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Structural and Functional Differences in Preterm Tracheal Epithelium Based on Mouse Airway Organoid Models

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Dear Editor,

Premature birth disrupts the natural progression of the respiratory system's development, resulting in adverse respiratory outcomes that extend into childhood [1]. Although the mechanisms underlying these adverse respiratory outcomes are not yet fully understood, inherent abnormalities in the airway epithelium may play a significant role. Recent studies on the tracheal epithelium have primarily focused on disease and infection models, with limited attention given to the developmental aspects of the airway epithelium. Furthermore, there is no consensus regarding the differences in functional cell populations between preterm and full-term infants, nor their potential association with the increased susceptibility to respiratory diseases observed in preterm infants [1–4]. However, the loss of balance of airway functional cells can be associated with many diseases including pneumonia, airway obstruction and progressive interstitial lung disease [5, 6], which could be associated with the vulnerability of the immature airway. As early as 2014, the emergence of human-induced pluripotent stem cell (iPSC) technology enables the generation of personalized laboratory models [7]. Subsequently, more rapidly generated adult stem cell (AdSC) models and various culture media have also emerged to form organoids models. Although mouse lung organoids have been used as disease models in several studies, their application in preterm and full-term settings is yet to be explored [8, 9]. Herein, we aimed to assess the airway characteristics of preterm and term neonatal mice, by detecting cell subtypes and gene expression in preterm and full-term airways at homeostasis, as well as functional and inflammatory responses to viral intervention.

C57/BL mice (E16.5 and newborn P0 pups) were used to isolate tracheal epithelial cells for airway organoid cultivation (Fig. 1A). E16.5 is a time point typically used for studies related to immature mice [10–12] and is reported to correspond to 16–26 weeks of human lung development [13], whereas P0 represents the full-term control group. The tracheal tissue was digested using medium containing 400 U/mL collagenase I (Sigma, 9001-12-1), 0.25 mg/mL Protease E (Sigma, P5147), 10 µM Y27632 (Selleck, S6390), and 10 U/ml Deoxyribonuclease (DNase) I (Sigma, 10104159001) in AdDF+++ buffer (Advanced DMEM/F12 containing 1 × Glutamax, 10 mM HEPES, and antibiotics). The cell pellet was washed with AdDF+++ and embedded in Matrigel (Corning, 356231). Then, added culture medium comprised AdDF+++, 1 × B27 (Gibco, 0080085SA), 5 mM nicotinamide (Sigma, N0636), 1.25 mM N-acetylcysteine (Sigma, A0737), 500 ng/mL R-spondin1 (R&D, 4645), 25 ng/mL recombinant human FGF7 (PeproTech, 450−61), 100 ng/mL recombinant human FGF10, 100 ng/mL recombinant

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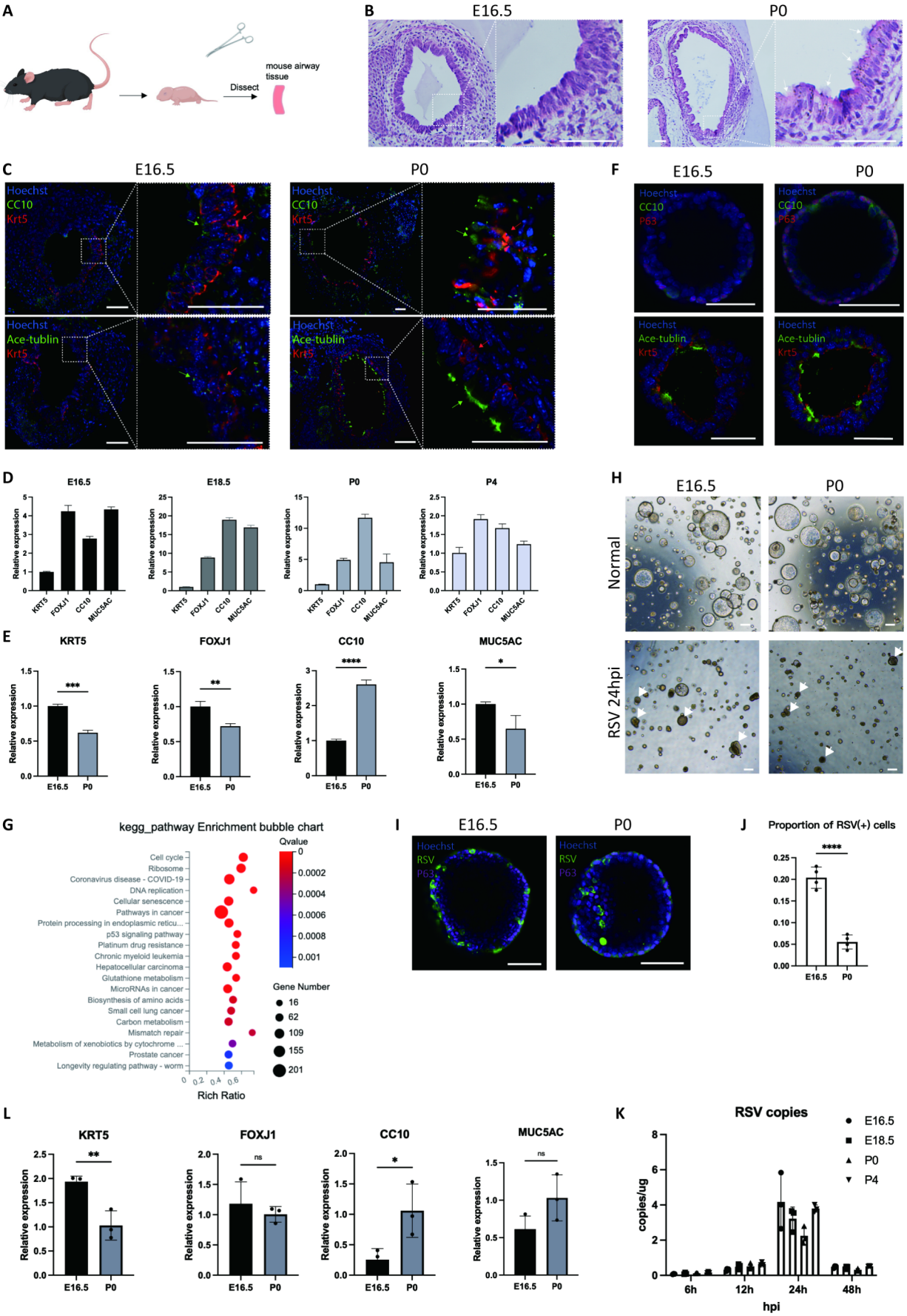
A
mouse airway tissue
Dissect
B
E16.5
P0
C
E16.5
P0
Hoechst
CC10
Krt5
Ace-tublin
F
E16.5
P0
Hoechst
CC10
P63
Ace-tublin
Krt5
D
E16.5
E18.5
P0
P4
Relative expression
KRT5
FOXJ1
CC10
MUC5AC
E
KRT5
FOXJ1
CC10
MUC5AC
H
E16.5
P0
Normal
RSV 24hpi
G
kegg_pathway Enrichment bubble chart
Qvalue
Gene Number
Cell cycle
Ribosome
Coronavirus disease - COVID-19
DNA replication
Cellular senescence
Pathways in cancer
Protein processing in endoplasmic reticu...
p53 signaling pathway
Platinum drug resistance
Chronic myeloid leukemia
Hepatocellular carcinoma
Glutathione metabolism
MicroRNAs in cancer
Biosynthesis of amino acids
Small cell lung cancer
Carbon metabolism
Mismatch repair
Metabolism of xenobiotics by cytochrome ...
Prostate cancer
Longevity regulating pathway - worm
Rich Ratio
I
E16.5
P0
Hoechst
RSV
P63
J
Proportion of RSV(+) cells
L
KRT5
FOXJ1
CC10
MUC5AC
K
RSV copies
E16.5
E18.5
P0
P4
copies/ug
6h
12h
24h
48h
hpi

Fig. 1 **A** Schematic illustration of mice tracheal tissue extraction; **B** HE staining of tissue (White arrow: cilia structure. Scalebar = 50 μm); **C** IF staining of tissue (Hoechst33342, blue; *CC10* and *ace-tublin*, green; *Krt5*, red. Scalebar = 50 μm); **D** Relative mRNA expression of marker genes in four groups of tissue. Basal cell (*KRT5*), ciliated cell (*FOXJ1*), Club cell (*CC10*), goblet cell (*MUC5AC*). $n = 3$ replicates; **E** Relative mRNA expression of marker genes between E16.5 (preterm) and P0 (full-term) groups of tissue. Basal cell (*KRT5*), ciliated cell (*FOXJ1*), Club cell (*CC10*), goblet cell (*MUC5AC*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns: no significance; $n = 3$ replicates; **F** IF staining of preterm and term organoids (Hoechst33342, blue; *CC10* and *ace-tublin*, green; *P63* and *Krt5*, red. Scalebar = 50 μm); **G** Transcriptomic profiling of preterm and full-term mouse airway organoids under steady-state condition; **H** Bright-view images of normal and RSV infected organoids (Scalebar = 200 μm); **I** IF staining of RSV infected organoids (Hoechst33342, blue; RSV, green; *p63*, purple. Scalebar = 50 μm); **J** Proportion of RSV(+) cells in infected preterm and full-term mouse airway organoids; **K** ddPCR results of absolute RSV copies in infected organoids at different time points (6, 12, 24, 48 h) in each group (E16.5, E18.5, P0, P4 group); **L** Relative mRNA expression of marker genes in infected organoids between E16.5 (preterm) and P0 (full-term) groups. Basal cell (*KRT5*), ciliated cell (*FOXJ1*), club cell (*CC10*), goblet cell (*MUC5AC*). * $p < 0.05$, ** $p < 0.01$; ns: no significance; $n = 3$ replicates

human Noggin (R&D, 6057), 5 μM Y27632 (CS, 13624), 500 nM SB202190 (Selleck, S1077), and 500 nM A-8301 (Selleck, S8301). After the same period of cultivation of those organoids, hematoxylin-eosin (HE) and immunofluorescence (IF) staining revealed growth in the perimeter according to the gestational age (Fig. 1B and C). Cilia were clearly identified following the staining, and all functional cells, including club and ciliated cells, were arranged around the epithelium of the airway, with KRT5 + basal cells encircling the basal layer (Fig. 1C). In addition, expression of classic genes for epithelial cells including *FOXJ1* (ciliated cell), *MUC5AC* (goblet cell) and *CC10* (Club cell) were conducted using CFX96 real-time PCR system (Bio-Rad, USA). In the E16.5 preterm tissues, expressions of *FOXJ1* and *MUC5AC* were higher than *CC10*. As gestational age increases, *CC10* expression levels elevated and exceeded those of *FOXJ1* and *MUC5AC*, whereas they reached a relative balance in P4 group (Fig. 1D). Basal cells and ciliated cells had higher marker expressions in the preterm group than that of full-term group, whereas *CC10* had lower expressions in the premature group (Fig. 1E). The results of classic genes and cells using IF staining in the organoid cells were consistent with that in airway tissues (Fig. 1F). Transcriptomic profiling of E16.5 and P0 mouse samples revealed differentially expressed genes (DEGs) between the two group. DEGs in preterm samples were related to inflammatory responses and the PI3K-Akt signaling pathway. In contrast, the expression of *Wnt* family was lower than that in the full-term group, indicating lower *Wnt* signaling activity in our model. This may indicate a higher tendency for differentiation toward the ciliated cell fate, potentially as a compensatory response to the underdevelopment of cilia [14]. It also showed DEGs enrichments in basal cell proliferation (*p53* signaling pathways [15]) and virus response (COVID-19) (Fig. 1G), which is consistent with a previous study reporting higher susceptibility to SARS-CoV-2 in preterm infants [16].

To test whether preterm airways have a hyperactive viral response phenotype, we evaluated the viral reaction using Respiratory syncytial virus (RSV) (ATCC, VR-1540) propagated in HEp-2 cells (ATCC) [17]. The infected organoids became consolidated after virus infection (Fig. 1H). IF staining showed that preterm infection was more severe than term infections, presenting with a higher RSV signal and RSV-positive cell number under confocal 3D scanning (Fig. 1I-J, Video S1a-b). Droplet Digital PCR (ddPCR) was also performed on E16.5, E18.5, P0, and P4 mouse airway organoid to monitor their RSV copies. The results showed that the mouse airway organoids were most severely affected at 24 h post-infection (hpi) (Fig. 1K). However, we found that the infection severity decreased with an increase in pup age (in the E16.5, E18.5, and P0 groups), demonstrating that the earlier the gestational age at birth, the more serious the pups' infection. The discrepancy in the P4 group may reflect differences in cellular composition or immune responses not fully recapitulated in the organoid model. Further studies are needed to clarify this observation [18]. Quantitative real-time polymerase chain reaction (qRT-PCR) showed that preterm airway organoids showed a significantly higher levels of basal cell expression. Nevertheless, functional cell markers, including *CC10* for Club cell and *MUC5AC* for goblet cell, both presented with lower transcription levels than full-term airway organoids (Fig. 1L). For immune responses upon viral infection, qRT-PCR results showed the full-term organoids exhibited a higher transcriptomic level of both IL-6 ($p < 0.05$) and TNF than preterm group (Fig. S1), whereas no specific difference was found upon liquid chip immunoassay for measuring inflammatory factors in the supernatant of culture media. Further studies and more samples are necessary to examine the association between these results and preterm airway diseases, as the signaling pathways and mechanisms behind these phenomena remain unknown. Taken together, our study established a preterm mouse airway organoid model that reveals structural and functional differences between preterm and term airway epithelium, providing novel insights into respiratory disease susceptibility in premature infants.

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Author Contributions W Yang and Y Liu conceived and designed research. Y Li performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript. W Yang, J Luo, L Zhu, and D Lu helped perform

experiments and data analysis. Y Zhang and Q Hu helped interpret the results of experiments. X Zhang revised the manuscript. Y Liu edited and revised the manuscript. H Liu and F Yang supervised the study. All authors read and approved the final manuscript.

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Data Availability Private sharing link for transcriptomic profiling data in NCBI database: https://www.ncbi.nlm.nih.gov/sra/PRJNA1056296. Other source data can be found on contacting the corresponding authors.

Declarations

Ethics Approval Ethics All animal experiments were conducted with the approval of Experimental Animal Management and the Institutional Animal Care of West China Second University Hospital, adhering to the highest ethical standards. Animal Ethics Approval Number: 2023, No.021). Date of Approval: June 26, 2023.

Conflict of Interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Looi, K. (2019). Preterm birth: Born too soon for the developing airway epithelium? *Paediatric Respiratory Reviews*, *31*, 82–88.
2. Haworth, A. A. H. S. G. (1989). Airway size and structure in the normal fetal and infant lung and the effect of premature delivery and artificial ventilation.
3. Groves, et al. (2018). Characterisation of morphological differences in well-differentiated nasal epithelial cell cultures from preterm and term infants at birth and one-year. *PLoS One*, *13*(12), e0201328.
4. Martinu, T., Todd, J. L., Gelman, A. E., Guerra, S., & Palmer, S. M. (2023). Club cell secretory protein in lung disease: Emerging concepts and potential therapeutics. *Annual Review of Medicine*, *74*, 427–441.
5. Rogers, D. F. (2002). Airway goblet cell hyperplasia in asthma: Hypersecretory and anti-inflammatory? *Clinical and Experimental Allergy*, *32*(8), 1124–1127.
6. Reynaud, P., Ahmed, E., Serre, I., Knabe, L., Bommart, S., Suehs, C., Vachier, I., Berthet, J. P., Romagnoli, M., Vernisse, C., et al. (2021). Club cell loss as a feature of bronchiolization in ILD. *Frontiers in Immunology*, *12*, 630096.
7. McCauley, H. A., & Wells, J. M. (2017). Pluripotent stem cell-derived organoids: Using principles of developmental biology to grow human tissues in a dish. *Development*, *144*(6), 958–962.
8. Laube, M., Pietsch, S., Pannicke, T., Thome, U. H., & Fabian, C. (2021). Development and functional characterization of fetal lung organoids. *Front Med (Lausanne)*, *8*, 678438.
9. Zheng, R., Yang, W., Wen, Y., Xie, L., Shi, F., Lu, D., Luo, J., Li, Y., Zhang, R., Chen, T. (2022). Dnah9 mutant mice and organoid models recapitulate the clinical features of patients with PCD and provide an excellent platform for drug screening. *Cell Death & Disease*, *13*(6), 1–14.
10. Yang, H., Du, L., Wu, G., Wu, Z., & Keelan, J. A. (2018). Murine exposure to gold nanoparticles during early pregnancy promotes abortion by inhibiting ectodermal differentiation. *Molecular Medicine (Cambridge Mass)*, *24*(62), 1–16.
11. Suff, N., Karda, R., Diaz, J. A., Ng, J., Baruteau, J., Perocheau, D., Taylor, P. W., Alber, D., Buckley, S. M. K., Bajaj-Elliott, M. (2020). Cervical gene delivery of the antimicrobial peptide, human β-Defensin (HBD)-3, in a mouse model of ascending infection-related preterm birth. *Frontiers in Immunology*, *11*(106), 1–14.
12. Ljungberg, M. C., Sadi, M., Wang, Y., Aronow, B. J., Xu, Y., Kao, R. J., Liu, Y., Gaddis, N., Ardini-Poleske, M. E., Umrod, T., et al. (2019). Spatial distribution of marker gene activity in the mouse lung during alveolarization. *Data Brief, 22*, 365–372.
13. Schittny, J. C. (2017). Development of the lung. *Cell and Tissue Research*, *367*(3), 427–444.
14. Aros, C. J., Pantoja, C. J., & Gomperts, B. N. (2021). Wnt signaling in lung development, regeneration, and disease progression. *Commun Biol*, *4*(1), 601.
15. Mou, H., Yang, Y., Riehs, M. A., Barrios, J., Shivaraju, M., Haber, A. L., Montoro, D. T., Gilmore, K., Haas, E. A., Paunovic, B., et al. (2021). Airway basal stem cells generate distinct subpopulations of PNECs. *Cell Rep*, *35*(3), 109011.
16. Yee, M., David Cohen, E., Haak, J., Dylag, A. M., & O'Reilly, M. A. (2020). Neonatal hyperoxia enhances age-dependent expression of SARS-CoV-2 receptors in mice. *Scientific Reports*, *10*(1), 22401.
17. McKimm-Breschkin, J. L. (2004). A simplified plaque assay for respiratory syncytial virus–direct visualization of plaques without immunostaining. *Journal of Virological Methods*, *120*(1), 113–117.
18. Li, Y. (2022). Global, regional, and National disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in children younger than 5 years in 2019: A systematic analysis. *Lancet*, *399*(10340), 2047–2064.