*Commentary*

# Dual Genetic Tracing Reveals the Origin of Alveolar Stem Cells after Lung Injury

Kuo Liu [1,2] and Bin Zhou [1,2,3,*]

1   Key Laboratory of Systems Health Science of Zhejiang Province, School of Life Science, Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, Hangzhou 310024, China; liukuo2015@sibcb.ac.cn (K.L.)

2   New Cornerstone Science Laboratory, Key Laboratory of Multi-Cell Systems, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai 200031, China

3   School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China

*   Corresponding author. E-mail: zhoubin@sibs.ac.cn (B.Z.)

**ABSTRACT:** As alveolar epithelial stem cells, alveolar type II (AT2) cells play a pivotal role in sustaining alveolar homeostasis and facilitating repair processes. However, the sources of AT2 cell regeneration have remained contentious due to the non-specific labeling limitations of traditional single recombinase-based lineage tracing techniques. To address this issue, we employed dual recombination systems to develop more precise lineage tracing methodologies, effectively bypassing the shortcomings of conventional approaches and enabling specific labeling of lung epithelial cells. Our findings demonstrate that, following lung injury, regenerated AT2 cells do not originate from alveolar type I (AT1) cells, but instead derive from bronchiolar club cells and bronchioalveolar stem cells (BASCs), alongside the self-renewal of resident AT2 cells. Furthermore, we discovered that the transition of club cells and BASCs into AT2 cells is distinctly modulated by the Notch signaling pathway. This study not only provides novel insights into lung regeneration, but the innovative lineage tracing technology developed herein also holds promise as a technical support for research in diverse fields.

**Keywords:** Genetic lineage tracing; Lung regeneration; Alveolar stem cell; Cre-loxP; Dre-rox

The alveolar epithelium of the lung comprises AT1 cells and AT2 cells. As alveolar stem cells, AT2 cells are capable of self-renewal and differentiation into AT1 cells during homeostasis and repair [1]. Identifying the cellular origins of AT2 cells following lung damage is vital for uncovering new therapeutic targets for various lung diseases to enhance tissue regeneration. However, the sources of AT2 cells have been subject to debate due to potential non-specific labeling by traditional genetic lineage tracing methods. Site-specific DNA recombination systems like the Cre-loxP system are frequently employed in cell lineage studies. The accuracy of promoter-driven Cre expression is critical for ensuring cell-specific labeling. If Cre recombinase is expressed under a promoter active in the target cells but also in unintended or "unwanted" cell types, then both cell types will be labeled following Cre-loxP recombination. This co-labeling can result in misleading conclusions, as it suggests a lineage relationship where none may exist. It is crucial to ensure the specificity of promoter activity to avoid such inaccuracies in cell fate-mapping studies [2]. Therefore, it is essential to validate the specificity of lineage tracing tools prior to their use in cell fate-mapping studies.

Recent lineage tracing studies have indicated that Hopx-CreER-labeled AT1 cells [3–7] and *Scgb1a1-CreER*-labeled club cells [8,9] are progenitors of AT2 cells following alveolar damage. However, the labeling specificity of these genetic tools has not been rigorously evaluated. In our recent study, "Tracing the origin of alveolar stem cells in lung repair and regeneration" [10], we undertook a detailed examination of the non-specific labeling associated with conventional *Hopx-CreER* and *Scgb1a1-CreER* tools. Moreover, we employed dual recombinase-mediated genetic strategies to re-examine the cellular origin of AT2 cells during lung repair, enhancing the accuracy of lineage tracing in this context (Figure 1).

Our study uncovered that the *Hopx-CreER* genetic tool could inappropriately label unintended or "unwanted" cell types, including club cells, AT2 cells, bronchioalveolar stem cells (BASCs), and ciliated cells, besides the intended AT1 cells. Both AT2 cells and BASCs have been previously identified as stem/progenitor cells for regenerating AT2 cells post-lung injury [11–17]. Similarly, another tool, *Ager-CreER*, also targeted AT2 cells and a subset of BASCs, in addition to AT1 cells. Likewise, the *Scgb1a1-CreER* tool could ectopically label BASCs and a subset of AT2 cells, in addition to its intended target, club cells [18]. Currently, there are no such specific Cre tools available that completely avoid this issue of non-specific labeling.

To address this, we utilized the Dre-rox system, another homologous recombinase [19], in conjunction with the Cre-loxP system. By exploiting the mutual exclusion of ectopic labeling between *Ager-CreER* and *Hopx-2A-DreER*, we achieved an 83% efficiency in labeling Ager$^+$Hopx$^+$ AT1 cells using an intersectional dual genetic reporter system (*R26-RSR-LSL-tdT*). Additionally, we employed another dual recombination system (R26-NR2), which specifically traced Hopx$^+$ AT1 cells with approximately 98% efficiency. Fate-mapping analysis confirmed that AT1 cells are terminally differentiated and do not de-differentiate into AT2 cells during injuries caused by pneumonectomy (PNX), bleomycin, or hyperoxia. These findings align with recent studies indicating that Hopx$^+$Igfbp2$^+$ AT1 cell subpopulations do not de-differentiate into AT2 cells in PNX-injured lungs [20]. The potential for AT1 cells to convert to AT2 cells under extreme conditions remains an area for further investigation. Such a transformation might require a stronger impetus, likely through genetic manipulation, to trigger a de-differentiation program in these terminally differentiated cells [21,22]. Exploring these conditions could open new pathways for enhancing lung repair and regeneration, offering promising avenues for therapeutic interventions in lung diseases.

For club cells, AT2 cells, and BASCs, we employed an alternative intersectional dual genetic system (*R26-TLR*), which allowed for the simultaneous and specific labeling of these three distinct cell populations within a single mouse. This approach facilitated precise elucidation of their contributions to AT2 cell regeneration following lung injury. Utilizing various injury models revealed that the cellular plasticity of these cells varied depending on the type of lung injury encountered. In the PNX injury model, the regeneration of AT2 cells predominantly occurred through self-renewal, with minimal contribution from club cells and BASCs. However, in the bleomycin-induced injury model, both club cells and BASCs significantly contributed to the regeneration of a substantial portion of AT2 and AT1 cells.

To further explore the regenerative potential of club cells, we constructed two severe alveolar injury models. In the first model, we impaired the regenerative capacities of AT2 cells and bronchioalveolar stem cells (BASCs) in a bleomycin-injury scenario by ectopically expressing p21. In the second, we induced the depletion of AT2 cells and BASCs through diphtheria toxin (DT)-mediated cell death. Remarkably, club cells exhibited substantial plasticity and regenerative potential, contributing to the reconstitution of the majority of AT2 cells in some lung lobes following severe alveolar injury. This finding suggests that when the alveolar epithelium's native repair mechanisms are inhibited, club cells in the airway region are capable of completely reconstructing the alveolar structures in certain damaged areas. Employing combined single-cell RNA sequencing (scRNA-seq), cell-specific gene knockouts, and multiple cell lineage tracing techniques, we identified that Notch signaling has divergent regulatory effects on the differentiation of club cells and BASCs into AT2 cells post-injury. Specifically, inhibition of Notch signaling enhanced the conversion of BASCs into AT2 cells, whereas its activation prevented this transformation. Conversely, inhibition of Notch signaling limited the differentiation of club cells into AT2 cells, instead favoring their development into ciliated cells.

In pulmonary diseases such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and lung diseases induced by smoking, the damage to or senescence of AT2 cells significantly impairs the repair and regeneration of lung epithelial tissues. The transplantation of lung progenitor or stem cells presents a critical therapeutic strategy for treating these diseases [23]. Given the remarkable plasticity of club cells and BASCs in alveolar regeneration, directly delivering these progenitor cells into the alveolar region might facilitate immediate cell fate conversion, potentially enhancing the effectiveness of cell-based therapies. Furthermore, pharmacologically targeting pathways such as Notch signaling to modulate this plasticity offers an additional promising approach to develop successful treatments for pulmonary diseases.

It should be noted that our findings appear to contradict a recent study, which suggested that inhibition of Notch signaling facilitates the transition of club cells to AT2 cells during repair processes [9]. This discrepancy may arise because the previous study employed *Scgb1a1-CreER* to trace both club cells and BASCs, complicating the differentiation of the effects of Notch signaling in each cell type [9]. Moreover, another study identified a distinct population of club cells, termed respiratory airway secretory cells (RAS), located in the human distal airways [24]. These cells exhibited a hybrid expression pattern characteristic of both club and AT2 cells, suggesting their role as progenitors in AT2 cell regeneration in pulmonary diseases [24]. In vitro experiments demonstrated that inhibition of

Notch signaling promotes the differentiation of RAS into AT2 cells [24], aligning with our findings concerning the regulation of BASCs by Notch. Given the similar features between BASCs and RAS, BASCs may provide an ideal in vivo model for studying the molecular and cellular mechanisms involved in lung repair.

In conclusion, by employing dual recombinases-mediated genetic tracing, we have precisely labeled multiple types of pulmonary epithelial cells and revealed their cell capacity for contributing to AT2 cell regeneration following lung injury. The dual genetic strategies developed in this study surpass the conventional Cre-loxP system in targeting specificity and, in conjunction with cell lineage tracing, facilitate a more accurate investigation of molecular mechanisms. This advanced genetic lineage tracing technique will offer significant potential for exploring cell plasticity and fate decisions in various organs across development, disease states, and regenerative processes.
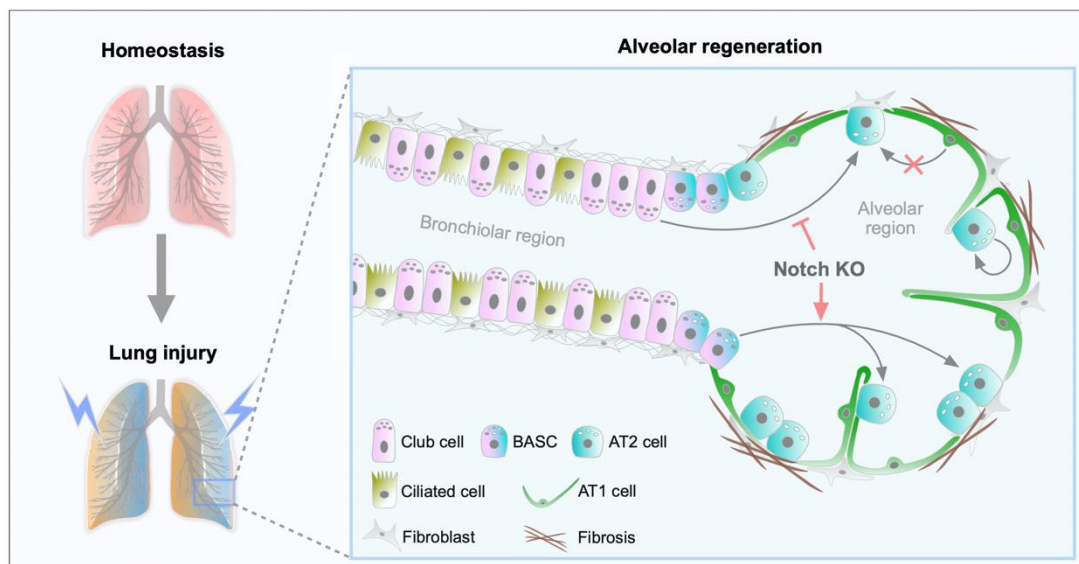


**Figure 1.** The cellular origin of AT2 stem cells is diverse after lung injury. In the bleomycin-induced alveolar injury model, the regenerated AT2 cells originate from multiple sources, including club cells, BASCs, and the self-renewal of existing AT2 cells, but not from AT1 cells. Furthermore, Notch signaling exhibits opposite regulatory functions in the differentiation of club cells and BASCs into AT2 cells post-injury. Specifically, inhibition of Notch signaling decreases the conversion of club cells into AT2 cells, whereas promoting BASCs differentiation into AT2 cells.

## Acknowledgments

## Author Contributions

K.L. and B.Z. designed the study and wrote the paper.

## Ethics Statement

All mouse experiments in this study were strictly performed within the committee's guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences.

## Informed Consent Statement

Not applicable.

## Funding

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## References

1. Basil MC, Katzen J, Engler AE, Guo M, Herriges MJ, Kathiriya JJ, et al. The cellular and physiological basis for lung repair and regeneration: Past, present, and future. *Cell Stem. Cell* **2020**, *26*, 482–502.
2. Liu K, Jin H, Zhou B. Genetic lineage tracing with multiple DNA recombinases: A user's guide for conducting more precise cell fate mapping studies. *J. Biol. Chem*. **2020**, *295*, 6413–6424.
3. Shiraishi K, Shah PP, Morley MP, Loebel C, Santini GT, Katzen J, et al. Biophysical forces mediated by respiration maintain lung alveolar epithelial cell fate. *Cell* **2023**, *186*, 1478–1492.
4. Juul NH, Yoon JK, Martinez MC, Rishi N, Kazadaeva YI, Morri M, et al. Kras(g12d) drives lepidic adenocarcinoma through stem-cell reprogramming. *Nature* **2023**, *619*, 860–867.
5. Penkala IJ, Liberti DC, Pankin J, Sivakumar A, Kremp MM, Jayachandran S, et al. Age-dependent alveolar epithelial plasticity orchestrates lung homeostasis and regeneration. *Cell Stem. Cell*. **2021**, *28*, 1775–1789.
6. Liberti DC, Libert WA, Kremp MM, Penkala IJ, Cardenas-Diaz FL, Morley MP, et al. Klf5 defines alveolar epithelial type 1 cell lineage commitment during lung development and regeneration. *Dev. Cell*. **2022**, *57*, 1742–1757.
7. Jain R, Barkauskas CE, Takeda N, Bowie EJ, Aghajanian H, Wang QH, et al. Plasticity of hopx(+) type i alveolar cells to regenerate type ii cells in the lung. *Nat. Commun*. **2015**, *6*, 6727.
8. Kathiriya JJ, Brumwell AN, Jackson JR, Tang X, Chapman HA. Distinct airway epithelial stem cells hide among club cells but mobilize to promote alveolar regeneration. *Cell Stem. Cell*. **2020**, *26*, 346–358.
9. Choi J, Jang YJ, Dabrowska C, Iich E, Evans KV, Hall H, et al. Release of notch activity coordinated by il-1beta signalling confers differentiation plasticity of airway progenitors via fosl2 during alveolar regeneration. *Nat. Cell. Biol*. **2021**, *23*, 953–966.
10. Liu K, Meng X, Liu Z, Tang M, Lv Z, Huang X, et al. Tracing the origin of alveolar stem cells in lung repair and regeneration. *Cell* **2024**, *187*, 1–18.
11. Barkauskas CE, Cronce MJ, Rackley CR, Bowie EJ, Keene DR, Stripp BR, et al. Type 2 alveolar cells are stem cells in adult lung. *J. Clin. Invest*. **2013**, *123*, 3025–3036.
12. Nabhan AN, Brownfield DG, Harbury PB, Krasnow MA, Desai TJ. Single-cell wnt signaling niches maintain stemness of alveolar type 2 cells. *Science* **2018**, *359*, 1118–1123.
13. Zacharias WJ, Frank DB, Zepp JA, Morley MP, Alkhaleel FA, Kong J, et al. Regeneration of the lung alveolus by an evolutionarily conserved epithelial progenitor. *Nature* **2018**, *555*, 251.
14. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* **2005**, *121*, 823–835.
15. Liu Q, Liu K, Cui G, Huang X, Yao S, Guo W, et al. Lung regeneration by multipotent stem cells residing at the bronchioalveolar-duct junction. *Nat. Genet*. **2019**, *51*, 728–738.
16. Liu K, Tang M, Liu Q, Han X, Jin H, Zhu H, et al. Bi-directional differentiation of single bronchioalveolar stem cells during lung repair. *Cell Discov*. **2020**, *6*, 1.
17. Salwig I, Spitznagel B, Vazquez-Armendariz AI, Khalooghi K, Guenther S, Herold S, et al. Bronchioalveolar stem cells are a main source for regeneration of distal lung epithelia in vivo. *EMBO J*. **2019**, *38*, e102099.
18. Rawlins EL, Okubo T, Xue Y, Brass DM, Auten RL, Hasegawa H, et al. The role of scgb1a1+ clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. *Cell Stem. Cell* **2009**, *4*, 525–534.
19. He LJ, Li Y, Li Y, Pu WJ, Huang XZ, Tian XY, et al. Enhancing the precision of genetic lineage tracing using dual recombinases. *Nat. Med*. **2017**, *23*, 1488.
20. Wang Y, Tang Z, Huang H, Li J, Wang Z, Yu Y, et al. Pulmonary alveolar type i cell population consists of two distinct subtypes that differ in cell fate. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 2407–2412.
21. Yang J, Hernandez BJ, Martinez Alanis D, Narvaez del Pilar O, Vila-Ellis L, Akiyama H, et al. The development and plasticity

of alveolar type 1 cells. *Development* **2016**, *143*, 54–65.

22. Little DR, Lynch AM, Yan Y, Akiyama H, Kimura S, Chen J. Differential chromatin binding of the lung lineage transcription factor nkx2-1 resolves opposing murine alveolar cell fates in vivo. *Nat. Commun*. **2021**, *12*, 2509.

23. Herriges MJ, Yampolskaya M, Thapa BR, Lindstrom-Vautrin J, Wang F, Huang J, et al. Durable alveolar engraftment of psc-derived lung epithelial cells into immunocompetent mice. *Cell Stem. Cell* **2023**, *30*, 1217–1234.

24. Kadur Lakshminarasimha Murthy P, Sontake V, Tata A, Kobayashi Y, Macadlo L, Okuda K, et al. Human distal lung maps and lineage hierarchies reveal a bipotent progenitor. *Nature* **2022**, *604*, 111–119.