Genetic lineage tracing is widely used to study organ development and tissue regeneration. Multicolor reporters are a powerful platform for simultaneously tracking discrete cell populations. Here, combining Dre-rox and Cre-loxP systems, we generated a new dual-recombinease reporter system, called Rosa26 traffic light reporter (R26-TLR), to monitor red, green, and yellow fluorescence. Using this new reporter system with the three distinct fluorescent reporters combined on one allele, we found that the readouts of the two recombinases Cre and Dre simultaneously reflect Cre+Dre−, Cre−Dre+, and Cre+Dre+ cell lineages. As proof of principle, we show specific labeling in three distinct progenitor/stem cell populations, including club cells, AT2 cells, and bronchioalveolar stem cells, in Sftpc-DreER; Scgb1a1-CreER;R26-TLR mice. By using this new dual-recombinease reporter system, we simultaneously traced the cell fate of these three distinct cell populations during lung repair and regeneration, providing a more comprehensive picture of stem cell function in distal airway repair and regeneration. We propose that this new reporter system will advance developmental and regenerative research by facilitating a more sophisticated genetic approach to studying in vivo cell fate plasticity.

Mammalian organs are complex, dynamic tissues whose maintenance and repair depend on diverse stem/progenitor cell populations. During multiple types of organogenesis, such as heart, lung, and intestine, one stem/progenitor cell population can contribute to several cell types. For example, the epicardium and endocardium of the heart (1–4), basal cells of the lungs (5–7), and Lgr5+ stem cells of intestinal crypts (8) can give rise to multiple cell types during homeostasis, growth, and regeneration. Some differentiated cell types can also originate from multiple stem/progenitor cell sources. For instance, during heart development, coronary vascular endothelial cells, fibroblasts, pericytes, and adipocytes are derived from both epithelial and endocardial cells, albeit with different proportions of contribution (9–12). Additionally, cardiomyocytes in the developing heart are derived from both the primary and secondary heart field (13, 14). Multiple different epithelial cells in the distal airway are derived from club cells or alveolar type 2 cells and bronchioalveolar stem cells (BASCs)2 after lung injury (15, 16). Fate mapping of multiple cellular origins for their contribution of discrete cell lineages provides critical information for understanding tissue homeostasis renewal and regeneration after injury.

The ability to study multiple cell populations in one tissue is important to understand the complex biological process underlying development, tissue homeostasis, and regeneration. Genetic lineage tracing mediated by DNA site-specific recombination systems has been widely used for cell lineage and fate studies (17). Similar to the Cre-loxP system, multiple site-specific recombination systems, such as Flpe-Frt, Dre-rox, and Nigri-nox, have been developed and used for cell fate tracing studies (17–21). Two types of reporter systems have been developed and used for cell fates analysis. One type is conventional single-color reporter systems, such as Rosa26-taTomato, Rosa26-LacZ, or Rosa26-GFP, which are used to track one cell lineage in one color (22–24), or multicolor systems, such as Brainbow or Confetti, used for clonal analysis of one cell lineage (25, 26). Another type is multicolor reporter systems that combine two or more recombination systems for genetic targeting of more than one cell population (usually two). Intersectional dual-reporter systems, such as RC::Fela, R26::FLAP, RC::RlTg, and R26N2CG, can be used to label two distinct cell populations (27–30). In addition, sequential and exclusive double-reporter systems are also explored for labeling specific cell types more precisely (31–33). However, because of the exclusive nature of recombination design, these genetic tools only allow simultaneous labeling of two (sub)populations in tissue. Development of a multicolor reporter system capable of labeling three distinct cell populations by noninterfering recombination is useful.

This work was supported by National Key Research and Development Program of China Grants 2018YFA0108100, 2019YFA0110400, 2018YFA0107900, 2016YFC1000600, and 2017YFC1001303; Strategic Priority Research Program of the Chinese Academy of Sciences Grants XDA16010507 and XDB19000000; and National Science Foundation of China Grants 317703112, 91639302, 31625019, 91849202, 81761138040, 81872241, 31701292, and 31922032. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains refs 25 and Table S1.

1 Present address: 320 Yueyang Rd., Life Science Research Bldg. A-2112, 200031 Shanghai, China. To whom correspondence should be addressed: Tel: 86-21-54920974; Fax: 86-21-54920974; E-mail: zhoubin@sibs.ac.cn.

2 The abbreviations used are: BASC, bronchioalveolar stem cell; E13.5, embryonic day 13.5; BADJ, bronchioalveolar duct junction; WPRe, woodchuck hepatitis virus posttranscriptional regulatory element; CAG, hybrid construct consisting of the cytomegalovirus enhancer fused to the chicken beta-actin promoter; ACTB, beta-actin; PECAM, platelet endothelial cell adhesion molecule; PDGFRα, platelet-derived growth factor receptor alpha; PDGFRβ, platelet-derived growth factor receptor beta; SCP, surfactant protein C.
for studying the behavior of more diverse cell types simultaneously in vivo.

In this study, we generated a new dual genetic system that incorporates both the Cre-loxP and Dre-rox recombination systems. Different from previous dual-recombinase reporters that reflect combination readout of Cre/Dre recombinases, the current reporters are parallel for two recombination readouts. Thus, this new dual system has the capacity to label three distinct cell populations simultaneously: Dre+Cre−, Dre−Cre+, and Dre−Cre− cell populations. As proof of principle, we traced cardiomyocytes and endothelial cells simultaneously during heart development. Additionally, we used the system to label three stem cell populations (club cells, AT2 cells, and BASCs) in the lungs at homeostasis and to track their cell fate after injury. Our new mouse reporter R26-TLR extents the scope of cell type labeling with one reporter allele, which could be broadly used for diverse cell origin and cell fate studies in development, disease, and regeneration.

Results
Generation and characterization of R26-TLR

The Cre-loxP system is a widely used site-specific recombinase-based system. Like the Cre-loxP system, Dre recombinase specifically targets its recombination site rox (19). For genetic lineage studies, cell-specific promoter-derived Cre (or Dre) removes a loxP- or rox-flanked transcriptional stop cassette (stop) after the Rosa26 promoter, leading to permanent constitutively active expression of the following reporter in Cre- or Dre-expressing cells and their descendants (4). To genetically label multiple cell lineages, we generated a new lineage tracing reporter system that incorporates both the Cre-loxP and Dre-rox recombinations. This reporter was generated by knocking the CAG-rox-stop-rox-ZsGreen-WPRE-pA-Frt-Neo-Frt sequence into exon1 of the Rosa26 gene locus and knocking the insulator-CAG-rox-stop-rox-tdTomato-WPRE-pA sequence into exon2 of the Rosa26 gene locus through two homologous recombinations (Fig. 1A). Whether ZsGreen-WPRE-poly(A) after the Dre-rox recombination event can affect tdTomato gene expression by Cre-loxP recombination in the same allele is unknown. We therefore intentionally inserted the insulator between them to block the potential influence between them and ensure that the readouts of Dre-rox and Cre-loxP are specific and efficient. The insulator sequence is in Table S1. Dre and Cre recombinases driven by two different promoters recombine rox or loxP sites, resulting in ZsGreen or tdTomato reporter expression, respectively. Notably, when the two promoters are both active in a cell, the cell expresses ZsGreen and tdTomato at the same time, yielding yellow fluorescence (Fig. 1, B and C). We named this reporter line Rosa26 traffic light reporter (R26-TLR).

To characterize R26-TLR, we crossed the R26-TLR mouse line with CAG-Dre and ACTB-Cre lines. CAG and ACTB are gene promoters broadly active in almost all tissues. We acquired four littersmate genotypes: R26-TLR, CAG-Dre;R26-TLR, ACTB-Cre;R26-TLR, and CAG-Dre;ACTB-Cre;R26-TLR. As expected by design (Fig. 1A), whole-mount epifluorescence analysis showed that neither ZsGreen nor tdTomato was detected in embryonic day 13.5 (E13.5) R26-TLR embryos, indicating no leakiness of R26-TLR without any recombinase. ZsGreen+ but not tdTomato+ signals were detected in E13.5 CAG-Dre;R26-TLR embryos, whereas tdTomato+ but not ZsGreen+ signals were detected in E15.5 ACTB-Cre;R26-TLR embryos. In CAG-Dre;ACTB-Cre;R26-TLR triple-positive embryos, both ZsGreen+ and tdTomato+ signals were detected (Fig. 1D). Immunostaining of ZsGreen and tdTomato on four genotyped embryonic sections showed that no ZsGreen or tdTomato was detected in cells in R26-TLR embryonic sections. All cells were ZsGreen+tdTomato− in the CAG-Dre;R26-TLR line, whereas all cells were ZsGreen− tdTomato+ in the ACTB-Cre;R26-TLR line, indicating that Dre or Cre recombinase specifically recombined its own target site (Fig. 1E). In the CAG-Dre;ACTB-Cre;R26-TLR triple-positive line, all cells expressed both ZsGreen and tdTomato, yielding yellow fluorescence in those Dre−Cre− cells. Taken together, these data demonstrated that the new reporter R26-TLR could be used for tracking different cell populations simultaneously in vivo.

Tracing distinct cell populations simultaneously in the heart

To further prove that R26-TLR can be used to trace different/diverse cell populations simultaneously, we crossed the R26-TLR mouse line with the Tnni3-Dre and Tie2-Cre mouse lines, which specifically target cardiomyocytes and endothelial cell lineages, respectively (32, 35). As shown in the design, Dre expression driven by the Tnni3 promoter resulted in ZsGreen gene expression, and Tie2-derived Cre led to tdTomato gene expression (Fig. 2, A and B). For analysis, we collected hearts from E16.5 embryos of three genotypes: Tnni3-Dre;R26-TLR, Tie2-Cre;R26-TLR, and Tnni3-Dre;Tie2-Cre;R26-TLR (Fig. 2C). Whole-mount epifluorescence imaging showed ZsGreen+ but not tdTomato+ signals detected in Tnni3-Dre;R26-TLR hearts. In contrast, tdTomato+ but not ZsGreen+ signals in vascular patterns were detected in Tie2-Cre;R26-TLR hearts. As expected, both ZsGreen+ and tdTomato+ signals were detected in Tnni3-Dre;Tie2-Cre;R26-TLR triple-positive hearts (Fig. 2D). To further verify the labeling specificity, we performed immunostaining on heart sections with the cardiomyocyte marker Tnni3 and the endothelial cell marker Pecam. Immunostaining for ZsGreen, tdTomato, and Tnni3 on Tnni3-Dre;Tie2-Cre;R26-TLR triple-positive heart sections showed that over 99% of all cardiomyocytes were ZsGreen+ tdTomato− (Fig. 2, E and F). We also performed immunostaining for ZsGreen, tdTomato, and Pecam on triple-positive heart sections and detected that more than 99% of endothelial cells were tdTomato+ZsGreen− (Fig. 2, G and H). The distributions of ZsGreen+ cardiomyocytes and tdTomato+ endothelial cells were interleaved and connected to each other. Furthermore, we could hardly detect any yellow signals, indicating that the promoters of Tnni3 and Tie2 were less likely to be active in the same cell types (Fig. 2, E and G). Additionally, immunostaining data from the Tnni3-Dre;R26-TLR and Tie2-Cre;R26-TLR double-positive line confirmed no cross-talk between these two Dre-rox and Cre-loxP recombinations (Fig. S1, A and B). Together, these data demonstrate that R26-TLR can be used for simultaneous labeling of distinct cell populations.
Figure 1. Generation and characterization of the R26-TLR mouse line. A, schematic showing the knock-in strategy of R26-TLR by homologous recombination.

B and C, schematics showing the results after Dre-rox or Cre-loxP recombination. D, whole-mount bright-field and epifluorescence images of E13.5 embryos of R26-TLR, CAG-Dre;R26-TLR, CAG-Dre;ACTB-Cre;R26-TLR, and E15.5 embryos of ACTB-Cre;R26-TLR. E, immunostaining for ZsGreen and tdTomato on the indicated embryos sections in D. Yellow scale bars = 1 mm; white scale bars = 100 μm. Each image is representative of five individual samples.
Figure 2. Simultaneous labeling and tracing of cardiomyocytes and endothelial cells in Tnni3-Dre;Tie2-Cre;R26-TLR mice. A and B, schematics showing the results after Dre-rox and Cre-loxP recombination in Tnni3-Dre;Tie2-Cre;R26-TLR mice. C, schematic showing the time point for tissue analysis. D, whole-mount bright-field and epifluorescence images of E16.5 embryos of Tnni3-Dre;R26-TLR, Tie2-Cre;R26-TLR, and Tnni3-Dre;Tie2-Cre;R26-TLR. E, immunostaining for ZsGreen, tdTomato, and TNNI3 on E16.5 Tnni3-Dre;Tie2-Cre;R26-TLR heart sections shows that TNNI3−/− cardiomyocytes are ZsGreen−/− tdTomato−/−. F, quantification of the percentage of TNNI3−/− cardiomyocytes labeled by Tnni3-Dre;Tie2-Cre;R26-TLR. Data are mean ± S.D., *, p < 0.001 by two-tailed t test. G, immunostaining for ZsGreen, tdTomato, and PECAM on E16.5 Tnni3-Dre;Tie2-Cre;R26-TLR heat sections shows that PECAM−/− endothelial cells are tdTomato−/− ZsGreen−. H, quantification of the percentage of PECAM−/− endothelial cells labeled by Tnni3-Dre;Tie2-Cre;R26-TLR. Data are mean ± S.D., *, p < 0.001 by two-tailed t test. LV, left ventricle; RV, right ventricle; VS, ventricular septum. Yellow scale bars = 1 mm; white scale bars = 100 μm. Each image is representative of five individual samples.
Triple-cell lineage tracing

Genetic tracing of diverse epithelial cell types simultaneously in the lungs

The lungs are a multifunctional organ consisting of pulmonary vasculature and a respiratory epithelial system that are essential for mammalian survival (36). Multiple stem/progenitor cells participate in the maintenance of lung function during homeostasis and repair (37). In the respiratory system, diverse resident epithelial stem cell populations have been identified from the proximal to the distal airway, which consists of four regions: the trachea, bronchi, bronchioles, and alveoli. The tracheal and bronchial epithelia predominantly consist of basal cells, club cells, ciliated cells, and goblet cells, whereas bronchioles mainly contain ciliated cells, club cells, and neuroendocrine cells (38). The alveolar epithelium includes squamous alveolar type I (AT1) and cuboidal alveolar type II (AT2) cells. These types of epithelial cells are vital for maintenance of the respiratory tract, and they are maintained by self-renewal and differentiation from stem cells (37, 39), e.g. club cells give rise to ciliated cells, and AT2 cells generate AT1 cells. Notably, a multipotent stem cell population has been reported to reside at bronchioalveolar duct junctions (BADJs); these BASCs coexpress the club cell maker CC10 and the AT2 cell maker SPC (40). In vivo genetic fate mapping studies have recently demonstrated that CC10+ SPC+ BASCs can proliferate and differentiate into bronchiole epithelial club cells and ciliated cells and also alveolar epithelial AT1 and AT2 cells in distinct lung injury models (15, 16).

To understand the contribution of club cells, AT2 cells, and BASCs to lung repair in the same context, we employed R26-TLR to simultaneously label CC10+ club cells, SPC+ AT2 cells, and CC10+ SPC+ BASCs in one mouse. We crossed R26-TLR with Sftpc-DreER and Scgb1a1-CreER lines to generate the Sftpc-DreER;Scgb1a1-CreER;R26-TLR triple-positive line. We expected tamoxifen treatment to induce ZsGreen gene expression in SPC+ AT2 cells by Dre-rox recombination, tdTomato gene expression in CC10+ cell bodies by Cre-loxP recombination, and ZsGreen and tdTomato gene expression in CC10+ SPC+ BASCs by Dre-rox and Cre-loxP recombination (Fig. 3A). We administered tamoxifen to 7-week-old of Sftpc-DreER;Scgb1a1-CreER;R26-TLR triple-positive mice and collected the lungs after 1 week (Fig. 3B). Whole-mount epifluorescence data showed that ZsGreen+ and tdTomato+ signals were detected in triple-positive lungs. Furthermore, immunostaining for ZsGreen, tdTomato, and CC10 or SPC on triple-positive lung sections showed that tdTomato+ cells at bronchioles were CC10+ club cells, ZsGreen+ cells at alveolar region were SPC+ AT2 cells, and ZsGreen+ tdTomato+ cells at BADJs were CC10+ SPC+ BASCs (Fig. 3, D, E, and H). After 1 week of tamoxifen treatment (dose/weight, 0.2 mg/g), 2.94 ± 0.23 ZsGreen+ tdTomato+ cells were detected in each BADJ field of tissue sections (Fig. 3F). Furthermore, 87.00% ± 1.71% of ZsGreen+ tdTomato+ cells were located in BADJ regions, and 12.33% ± 1.26% of ZsGreen+ tdTomato+ cells were located in alveolar regions (Fig. 3G), indicating that the majority of double-positive cells labeled by our system were specific. These data demonstrate that R26-TLR has the capability of labeling three distinct cell populations simultaneously in vivo.

Tracing diverse epithelial cell types simultaneously in lung repair and regeneration

To monitor diverse epithelial cell behaviors labeled by R26-TLR after lung injury, we used two lung injury models: the naphthalene-induced bronchiolar injury model and the bleomycin-induced alveolar injury model. To study the fates of club cells, AT2 cells, and BASCs simultaneously in the bronchiolar injury model, Sftpc-DreER;Scgb1a1-CreER;R26-TLR triple-positive mice were treated with tamoxifen at 7 weeks and then with naphthalene or vehicle (corn oil) 10 days after tamoxifen induction (Fig. 4A). 8 weeks after injury, lung tissues were collected, and sectional immunostaining of ZsGreen, tdTomato, and the club cell maker CC10 or the ciliated cell maker Acetylated tubulin showed that both ZsGreen+ tdTomato+ BASCs and ZsGreen+ tdTomato+ club cells expanded and contributed to bronchiole epithelial cells, including club cells and ciliated cells, for repair of the distal airway (Fig. 4, B, D, and F). The alveolar region was largely undamaged, and we did not detect a significant contribution of ZsGreen+ tdTomato+ AT2 cells to ZsGreen+ tdTomato+ club cells by naphthalene-induced injury (Fig. 4, B and D). Interestingly, ZsGreen+ tdTomato+ BASCs were mainly responsible for repair of more terminal bronchioles. Quantification data showed a significant increase in bronchiolar ZsGreen+ tdTomato+ CC10+ cells (28.92 ± 2.07 naphthalene versus 3.72 ± 0.39 vehicle) and ZsGreen+ tdTomato+ acetylated tubulin+ cells (4.80 ± 0.66 naphthalene versus 0.11 ± 0.07 vehicle) after bronchiolar injury (Fig. 4, C and E).

Next we performed alveolar injury to study the fates of these three types of epithelial cells (Fig. 4G). After bleomycin-induced alveolar injury, ZsGreen+ tdTomato+ BASCs mainly gave rise to SPC+ AT2 cells and T1a+ AT1 cells surrounding BADJ regions (Fig. 4, H and J). Quantification data showed a significant increase in alveolar ZsGreen+ tdTomato+ SPC+ cells (49.60 ± 5.87 bleomycin versus 3.96 ± 0.51 vehicle) and ZsGreen+ tdTomato+ T1a+ cells (26.40 ± 1.97 bleomycin versus 0.09 ± 0.03 vehicle) after alveolar injury (Fig. 4, I and K). Conversely, AT2 cells and AT1 cells far from BADJ regions were derived from ZsGreen+ tdTomato+ AT2 cells (Fig. 4, H, J, and L). The fates of BASCs after distinct injury models were consistent with previous reports (15, 16). Taken together, using lung epithelial cells as an example, the above results demonstrate that R26-TLR can be used to study diverse cell origins and fates during development, disease, and regeneration.

Discussion

In this study, we developed a new genetic tool, R26-TLR, for readout of Cre-loxP and Dre-rox recombination. How different recombination sites are arranged with reporter genes is critical for its use in tracing multiple cell lineages in vivo. The arrangement between recombination sites and reporter genes of R26-TLR is different from all previously reported intersectional dual-reporter systems, such as RC::Fela, R26::FLAP, RC::RLTG, and R26NZG (27–30). The model of previous intersectional dual systems is CAG-site1-stop-site1-site2-reporter1-stop-site2-reporter2. Recombination of recombinase1-site1 results in reporter1 expression. Expression of reporter2 needs recombination of recombinase1-site1 and recombinase2-site2. Because
of the exclusive nature of two reporters in recombinase-targeted cells, reporter2 can only reflect a subpopulation of reporter1-labeled populations (33). The model of our newly generated R26-TLR is CAG-site1-stop-site1-reporter1-CAG-site2-stop-site2-reporter2, which permits parallel expression of reporters by two different recombination events. We previously reported a dual system, R26-NLR, which was generated based on Nigri-nox and Cre-loxP systems (20). Driven by different cell-specific promoters, e.g., promoter A and promoter B, R26-NLR can be applied to label two distinct A−/H11001B+/H11002 and A+/H11002B−/H11001 cell populations simultaneously in the same mouse. However, R26-NLR cannot clearly distinguish the double A+/H11001B+/H11001 cell population from the A+/H11001B−/H11002 or A+/H11002B+/H11001 cell population. In this study, because of the different design strategy, R26-TLR integrates the

Figure 3. Simultaneous labeling of CC10+/H11545 club cells, SPC+/H11545 AT2 cells, and CC10+/H11545 BASCs in Sftpc-DreER;Scgb1a1-CreER;R26-TLR mice. A, schematic showing the strategy for simultaneous labeling of club cells, AT2 cells, and BASCs using Sftpc-DreER;Scgb1a1-CreER;R26-TLR mice. B, schematic showing the experimental strategy. C, whole-mount bright-field and epifluorescence images of lungs collected from Sftpc-DreER;R26-TLR mice, Scgb1a1-CreER;R26-TLR mice, and the club cell maker CC10 on Sftpc-DreER;Scgb1a1-CreER; R26-TLR lung section shows that tdTomato+ cells specifically label CC10+ club cells (white arrowheads). Yellow arrowheads, BASCs. E, immunostaining for ZsGreen, tdTomato, and the club cell maker CC10 on Sftpc-DreER;Scgb1a1-CreER;R26-TLR lung section shows that tdTomato+ cells specifically label CC10+ club cells (white arrowheads). Yellow arrowheads, BASCs. F, quantification of ZsGreen+/H11001 tdTomato+/H11001 cells in each BADJ field. Data are mean ± S.D., *, p < 0.01 by two-tailed t test. G, quantification of the percentage of ZsGreen+/tdTomato+ cells located in BADJ or alveolar regions. Data are mean ± S.D., *, p < 0.01 by two-tailed t test. H, cartoon showing the labeling result after recombination by tamoxifen (Tam). Yellow scale bars 1 mm, white scale bars 100 μm. Each image is representative of five individual samples.
advantages of previously reported intersectional reporters and our previous R26-NLR tool. It can be used to label two distinct cell populations and also to label their intersectional subpopulations. The new dual R26–TLR system could be used to label all three cell populations: A⁻B⁻, A⁻B⁺, and A⁺B⁺ (double-positive population). It is a better system than our previous R26-
triple-positive line to demonstrate that and endothelial cells simultaneously in the heart. In the third we showed that the reaction between the Dre-rox and Cre-loxP systems. Further, these data demonstrate that there is no cross-reaction between the Dre-rox and Cre-loxP systems. Moreover, using the strategy of an insulator to permit parallel expression of two recombination results, we can expand them into three or more and, in theory, provide an approach to genetically study multiple cell lineages simultaneously in vivo.

In summary, our newly generated R26-TLR eases the number of mice crossing of different Cre/Dre reporter lines and permits simultaneous tracing of three distinct cell lineages in vivo. Notably, this mouse line extends the capacity of previously reported dual-recombinase-mediated genetic cell labeling and can be used widely to study the origin and fate of diverse cell populations simultaneously in development, disease, and regeneration.

**Experimental procedures**

**Mice**

All animal procedures were reviewed and approved by the institutional Animal Care and Use Committee of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Experimental procedures were performed strictly within the committee’s guidelines. The ACTB-Cre, CAG-Dre, Tnni3-Dre, Tie2-Cre, Sftpc-DreER, and Sgcb1a1-CreER mice lines were described previously (15, 19, 32, 34, 35). The R26-TLR mouse line was created by knocking the CAG-rox-stop-rox-ZsGreen-WPRE-pA-Frt-Neo-Frt sequence into exon1 of the Rosa26 locus and knocking the insulator-CAG-loxP-stop-loxP-tdTomato-WPRE-pA sequence into exon2 of the Rosa26 gene locus using traditional targeted mutation through homologous recombinant strategies. Stop is a transcriptional stop cassette. rox and loxP are recombinase recognition sites. WPRE is a woodchuck hepatitis virus posttranscriptional regulatory element for enhancing the stability of RNA transcription. Neo is a screening gene in mouse generation. An insulator was used to block the potentially mutual influence between the two recombination events. ZsGreen and tdTomato are two kinds of fluorescent protein genes. The obtained chimeric mouse lines were crossed to C57BL/6 lines for germline transmission. Generation of R26-TLR was supported by Shanghai Model Organisms Center, Inc. All mice used in this study were kept at C57BL6 backgrounds. The dosage of tamoxifen (Sigma, T5648) treatment was 0.2 mg/g by oral gavage.
Triple-cell lineage tracing

Genomic PCR

Genomic DNA was extracted from mouse tails. Briefly, the tail tissues were lysed in the lysis buffer for 8 h at 55 °C, and then the mixture was centrifuged at 15,000 rpm for 5 min to obtain a supernatant solution of genomic DNA. Next we used isopropanol to precipitate the DNA and then washed the DNA in 70% ethanol by centrifugation at 15,000 rpm for 3 min. Finally, the DNA was dissolved in double-distilled H2O.

Tissue collection and immunofluorescent staining

The immunostaining protocol was performed as described previously (20). Briefly, the tissues were collected after euthanasia of the mice and then fixed in 4% paraformaldehyde (Sigma) for 30–60 min at 4 °C according to tissue size. The tissues were washed three times in PBS, and then the tissues were dehydrated at 30% sucrose (dissolved in PBS) at 4 °C. After the tissues sank to the bottom of the solution, they were embedded in optimum cutting temperature (Sakura) and stored at −80 °C. 10-μm frozen sections were collected on slides. For immunofluorescent staining, the tissue sections were first dried at room temperature and then placed in PBS to remove optimum cutting temperature. Next, the tissue sections were blocked in 5% PBSST (5% donkey serum in PBS and 0.1% Triton X-100 in PBS) for 30 min at room temperature. Then the blocking buffer was discarded, and tissue sections were incubated with primary antibodies at 4 °C overnight in the dark. The following primary antibodies and dilutions were used: PECAM (BD Biosciences, 553370, 1:500), ZsGreen (Clontech, 632474, 1:1000), tdTomato (Rockland, 600-401-379, 1:1000), CC10 (Santa Cruz Biotechnology, SC-9772, 1:200), acetylated tubulin (Abcam, ab56357, 1:200), and T1a (DSHB, 8.1.1, 1:200). The next day, the slides were washed in PBS to remove primary antibodies and then incubated with secondary antibodies for 40 min at room temperature in the dark. Next, the slides were washed in PBS several times. Then the slides were incubated with 4′,6-diamidino-2-phenylindole (Vector Laboratories) and mounted with mounting medium. The secondary antibodies were Alexa donkey anti-rabbit 555 (Invitrogen, A31572, 1:1000), donkey anti-rat 647 (Abcam, ab150155, 1:1000) Alexa donkey anti-mouse 647 (Invitrogen, A31571, 1:1000), biotin-sp-a-rabbit IgG (JIR, 711-065-152, 1:200), Biotin-sp-a-goat IgG (JIR, 705-065-147, 1:200), donkey anti-goat IgG (Invitrogen, A21447, 1:1000), biotin-sp-goat anti-Syrian hamster IgG(H+L) (JIR, 107-065-142, 1:200), Dylight 647-streptavidin (JIR, 016-490-084, 1:200), and Immpress goat-anti rat (Vector Laboratories, MP-7444, 1:3). An Olympus confocal microscope (FV1200) was used to acquire immunostaining images. Images were analyzed using ImageJ (National Institutes of Health) software.

Bronchiolar injury

Bronchiolar injury was achieved as described previously (15). Naphthalene (Sigma, 84679) was dissolved in sterile corn oil (25 mg ml−1). For bronchiolar injury, Sftpce-DreER;Scgb1a1-CreER;R26-TLR mice were treated with tamoxifen at 7 weeks and then with 250 mg kg−1 naphthalene or vehicle (corn oil) by naphthalene intraperitoneal injection after 10 days. The lung tissues were collected after 8 weeks of recovery. For analysis, fluorescence+ CC10+ club cell number or fluorescence+ acetylated-tubulin+ ciliated cell number was quantified at BADJ field.

Alveolar injury

Alveolar injury was induced by bleomycin as described previously (15). Bleomycin (Sigma, B8416) was freshly dissolved in PBS (10 units ml−1) and stored at −80 °C. 10 units ml−1 bleomycin was diluted to 1 unit ml−1 with PBS before use. For alveolar injuries, Sftpce-DreER;Scgb1a1-CreER;R26-TLR mice were treated with tamoxifen at 7 weeks and then with 2 units kg−1 bleomycin or vehicle (PBS) by intratracheal instillation after 10 days. The lungs were collected after 8 weeks. For analysis, fluorescence+ SPC+ AT2 cells or fluorescence+ T1a+ AT1 cells were quantified at BADJ fields.

Statistical analysis

The data were acquired from five independent experiments and are presented as mean values ± S.D. Two-sided unpaired Student’s t test was used to compare the difference between two groups. p < 0.001 was considered statistically significant.

Author contributions—K. L., Q. L., L. X., X. Y., Y. L., L. Z., J. T., Z. L., H. W., H. Ji, and B. Z. resources; K. L. and M. T. data curation; K. L. and B. Z. software; K. L., M. T., and B. Z. formal analysis; K. L., H. Jin, L. H., H. Z., W. P., and B. Z. methodology; K. L. and B. Z. writing-original draft; K. L., M. T., and B. Z. writing-review and editing; L. H. and B. Z. supervision; B. Z. funding acquisition; B. Z. validation; B. Z. investigation; B. Z. visualization.

Acknowledgments—We thank Shanghai Model Organisms Center, Inc. for mouse generation and Baojin Wu, Guoyuan Chen, Zhonghui Weng, and Aimin Huang for animal husbandry. We also thank Wei Bian for technical assistance and members of National Center for Protein Science Shanghai for assistance with microscopy.

References

1. Chen, Q., Zhang, H., Liu, Y., Adams, S., Eilken, H., Stehling, M., Corada, M., Dejana, E., Zhou, B., and Adams, R. H. (2016) Endothelial cells are progenitors of cardiac pericytes and vascular smooth muscle cells. Nat. Commun. 7, 12422 CrossRef Medline
2. Zhou, B., Ma, Q., Rajagopal, S., Wu, S. M., Domian, I., Rivera-Feliciano, J., Jiang, D., von Gise, A., Ikeda, S., Chien, K. R., and Pu, W. T. (2008) Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454, 109–113 CrossRef Medline
3. Cai, C. L., Martin, J. C., Sun, Y., Cui, L., Wang, L., Ouyang, K., Yang, L., Bu, L., Liang, X., Zhang, X., Stallcup, W. B., Denton, C. P., McCulloch, A., Chen, J., and Evans, S. M. (2008) A myocardial lineage derives from Tbx18 progenitors of cardiac pericytes and vascular smooth muscle cells. Nat. Circ. Res. 116, 515–530 CrossRef Medline
4. Tian, X., Pu, W. T., and Zhou, B. (2015) Cellular origin and developmental program of coronary angiogenesis. Circ. Res. 116, 653–667.e5 CrossRef Medline
5. Lynch, T. J., Anderson, P. J., Rotti, P. G., Tyler, S. R., Croke, A. K., Choi, S. H., Montoro, D. T., Silverman, C. L., Shahin, W., Zhao, R., Jensen-Cody, C. W., Adamcakova-Dodd, A., Evans, T. I. A., Xie, W., Zhang, Y., et al. (2018) Submucosal gland myoepithelial cells are reserve stem cells that can regenerate mouse tracheal epithelium. Cell Stem Cell 22, 653–667.e6 CrossRef Medline
6. Tata, A., Kobayashi, Y., Chow, R. D., Tran, J., Desai, A., Massri, A. J., McCord, T. J., Gunn, M. D., and Tata, P. R. (2018) Myoepithelial cells of submucosal glands can function as reserve stem cells to regenerate airways after injury. Cell Stem Cell 22, 668–683.e6 CrossRef Medline
23. Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre re-
porter strain. Nat. Genet. 21, 70–71 CrossRef Medline

22. Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A.,
and Zeng, H. (2010) A robust and high-throughput Cre reporting and
based labeling to uncover cellular diversity. Development 142, 4385–4393

21. Buckingham, M. E., and Meilhac, S. M. (2011) Tracing cells for tracking
cell lineage and clonal behavior. Dev. Cell 21, 394–409 CrossRef Medline

20. Tian, X., Hu, T., Zhang, H., He, L., Huang, X., Liu, Q., Yu, W., He, L., Yang,
R., Pu, W., Zhang, L., He, L., Zhao, H., Wu, W., He, L., Zhang, Q., Zhang, H., Liu,
Q., Zhang, L., Zhao, H., Tang, J., Ji, H., Cai, D., Han, Z., et al. (2017) Enhancing the precision of genetic lineage tracing using dual recombi-
nases. Nat. Med. 23, 1488–1498 CrossRef Medline

19. Anastassiadis, K., Fu, J., Patsch, C., Hu, S., Weidlich, S., Schuchert, C.,
Guenther, S., Herold, S., Szibor, M., and Braun, T. (2019) Bronchial stem
cells are a main source for regeneration of distal lung epithelia in vivo.
EMBO J. 38, e102099 Medline

18. Rodríguez, C. I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala,
R., Stewart, A. F., and Stewart, A. F. (2009) Dre recombinase,
a dual-recombinase system for time- and host-specific targeting of pancre-
ic cancer. Nat. Med. 15, 123–138 CrossRef Medline

17. Kretzschmar, K., and Watt, F. M. (2012) Lineage tracing. Cell Res. 22,
1157–1177 CrossRef Medline

16. Tan, L., Li, Y., Li, Y., Pu, W., Huang, X., Tian, X., Wang, N., Zhang, H., Liu,
Q., Zhang, L., Zhao, H., Tang, M., Ji, H., Ji, H., Cai, D., Han, Z., et al. (2017) Enhancing the precision of genetic lineage tracing using dual recombi-
nases. Nat. Med. 23, 1488–1498 CrossRef Medline

15. Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson,
J. A., and Yamasaki, M. (2001) Tie2-Cre transgenic mice: a new model
for endothelial cell-lineage analysis in vivo. Development 128, 1340–1347
CrossRef Medline

14. Buckingham, M. E., and Meilhac, S. M. (2003) Cryptic boundaries in roof plate and choroid plexus iden-
tified by intersectional gene activation. Nat. Neurosci. 6, 134–140 CrossRef Medline

13. Hogan, B. L., Barkauskas, C. E., Chapman, H. A., Epstein, J. A., Jain, R.,
Hsing, C. C., Niklason, L., Calle, E., Le, A. R., Randell, S. H., Rock, J., Smito, M.,
Krummel, M., Stripp, B. R., Vu, T., et al. (2014) Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. Cell Stem Cell 4, 525–534 CrossRef Medline

12. Tian, X., Hu, T., Zhang, H., He, L., Huang, X., Liu, Q., Yu, W., He, L., Yang,
R., Pu, W., Zhang, L., He, L., Zhao, H., Wu, W., He, L., Zhang, Q., Zhang, H., Liu,
Q., Zhang, L., Zhao, H., Tang, J., Ji, H., Cai, D., Han, Z., et al. (2017) Enhancing the precision of genetic lineage tracing using dual recombi-
nases. Nat. Med. 23, 1488–1498 CrossRef Medline

11. Liu, Q. Z., Huang, X., Oh, H. J., Lin, R. Z., Duan, S., Yu, Y., Yang, R., Qiu, J.,
Melero-Martín, J. M., Pu, W. T., and Zhou, B. (2014) EpidERM-to-fat
transition in injured heart. Cell Res. 24, 1367–1369 CrossRef Medline

10. Zhang, H., Hu, S., Liu, Q., He, L., Huang, X., Tian, X., Zhang, L., Nie, Y.,
Hu, S., Lui, K. O., and Zhou, B. (2016) Endocardium contributes to cardiac
fat. Circ. Res. 118, 254–265 CrossRef Medline

9. Zhang, H., Hu, S., Liu, Q., He, L., Huang, X., Tian, X., Zhang, L., Nie, Y.,
Hu, S., Lui, K. O., and Zhou, B. (2016) Endocardium contributes to cardiac
fat. Circ. Res. 118, 254–265 CrossRef Medline

8. Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A.,
and Zeng, H. (2010) A robust and high-throughput Cre reporting and
based labeling to uncover cellular diversity. Development 142, 4385–4393

7. Yang, Y., Riccio, P., Schotsaert, M., Mori, M., Lu, J., Lee, D. K., García-
Sastre, A., Xu, J., and Cardoso, W. V. (2018) Spatial-temporal lineage
restrictions of embryonic p63+ progenitors establish distinct stem cell
pools in adult airways. Dev. Cell 44, 752–761.e4 CrossRef Medline

6. Parker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Coizijnsen,
M., Hagebarth, A., Korving, J., Béthel, H., Peters, P. J., and Clevers, H.
(2007) Identification of stem cells in small intestine and colon by marker
gene Lgr5. Nature 449, 1003–1007 CrossRef Medline

5. Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre re-
porter strain. Nat. Genet. 21, 70–71 CrossRef Medline

4. Shin, S. M., Cardoso, W. V., and Muller, D. (2018) Tools for lineage tracing in
mammalian tissue. Annu. Rev. Pathol. 13, 133–140 CrossRef Medline

3. Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre re-
porter strain. Nat. Genet. 21, 70–71 CrossRef Medline

2. Srinivas, S., Watanabe, T., Lin, C.-S., William, C. M., Tanabe, Y., Jessell,
T. M., and Costantini, F. (2001) Cre reporter strains produced by targeted
insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. 1, 4 CrossRef Medline

1. Livet, J., Weissman, T. A., Kang, H., Draft, R. W., Lu, J., Bennis, R. A.,
Sanes, J. R., and Lichtman, J. W. (2007) Transgenic strategies for combi-
natorial expression of fluorescent proteins in the nervous system. Nature
450, 56–62 CrossRef Medline

Triple-cell lineage tracing
of the cervical and anorectal squamocolumnar junctions: a proposed model for anatomical differences in HPV-related cancer risk. *Mod. Pathol.* **28**, 994–1000 CrossRef Medline
44. Ksander, B. R., Kolovou, P. E., Wilson, B. J., Saab, K. R., Guo, Q., Ma, J., McGuire, S. P., Gregory, M. S., Vincent, W. J., Perez, V. L., Cruz-Guilloty, F., Kao, W. W., Call, M. K., Tucker, B. A., Zhan, Q., et al. (2014) ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature* **511**, 353–357 CrossRef Medline
45. Gonzalez, G., Sasamoto, Y., Ksander, B. R., Frank, M. H., and Frank, N. Y. (2018) Limbal stem cells: identity, developmental origin, and therapeutic potential. *Wiley Interdiscip. Rev. Dev. Biol.* 10.1002/wdev.303
46. Rawlins, E. L., Clark, C. P., Xue, Y., and Hogan, B. L. (2009) The Id2+ distal tip lung epithelium contains individual multipotent embryonic progenitor cells. *Development* **136**, 3741–3745 CrossRef Medline
47. Rockich, B. E., Hrycaj, S. M., Shih, H. P., Nagy, M. S., Ferguson, M. A., Kopp, J. L., Sander, M., Wellik, D. M., and Spence, J. R. (2013) Sox9 plays multiple roles in the lung epithelium during branching morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E4456–E4464 CrossRef Medline
48. Nabhan, A. N., Brownfield, D. G., Harbury, P. B., Krasnow, M. A., and Desai, T. J. (2018) Single-cell Wnt signaling niches maintain stemness of alveolar type 2 cells. *Science* **359**, 1118–1123 CrossRef Medline
49. Zacharias, W. J., Frank, D. B., Zepp, J. A., Morley, M. P., Alkhaleel, F. A., Kong, J., Zhou, S., Cantu, E., and Morrisey, E. E. (2018) Regeneration of the lung alveolus by an evolutionarily conserved epithelial progenitor. *Nature* **555**, 251–255 CrossRef Medline
50. Zuo, W., Zhang, T., Wu, D. Z., Guan, S. P., Liew, A. A., Yamamoto, Y., Wang, X., Lim, S. J., Vincent, M., Lessard, M., Crum, C. P., Xian, W., and McKeon, F. (2015) p63+Krt5+ distal airway stem cells are essential for lung regeneration. *Nature* **517**, 616–620 CrossRef Medline
51. Boström, H., Willetts, K., Pekny, M., Levéen, P., Lindahl, P., Hedstrand, H., Pekna, M., Hellström, M., Gebre-Medhin, S., Schalling, M., Nilsson, M., Kurland, S., Törnell, J., Heath, J. K., and Betsholtz, C. (1996) PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* **85**, 863–873 CrossRef Medline
52. Armulik, A., Genové, G., and Betsholtz, C. (2011) Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* **21**, 193–215 CrossRef Medline