Human Airway and Alveolar Organoids from BAL Fluid

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To the Editor:

Many lung diseases remain understudied because of a lack of experimental models. Lung organoids, which consist of self-organizing epithelial cells, provide versatile *in vitro* models for normal and abnormal biology, drug screening, gene editing, and personalized therapeutics (1). However, human organoids are generally derived from lung tissue, which is not commonly obtained, or induced pluripotent stem cells, which require complex manipulation. Recently, one study reported airway organoids from BAL fluid, although sample sizes and characterization were limited (2). Here, we demonstrate robust establishment of airway organoids from a variety of human BAL samples and show that these organoids contain diverse airway cell types. Furthermore, we report the development of BAL-derived alveolar organoids. These techniques significantly expand the scope of lung diseases that can be studied using safely accessible primary human cells. Some of these results were previously reported as an abstract (3).

We obtained deidentified BAL samples and lung tissue under protocols approved by the respective institutional review boards. Generally, 99% of cells in BAL fluid were immune (not shown) (4), and sorting for rare epithelial cells was not feasible. Instead, we refined a protocol for selective outgrowth of lung epithelial organoids from BAL (2). BAL samples from eight unique donors were used exclusively to optimize the methods. Detailed methods and donor characteristics can be found in the online supplement.

In brief, 5–10 ml of fresh BAL fluid were centrifuged and processed immediately or cryopreserved in Bambanker serum-free freezing media. Cells were dissociated using Liberase TM and DNase I, filtered, washed, and resuspended in growth factor–reduced Matrigel to a concentration of 100,000–125,000 live cells per 50 μl. Drops of 50 μl each were pipetted onto a prewarmed 12-well plate and allowed to solidify for 25 minutes at 37°C. Finally, 1 ml of either airway or alveolar medium was added to each well. We tested a variety of media components and ultimately used published recipes with minor modifications. For airway cultures (2), HRG1-β1 was added at 50 ng/ml for the first 3–4 days. For alveolar cultures (5, 6), 5 μM Y-27632 was included in the media, and IL-1β was added at 10 ng/ml for the first 3–4 days.

These methods yielded rapid and efficient outgrowth of epithelial organoids within 7–10 days. Airway organoids grew as spheres (Figure 1A). We quantified the success rate of organoid growth by applying the standardized culture methods to BAL from 21 additional donors, focusing on lung transplant patients undergoing surveillance bronchoscopies, together with three healthy control subjects. Airway organoids grew from 17 (94%) of 18 fresh and 4 (57%) of 7 cryopreserved human BAL samples, as well as 6 of 6 fresh murine BAL samples. Organoids were passaged by gently disrupting the Matrigel drop and incubating with TrypLE, then shearing through a pipette five times and replating. Airway organoids could be passaged for at least 6 months, as reported previously for airway organoids derived from lung tissue (2). Organoids could also be cryopreserved and regrown.

We characterized the cell types present in airway organoids. Immunofluorescence showed predominantly airway basal stem cells expressing TP63 and KRT5 (Figure 1A). Flow cytometry corroborated this finding, showing that $CD45^+$ immune cells were eliminated from culture, leaving $EpCAM^+$ epithelial cells, the majority of which expressed NGFR, a surface marker for basal cells (Figure 1B). This result was reproducible across all patient samples. In addition, BAL-derived organoids could be differentiated at the air–liquid interface using established methods (not shown) (2, 7).

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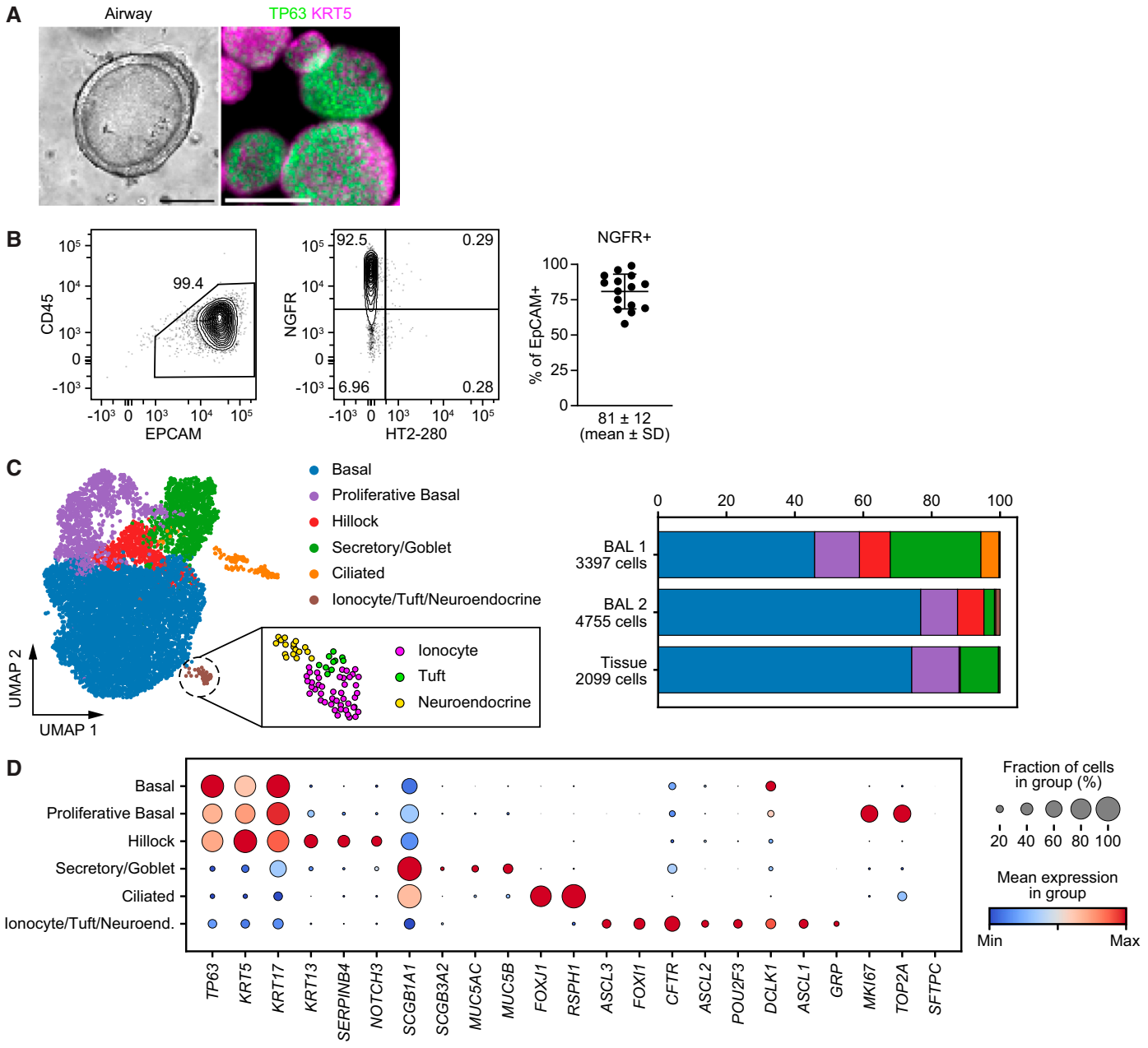

Figure 1. Airway organoids from BAL fluid. (*A*) Representative phase contrast image of an airway organoid 7–10 days after initial plating (passage 0). After expansion, immunofluorescence on whole organoids shows predominant expression of basal cell markers TP63 (green) and KRT5 (magenta). Scale bars, 100 μm. (*B*) Representative flow cytometry at passage 2 shows selective outgrowth of EpCAM$^+$ NGFR$^+$ airway basal cells. The NGFR$^+$ population formed a majority across all samples analyzed. (*C*) Uniform Manifold Approximation and Projection (UMAP) of airway organoids derived from two different human BAL samples and normal lung tissue, and distribution of cell populations in each airway organoid culture. Inset shows subclustering of the small ionocyte/tuft/neuroendocrine population. (*D*) Dot plot of canonical cell markers.

To uncover the full breadth of cell types present, we performed single-cell RNA sequencing, comparing two BAL-derived organoid lines with organoids derived in similar fashion from lung tissue. Single-cell libraries were generated using 10× Genomics Chromium Next GEM Single Cell *3′* version 3.1 kits and sequenced on NovaSeq. The data were processed using Cell Ranger version 7.1 and analyzed using Scanpy (8). After filtering and batch correction using BBKNN (9), data were referenced to the Human Lung Cell Atlas (10), then refined at different resolutions to arrive at biologically meaningful clusters.

Consistent with flow cytometry and immunofluorescence results, single-cell RNA sequencing revealed that most cells in airway organoids were basal cells, whereas secretory/goblet, ciliated, neuroendocrine, and tuft cells were present in lower abundance

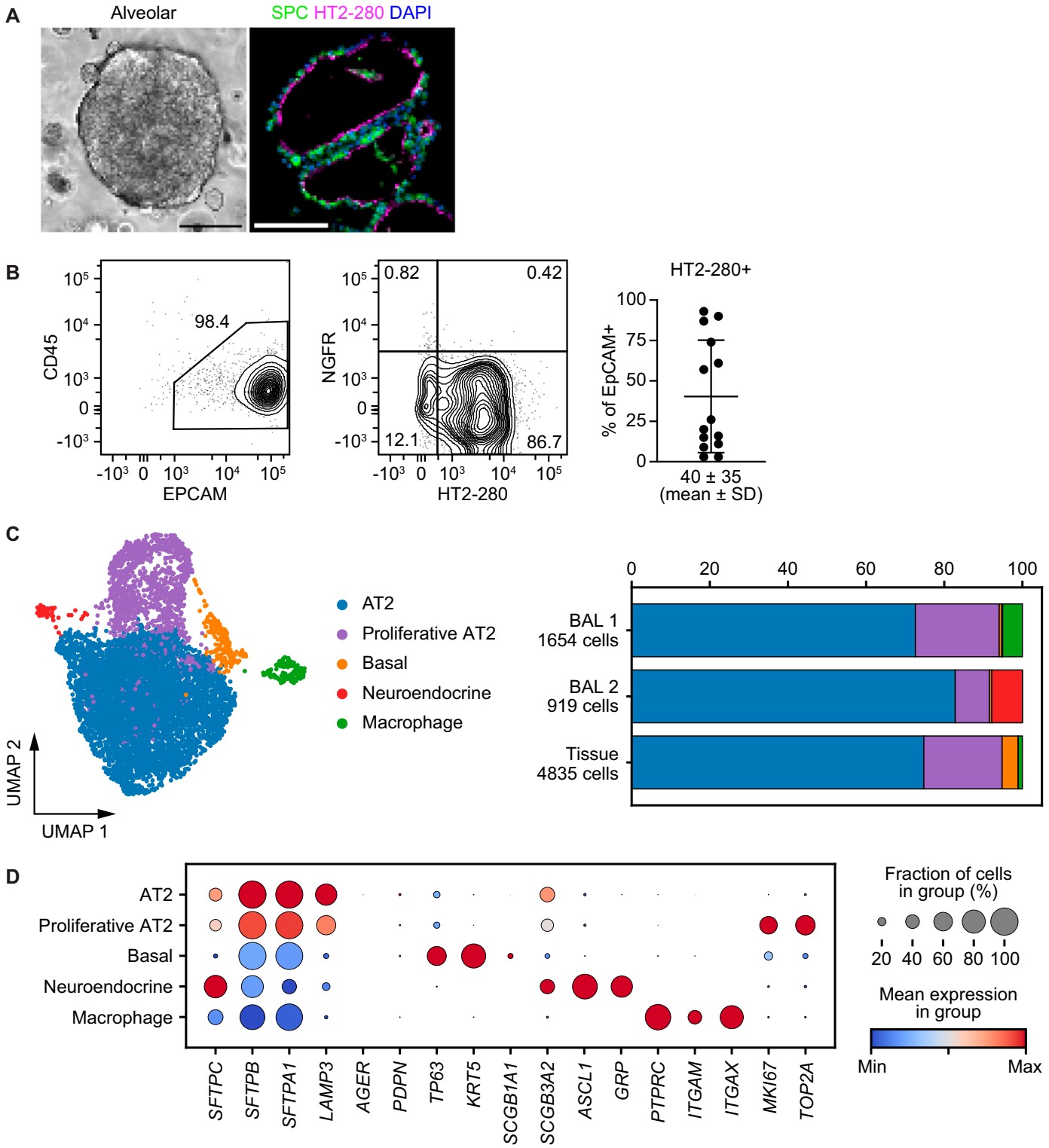

Figure 2. Alveolar organoids from BAL fluid. (*A*) Representative phase contrast image of an alveolar organoid 7–10 days after initial plating (passage 0). After expansion, immunofluorescence on sectioned organoids shows expression of the alveolar type 2 (AT2) cell markers surfactant protein C (SPC; green) and HT2-280 (magenta) on the expected apical surface. Scale bars, 100 μm. (*B*) Representative flow cytometry at passage 2 shows selective outgrowth of EpCAM$^+$ HT2-280$^+$ AT2 cells. The HT2-280$^+$ population was variable across all samples analyzed and could be sorted. (*C*) Uniform Manifold Approximation and Projection (UMAP) of alveolar organoids derived from two different human BAL samples and normal lung tissue, and distribution of cell populations in each alveolar organoid culture. BAL 1 is an unsorted alveolar organoid culture, whereas BAL 2 organoids were grown from HT2-280$^+$ sorted cells; tissue-derived organoids were not sorted. (*D*) Dot plot of canonical cell markers.

(Figures 1C and 1D). We also observed KRT13$^+$ basal cells similar to "hillock" cells that are believed to help repopulate airways after injury and FOXI1$^+$ CFTR$^+$ ionocytes, a cell type of interest in cystic fibrosis (11–13). This highlights the utility of our method for modeling airways from diverse patients and potentially studying many biologically relevant cell types.

We also derived alveolar organoids from BAL, which have not previously been described. Alveolar organoids grew from 16 (89%) of 18 fresh and 5 (71%) of 7 cryopreserved human BAL samples, as well as 7 of 7 fresh murine BAL samples. They typically had a dense appearance with an irregular border (Figure 2A). In line with the known difficulty of expanding alveolar type 2 (AT2) cells, we generally maintained alveolar organoids through four passages, with a maximum of seven to date. Immunofluorescence showed expression of surfactant protein C as well as the AT2 cell surface marker HT2-280 on the apical membrane (Figure 2A). Flow cytometry corroborated outgrowth of AT2 cells (Figure 2B). Notably, across 14 samples analyzed by flow cytometry, the proportion of AT2 cells in the alveolar cultures ranged widely, from 3% to 93% of epithelial cells; there were sometimes immune, mesenchymal, or airway cells remaining. This heterogeneity could be overcome by sorting for HT2-280$^+$ cells when needed. Finally, we analyzed the transcriptomic profile of the alveolar organoids—one unsorted (BAL 1) and one sorted (BAL 2)—alongside unsorted tissue-derived organoids. The predominant cell type was AT2 in all three samples (Figures 2C and 2D). No AT1 cells or transition states were observed.

In summary, we established and characterized airway and alveolar lung organoids from human BAL. Our serum-free, feeder-free methods carry several advantages: Initially unsorted, rare epithelial cells grow out within 7–10 days; all cells are from one person; many cell types are represented; expansion and differentiation are feasible; and organoids can be banked for future use. In preliminary studies, similar results can be obtained with lung tissue and human bronchial epithelial cells. Alveolar organoids from human BAL are more difficult to expand than airway organoids and sometimes benefit from downstream sorting for AT2 cells. Limitations include the variability of BAL techniques and fluid composition, which may impact organoid outgrowth, and a need to understand whether the cells that grow from BAL fluid are representative of patients' disease processes. Work is underway to examine how the properties of organoids correlate to specific lung conditions. Overall, these techniques enable new strategies for modeling diverse lung diseases using accessible primary human cells. ■

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References

1. Liberti DC, Morrisey EE. Organoid models: assessing lung cell fate decisions and disease responses. *Trends Mol Med* 2021;27: 1159–1174.
2. Sachs N, Papaspyropoulos A, Zomer-van Ommen DD, Heo I, Böttinger L, Klay D, *et al.* Long-term expanding human airway organoids for disease modeling. *EMBO J* 2019;38:1–20.
3. Liu MY, Garcia De Alba Rivas C, Movval Abdulla N, Sharma NS, Kim CF. Establishing airway lung organoids from human bronchoalveolar lavage fluid [abstract]. *Am J Respir Crit Care Med* 2023;207:A4690.
4. Maestre-Batlle D, Pena OM, Hirota JA, Gunawan E, Rider CF, Sutherland D, *et al.* Novel flow cytometry approach to identify bronchial epithelial cells from healthy human airways. *Sci Rep* 2017;7:42214.
5. Konishi S, Tata A, Tata PR. Defined conditions for long-term expansion of murine and human alveolar epithelial stem cells in three-dimensional cultures. *STAR Protoc* 2022;3:101447.
6. Katsura H, Sontake V, Tata A, Kobayashi Y, Edwards CE, Heaton BE, *et al.* Human lung stem cell-based alveolospheres provide insights into SARS-CoV-2-mediated interferon responses and pneumocyte dysfunction. *Cell Stem Cell* 2020;27:890–904.e8.
7. Wang R, Simone-Roach C, Lindstrom-Vautrin J, Wang F, Rollins S, Bawa PS, *et al. De novo* generation of pulmonary ionocytes from normal and cystic fibrosis human induced pluripotent stem cells. *Am J Respir Crit Care Med* 2023;207:1249–1253.
8. Wolf FA, Angerer P, Theis FJ. SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol* 2018;19:15.
9. Polański K, Young MD, Miao Z, Meyer KB, Teichmann SA, Park J-E. BBKNN: fast batch alignment of single cell transcriptomes. *Bioinformatics* 2020;36:964–965.
10. Sikkema L, Ramírez-Suástegui C, Strobl DC, Gillett TE, Zappia L, Madissoon E, *et al.*; Lung Biological Network Consortium. An integrated cell atlas of the lung in health and disease. *Nat Med* 2023; 29:1563–1577.
11. Deprez M, Zaragosi L-E, Truchi M, Becavin C, Ruiz García S, Arguel M-J, *et al.* A single-cell atlas of the human healthy airways. *Am J Respir Crit Care Med* 2020;202:1636–1645.
12. Montoro DT, Haber AL, Biton M, Vinarsky V, Lin B, Birket SE, *et al.* A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. *Nature* 2018;560:319–324.
13. Plasschaert LW, Žilionis R, Choo-Wing R, Savova V, Knehr J, Roma G, *et al.* A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte. *Nature* 2018;560:377–381.