not sufficient to define fetal AT2 identity and may not fully overlap with canonical fetal AT2 markers. To address this, we have removed reliance on HTII-280 as a stand-alone marker, now emphasize SFTPC as the primary (gold-standard) AT2 marker, supported by additional canonical markers such as ABCA3 and LAMP3 and have added SFTPC and ABCA3 immunostaining and strengthened these findings with bulk RNA-seq and deconvolution analysis of Stage 5 organoids. This provides a multi-modal validation (protein + transcript) of AT2-like differentiation that is better aligned with fetal lung biology.

3) Refinement of AT1 marker interpretation - We agree that PDPN and AQP5 are not specific indicators of fetal AT1 identity, as they can also be expressed in non-alveolar epithelial populations. Accordingly, we have incorporated additional AT1-associated markers (e.g., AGER, CAV1) at the transcript level and interpret AT1-like identity based on a combination of gene expression, morphology (flattened epithelial cells), and global transcriptional signatures derived from bulk RNA-seq

4) Clarification of bud tip progenitor identity - Consistent with the reviewer’s recommendation, we now explicitly define bud tip-like progenitors using SOX9/SOX2 co-expression, which is the established criterion for human distal epithelial progenitor identity (PMID:36493780). This has been clarified in the Results on line155-157, and corresponding figure legends for new Figure 1B.

5) Strengthening of multi-modal validation - To address the reviewer’s recommendation for more rigorous validation, we have strengthened the dataset by integrating i) expanded immunostaining for additional markers (SFTPC, ABCA3 for AT2; additional AT1-associated markers where applicable); and ii) performed and included bulk RNA-seq and deconvolution

analyses to support fetal-like distal epithelial trajectories across stages. In the description, we have improved the quantification clarity and marker interpretation. Together, these changes ensure that conclusions are not based on single-marker interpretation, but instead supported by convergent molecular and morphological evidence.

2. Scalable culture of differentiating lung epithelium including BPCs and fetal AT2: How much are the PSC-derived BPCs scalable in 3D? How many passages it can be propagated for scalable production of alveolar cells? The lung BPCs can be considered ‘proliferative’ but the authors need to provide the relevant data. Furthermore, this is directly linked to the stability of BPC state under in vitro condition.

We thank the reviewer for raising this important point regarding the scalability and stability of PSC-derived distal lung progenitors. We agree that these parameters are critical for enabling reproducible and scalable production of alveolar epithelial cells.

First, as revised throughout the manuscript, we no longer describe a discrete “bipotent progenitor cell (BPC)” state as an obligate intermediate in human lung development. Instead, we refer to these cells as distal bud tip (DBT) organoids enriched for late distal tip and late stalk-like epithelial progenitors, consistent with current human fetal lung models.

Second, in our system, PSC-derived DBT organoids are readily expandable in 3D organoids and support scalable production meaning: i) Organoids can be serially passaged for approximately 5–8 passages, ii) passaging is performed every 5–7 days, following dissociation and reseeded (~400 cells/μL in Matrigel) and, iii) across passages, cultures show robust growth and reproducible expansion, enabling scale-up for downstream applications. Current work and beyond the scope of this study, is trying to establish bioreactor systems to produce these organoids are much larger scale.

Third, across passages we observe that DBT organoids retain expression of distal progenitor markers (e.g., SOX2/SOX9 co-expression, NKX2.1), maintain morphological characteristics of bud tip-like epithelium and preserve the capacity to differentiate into AT1-like and AT2-like epithelial lineages under appropriate conditions. These observations indicate that the progenitor state is stable over multiple passages, rather than rapidly drifting or losing lineage identity.

Finally, while we did not explicitly quantify proliferation kinetics (e.g., EdU/Ki67 analysis) in this study, the ability to sustain serial passaging with consistent expansion demonstrates that this population is functionally proliferative *in vitro*. We have clarified this interpretation in the revised manuscript on lines 202-208. Overall, we agree with the reviewer that further detailed characterization of proliferation dynamics and long-term stability (e.g., extended passaging, clonal analysis) would be valuable, and this as an important direction for future work.

3. Surfactant protein expression: The authors compared several alveolar differentiation conditions, CK+DCI, CKD, CK, and CSBK to investigate the ideal alveolar induction in this context. However, they are missing surfactant genes when judging the differentiation efficiency – for example, lack of SFTPC, SFTPB, and LAMP3 expression was suggested. ABCA3 is also an important gene associated with alveolar differentiation, but the gold standard gene, SFTPC, at least must be included. Although detection of lamellar bodies by TEM analysis (Figure 2)

and one SFTPC staining image (in supplementary fig 5b) were provided, but a higher resolution of surfactant protein localization pattern will have to be evident.

We thank the reviewer for emphasizing the importance of incorporating surfactant-associated genes and proteins when evaluating alveolar differentiation efficiency. We fully agree that ABCA3 alone is insufficient, and that SFTPC, as a gold-standard AT2 marker, must be included alongside additional markers associated with lamellar body function. To address this, we have substantially strengthened both the molecular and imaging-based characterization of AT2-like differentiation across different media conditions tested (CK, CKD, CSBK) in the revised manuscript. First, we have now added Western blot analysis for SFTPC across key differentiation conditions (CK, CKD, CSBK), presented in new Supplementary Figure 4a, providing a direct and quantitative surfactant protein readout for comparison of differentiation efficiency. This complements existing analyses and ensures that surfactant production is explicitly incorporated into condition ranking. In addition, we include ABCA3 flow cytometry quantification (new Supplementary Figure 4b) as a complementary readout of lamellar body-associated maturation, interpreted alongside SFTPC rather than as a stand-alone marker

Second, we have reprocessed and/or reacquired immunofluorescence images with improved resolution and optimized contrast for surfactant protein localization. We have added high-magnification inset panels to better resolve intracellular localization patterns of SFTPC and ABCA3 (New Figure. 2H).

Third, we now present Stage 4 DBT and Stage 5 organoid TEM images side-by-side in new Figure 2K, acquired under matched imaging conditions for direct comparison. Lamellar body-like structures are shown more clearly and are now quantified using a defined, cell-based analysis approach, which is explicitly described in the Methods lines 845-847. These ultrastructural observations are interpreted in conjunction with SFTPC protein expression, providing complementary morphological and molecular evidence of AT2-like maturation

4. Positive control (adult human lung) for evaluating AT1 fate differentiation: The authors used adult human lung (AL) as a control to compare AT1 marker expression levels in the differentiated organoids. However, the gene expression levels of AT1 markers upon AT1 differentiation seem not to be corresponded to those of whole adult lung. Did the authors use the purified cells when comparing the levels? The whole tissue lysates from adult and fetal lungs could not be optimal as a control. Therefore, it would be difficult to conclude that the upregulation of AT1 markers such as CAV1 and SPOCK2, as well as YAP1, TEAD1, and LATS1, is physiologically relevant to those in adult AT1 cells. - used whole adult lung RNA tissue (not purified), same for protein.

We agree that whole adult lung tissue is not an optimal control for quantitative comparison to purified AT1 cells, as bulk RNA and protein lysates reflect a heterogeneous mixture of epithelial, stromal, endothelial, and immune cell populations. This composition can dilute AT1-enriched signals and complicate direct comparisons. Specifically, we can confirm that in our study, the adult lung control samples were derived from whole tissue lysates (RNA and protein), not purified AT1 cells, and we agree that this limits their use as a strict quantitative benchmark for mature AT1 identity. To address this limitation, we have revised both our analysis and interpretation as follows: i) We no longer rely on whole lung comparisons as a definitive benchmark for adult AT1 equivalence. Instead, we assess AT1-like differentiation

using relative changes within our system, supported by upregulation of AT1-associated genes (e.g., *CAV1*, *SPOCK2*, *AGER*) and activation of Hippo/YAP pathway components (*YAP1*, *TEAD1*, *LATS1*); ii) we now benchmark these transcriptional changes against cell type-resolved human fetal and adult lung reference datasets, including published single-cell atlases (PMID: 36493756, PMID: 37291214), which provide a more appropriate and biologically relevant framework than whole-tissue comparisons. Importantly, we have revised the manuscript to clarify that we do not interpret these cells as equivalent to mature adult AT1 cells. Rather, we conclude that the observed gene expression changes reflect progression toward an AT1-like transcriptional program within a developmental (fetal-like) context on lines 239-255.

Additionally, *HOPX* is considered as an AT2 marker in human, in contrast to mouse lungs where it is commonly used as an AT1 marker. Therefore, the images on Figure 3F could be strong evidence that the AT1 differentiation is yet fully achieved in this manuscript. As a minor, the quality of western blotting image in Figure 3 also needs to be improved as well. - add reference of *HOPX* in AT1 in humans.

We thank the reviewer for raising this important point regarding species-specific differences in marker interpretation, particularly for *HOPX*, and we agree that careful contextualization within human lung biology is necessary. We acknowledge that *HOPX* has been reported with differing associations across species and developmental stages. However, analysis of human fetal lung single-cell datasets indicates that *HOPX* is enriched in AT1-associated populations during human lung development, rather than being restricted to AT2 identity. To address this directly, we have incorporated a new analysis in New Supplementary Figure 3e, where we query the human fetal lung atlas (He et al., 2022). This analysis shows that *HOPX* expression aligns more strongly with fetal AT1-like epithelial populations, supporting its interpretation as an AT1-associated marker in this human developmental context. Based on this, we interpret the *HOPX* signal observed in New Figure. 3G as consistent with AT1-leaning (AT1-like) differentiation, rather than evidence that AT1 differentiation has not been achieved.

We also agree that reliance on a single marker can be misleading, particularly for developmentally regulated genes. Therefore, in the revised manuscript, we avoid overinterpreting any single marker (including *HOPX*) and emphasize a multi-marker framework for AT1-like identity; such as bisque bulk-RNA-seq deconvolution identification of AT1 population (New Figure 3C), HT1-56, a marker associated with more advanced (late fetal) AT1 differentiation (New Figure 3E, 3F) and canonical AT1-associated transcriptional signatures (e.g., *AGER*, *CAV1*). We have also included morphological features (flattened epithelial morphology from TEM images, New Figure 2M) and bulk RNA-seq signatures (New Figure 2I) to support AT1-like differentiation. Accordingly, we now define our cells as progressing toward a late fetal/advanced AT1-like state, rather than claiming fully mature adult AT1 identity.

Finally, concerning the quality of the Western blot images in the original Figure 3 (now New Figure 3E), we have optimized the exposure settings to improve visibility of bands, ensured consistent lane alignment and labeling, and provided uncropped original blot images

(See Response to Reviewer #2 Question 3) as Source Data to enhance transparency and reproducibility.

5. Heterogeneity on 2D stretched condition: Nuclear YAP localization needs to be properly suggested, which is the direct evidence of active YAP signaling. In line with this, the nuclear YAP translocation shown in Figure 5A is very promising to show the active Yap signalling upon the stretching event. However, the percentage of cells and expression of the AT1 and AT2 markers seems highly heterogenous although they are plated on the same membrane. This could be because the authors used BPCs than fetal AT2-like cells. It would be informative if the authors addressed this issue.

We agree that nuclear localization of YAP is a key indicator of active YAP/TEAD signaling, and we interpret the nuclear YAP translocation observed in new Figure 5B as direct evidence that cyclic mechanical stretch activates Hippo-YAP mechanotransduction in our system. To strengthen this point, we have expanded the analysis in the revised manuscript to include quantification of the fraction of cells exhibiting nuclear YAP localization under cyclic stretch conditions (new Figure 5C), and provided high magnification images to clearly show nuclear vs cytoplasmic YAP distribution, enabling a more rigorous assessment of pathway activation (new Figure 5E-G)

We also acknowledge the reviewers' observation that AT1- and AT2-associated marker expression appears heterogeneous across the stretched epithelial sheet. Importantly, we interpret this heterogeneity as biologically meaningful rather than a limitation of the system. Specifically, the input population consists of distal bud tip (DBT)-derived epithelial cells enriched for late tip/late stalk-like progenitors, which inherently retain multilineage differentiation potential. Notably, in human fetal lung development, distal epithelial maturation proceeds through transitional and spatially patterned differentiation states (PMID: 29408236), rather than forming a fully homogeneous epithelium. Therefore, the observed mosaic pattern of AT1- and AT2-like marker expression likely reflects ongoing differentiation and self-organization under combined mechanical and stromal cues.

We also agree with the reviewers that the use of progenitor-derived populations contributes to this heterogeneity. This choice was intentional, as DBT-derived cells provide a developmentally appropriate starting population for modeling distal lung differentiation, and enable the emergence of both AT1- and AT2-like lineages under ALI Stretch conditions

We have revised the manuscript to clarify this rationale and to explicitly distinguish our system on lines 347-349.

Minor points

1. Immunostaining for ABCA3 in the supplementary fig. 3b is not convincing – it seems that the signal came from a necrotic core.

We thank the reviewer for this careful observation and agree that the ABCA3 signal shown in the old Supplementary Fig. 3b could be misinterpreted as arising from a necrotic core rather than representing specific intracellular staining. To address this concern, we have replaced new Figure 3H with newly acquired ABCA3 immunofluorescence images. In the revised panel, ABCA3 signal is clearly localized to intact cytoplasmic regions, and included higher-magnification inset images to better demonstrate subcellular localization.

2. In the supplementary fig. 6b, the DAPI/PDPN and HT2-280/AQP5/Merged images presented for the AVOD organoid group appear inconsistent and do not seem to depict the same fields.

We thank the reviewer for this careful observation regarding the apparent inconsistency in the DAPI/PDPN and HTII-280/AQP5 panels for the AVOD condition in the old Supplementary Fig. 6b. In response to this comment, we carefully re-evaluated both the figure presentation and the biological relevance of the AVOD condition within the overall study. Upon further analysis, including integration of bulk RNA-seq data, we found that the AVOD condition does not represent a physiologically relevant AT1 differentiation state in our system. Specifically, while AVOD promoted features resembling AT1-like cells at the marker level, transcriptomic profiling through bulk RNA-sequencing indicated upregulation of stress- and fibrosis-associated gene programs. Given these findings, we concluded that inclusion of the AVOD condition could lead to misinterpretation of AT1 differentiation as bona fide maturation, which is not aligned with the biological focus of this study. As our revised manuscript aims to define developmentally relevant and physiologically grounded alveolar differentiation pathways, we have therefore chosen to remove this condition from the analysis. Accordingly, the affected panel has been removed as part of a broader revision to streamline the manuscript and focus on conditions that more faithfully recapitulate fetal-to-mature alveolar transitions.

Reviewers' comments:

Reviewer #1:

We thank the authors for a thorough and substantial revision. The manuscript is considerably improved. The developmental frame has been aligned with current human data. Several claims have been appropriately tempered to match the evidence. Image quality and quantification have been strengthened, and the corresponding transcriptomic data now support imaging-based conclusions. The great majority of our previous comments have been addressed well.

One central issue remains unresolved, and it concerns the principal novelty of the manuscript (the microfluidic cyclic-stretch platform, Figure 6). Because this point is directly relevant to whether the study's core claim is experimentally supported, I recommend a further round of revision for the comment below. Minimal additional experiments are warranted.

Major comments:

1. The principal concern remains the biological validation of the cyclic stretch condition. The chosen cyclic-stretch condition is not justified as optimal for promoting alveolar maturation, because the authors state that no parameter validation was performed and defer it to future work. The new Supplementary Figure 6 confirms that the membrane reaches -5% strain under the applied pressure, but this is only an engineering calibration. It validates that the setpoint is reached, not that -5% is the biologically optimal magnitude for maturation.

We therefore request a minimal experiment to provide direct evidence that the maturation response is parameter-dependent around the chosen setpoint. Rather than a comprehensive optimization, it would be sufficient to compare the current condition with one lower and one higher strain (for example approximately 3%, 5%, and 8–10%), while maintaining the same frequency. Quantification of % ABCA3⁺ AT2, % HT1-56⁺/AGER⁺ AT1, % double-positive, and % double-negative cells would be helpful for readers to understand whether the reported maturation phenotype is specifically associated with the selected mechanical input rather than representing a stretch versus no-stretch effect.

We thank the reviewer for this thoughtful and important comment. We agree that Supplementary Figure 6 provides engineering validation that the applied pressure reproducibly generates the target membrane deformation but does not by itself demonstrate that 5% cyclic strain is the biologically optimal magnitude for alveolar maturation. In response, we have revised the manuscript to avoid describing the selected cyclic-stretch condition as "optimal" and instead characterize it as a physiologically motivated and technically validated mechanical stimulus. Specifically, the 5% strain magnitude was selected based on both physiological relevance and practical considerations of the ECM-based microfluidic platform. Previous studies of alveolar micromechanics have estimated that normal tidal breathing results in approximately 4% linear distension of the alveolar basement membrane (PMID: 21873445). Consistent with this physiological range, prior *in vitro* alveolar epithelial studies have used 5% cyclic strain to model normal breathing-associated mechanical cues, whereas higher strain magnitudes (e.g., 10%) are more commonly associated with pathological overdistension (PMID: 29434821). We have now added this rationale to the section describing the microfluidic ALI stretch system (**Line 798-816**) and further acknowledged the need for future parameter optimization in the Discussion (**Line 595-605**).

In addition, the achievable strain range in our system was constrained by the material properties and long-term biological stability of the ECM membrane. The membrane consisted of a Matrigel:collagen:elastin (1:2:0.5) formulation crosslinked with BS3, which was selected after evaluating multiple ECM compositions for cell attachment, epithelial expansion, and membrane integrity. Under the culture conditions used in this study, 5% strain represented the most reproducible and stable deformation that could be maintained throughout the full 8-day

culture period, including 4 days of submerged culture followed by 4 days of air-liquid interface culture, without membrane rupture or leakage. This consideration was particularly important given the potential for stromal cell-mediated ECM remodeling during prolonged culture. We have added this clarification to the Methods (**Line 808-817**).

We agree that a systematic comparison of lower and higher strain amplitudes would be valuable for determining whether the observed maturation response exhibits quantitative strain dependence. However, the primary objective of the present study was not to identify an optimal mechanical strain magnitude, but rather to establish that the combination of cyclic mechanical stimulation, stromal co-culture, and air-liquid interface culture promotes a more mature alveolar epithelial phenotype than static culture conditions. We therefore believe that a full strain-response study would extend beyond the scope of the current work.

To address the reviewer's concern as directly as possible, we have revised the manuscript in two ways. First, we added the physiological and technical rationale underlying the selection of the 5% cyclic-strain condition (**Lines 798-816**). Second, we explicitly acknowledge as a limitation that the present study does not determine the optimal strain magnitude for alveolar maturation and that future studies should systematically evaluate lower and higher strain amplitudes to define strain-response relationships and their effects on AT1, AT2, double-positive, and double-negative cell populations (**Lines 557-563, 595-605**).

We believe these revisions clarify the rationale for the selected stretch condition while appropriately defining the scope and limitations of the current study.

2. If the authors interpret YAP activation as a mechanistic driver of the observed phenotype rather than merely an associated event, this interpretation should either be experimentally supported (for example by pharmacological inhibition of YAP/TEAD signaling) or more carefully qualified throughout the manuscript.

We thank the reviewer for this important clarification. We agree that our data doesn't establish YAP activation as the sole mechanistic driver of the observed phenotype. Prior studies have shown that activation of YAP/TAZ signaling can promote AT1-like differentiation from human pluripotent stem cell-derived alveolar epithelial cells, supporting the plausibility of YAP involvement in AT1-like cell state transitions (PMID: 38642558 and PMID: 38552636). However, we also recognize that mechanical cues can engage multiple downstream pathways, including focal adhesion, actin cytoskeleton regulation, and ECM remodeling, in addition to YAP/TAZ. Therefore, we have revised the manuscript to present YAP activation as one potential mediator associated with the observed phenotype, rather than as a definitive or exclusive driver (**Line 504-532**). We have also added text noting that further perturbation experiments in future studies, such as YAP/TEAD inhibition, would be required to determine whether YAP activity is necessary for this response (**Line 528-532**).

Minor comment:

1. The strongest superiority data (the NKX2.1/SOX2/SOX9 comparison across the four protocols) now appears only in the rebuttal letter, not in the manuscript. Readers cannot evaluate the superiority claim from the paper itself. Please restore this comparison (at least to the Supplementary Material) so that the head-to-head advantage in transcription factor-defined FLP identity is part of the published record, alongside the retained CD47/PDX1 comparison.

We thank the reviewer for this helpful suggestion. We agree that the NKX2.1/SOX2/SOX9 comparison across the four differentiation protocols provides important supporting evidence for the improved foregut/lung progenitor identity generated by our protocol and should be included in the manuscript rather than only in the rebuttal letter. We have therefore included

this figure as part of **Supplementary Figure 2** and referenced this on **(Lines 169-172)**, alongside the CD47/PDX1 expression comparison.

Reviewer #4:

A majority of the concerns raised from my side have been adequately addressed in the revised manuscript. The authors have improved the presentation of their distal tip-like lung organoid model and provided clearer evidence of SOX9, SOX2, and NKX2.1 expression following modification of previously reported culture conditions.

Nevertheless, I remain unconvinced by the evidence supporting the presence of AT1-like cells in these organoids and under the stretched 2D ALI condition. For example, the stretched cell morphologies observed in the 2D cultures remain highly heterogeneous based on their quantification by HT1-56 and ABCA3 staining, suggesting that the induction of the AT1-like state is not yet well controlled in vitro.

We thank the reviewer for this important observation and agree that our data do not demonstrate the generation of a homogeneous or fully controlled AT1-like epithelial layer. Indeed, the persistence of heterogeneous HT1-56 and ABCA3 staining patterns indicates that AT1-like differentiation remains incomplete under the conditions examined. Importantly, the objective of the 2D ALI stretch experiments was not to generate pure AT1-like or AT2-like monolayers, but rather to determine whether cyclic mechanical stretch, in combination with air-liquid interface culture, could further promote maturation of DBT organoid-derived alveolar-like cells. The HT1-56 and ABCA3 markers were selected based on published studies of human alveolar epithelial cell isolation, in which these markers were reported to be among the most selectively expressed markers of adult primary AT1 and AT2 cells, respectively (PMID: 16982851; PMID: 9889249).

In light of this, we have revised the manuscript to more carefully interpret the findings and avoid overstating the extent of AT1-like differentiation. We modified the description of the Figure 6 results to indicate that the Ultrathin Extracellular-Matrix (UMM)-based ALI stretch condition generated a heterogeneous alveolar epithelial population with increased segregation of AT1- and AT2-associated marker expression, rather than a homogeneous AT1-like epithelial layer (**Line 449-456**). Stretch and ALI exposure reduced the proportion of cells co-expressing AT1- and AT2-associated markers and increased the fraction of cells exhibiting more restricted AT1-associated or AT2-associated marker profiles. We therefore interpret these findings as evidence of progressive alveolar epithelial maturation, lineage segregation, and partial structural organization, while recognizing that a substantial degree of cellular heterogeneity remains.

We also agree with the reviewer that achieving more homogeneous AT1 differentiation will require additional optimization. Therefore, we have revised the Discussion to indicate that future studies should examine a broader range of cyclic stretch parameters, multi-lineage co-culture conditions, ECM membrane properties, and signaling pathway modulation to achieve more controlled and homogeneous AT1 and AT2 epithelial differentiation (**Lines 595-605**). Overall, the main advance of this study is the development of an approach to differentiate expandable DBT cells and the demonstration that physiologically relevant mechanical stimulation combined with air-liquid interface culture promotes structural and molecular maturation, together with increased segregation of alveolar epithelial states.