Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury

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Broadly, tissue regeneration is achieved in two ways: by proliferation of common differentiated cells and/or by deployment of specialized stem/progenitor cells. Which of these pathways applies is both organ- and injury-specific1–4. Current models in the lung posit that epithelial repair can be attributed to cells expressing mature lineage markers5–8. By contrast, here we define the regenerative role of previously uncharacterized, rare lineage-negative epithelial stem/progenitor (LNEP) cells present within normal distal lung. Quiescent LNEPs activate a ΔNp63 (a p63 splice variant) and cytokertatin 5 remodelling program after influenza or bleomycin injury in mice. Activated cells proliferate and migrate widely to occupy heavily injured areas depleted of mature lineages, at which point they differentiate towards mature epithelium. Lineage tracing revealed scant contribution of pre-existing mature epithelial cells in such repair, whereas orthotopic transplantation of LNEPs, isolated by a definitive surface profile identified through single-cell sequencing, directly demonstrated the proliferative capacity and multipotency of this population. LNEPs require Notch signalling to activate the ΔNp63 and cytokertatin 5 program, and subsequent Notch blockade promotes an alveolar cell fate. Persistent Notch signalling after injury led to parenchymal 'micronuclear honeycombing' (alveolar cysts), indicative of failed regeneration. Lungs from patients with fibrosis show analogous honeycomb cysts with evidence of hyperactive Notch signalling. Our findings indicate that distinct stem/progenitor cell pools repopulate injured tissue depending on the extent of the injury, and the outcomes of regeneration or fibrosis may depend in part on the dynamics of Notch signalling.

Influenza infection challenges pulmonary regenerative capacity owing to the widespread ablation of epithelial cells in substantial areas of lung (Extended Data Fig. 1g, h). A robust expansion of regenerative cytokertatin-5-positive (Krt55) cells in the lung parenchyma after influenza infection has been observed in mice6, which we confirmed (Extended Data Fig. 1). In addition, we directly observed migration (Supplementary Videos 1, 2 and 3) and identified coexpression of integrin α6β4 (Extended Data Figs 1 and 2). These cells also appear variably after bleomycin injury, in which approximately one-third of the Krt55 cells resolved into type II pneumocytes by 50 days after injury (Extended Data Fig. 3). A cellular origin and mechanistic framework for expansion after influenza, and potential parallels in human lung injury, remain unknown.

To define the cell of origin, we lineage-traced mature cell types implicated in epithelial repair. Krt55 cells appearing by day 11 after influenza infection were essentially completely untraced using Clara cell-specific protein (CC10) and surfactant protein C (SPC) Cre-recombinase drivers (CC10-CreERT2 and SPC-CreERT2, respectively, containing tamoxifen-inducible Cre-modified oestrogen receptor fusion proteins) (Fig. 1b, e and Extended Data Fig. 1). Analysis at 7–8 days after injury confirmed mutual exclusivity of CC10-CreERT2-labelled and Krt55 cells (Fig. 1b). Conflicting results in other reports are probably caused by tamoxifen persistence (Supplementary Discussion and Extended Data Fig. 4).

A small fraction (13%) of expanded Krt55 cells bear the Krt5-CreERT2 lineage label (Fig. 1f, g), raising the possibility that tracheal basal cells might migrate distally during injury. We transplanted sections of fluorescent trachea into syngeneic animals and a non-fluorescent left lung into a fluorescent mouse7. Abundant Krt55 cells arose after infection but none were fluorescent (Fig. 1h and Extended Data Fig. 1j, k). Upper-airway basal cells therefore do not contribute to this phenomenon and instead implicate a lineage-negative epithelial progenitor (LNEP) as the major source of ΔNp63+ and Krt55 cells.

To characterize quiescent LNEPs, we used integrin β4 expression in CC10-CreERT2 mice to segregate LNEPs from club cells in uninjured lungs (Fig. 2a) and confirmed minimal expression of mature lineage markers (Extended Data Fig. 5c). The CC10− β4− (LNEP-containing) population uniquely expressed ΔNp63 (Extended Data Fig. 5c). ΔNp63+ cells were identified in situ scattered sporadically throughout distal airways (Fig. 2c). These cells did not express detectable Krt5 protein (Extended Data Fig. 5a). In a total of 65 small airways examined in two mice, we identified 24 ΔNp63+ cells. Only 7 of the 24 cells were labelled in Krt5-CreERT2 mice (Fig. 2c and Extended Data Fig. 5a), probably explaining the small fraction of post-injury Krt55 cells bearing the Krt5-CreERT2 lineage label (Fig. 1f, g).

Given the infrequency of ΔNp63+ cells, we suspected progenitor activity of the CC10− β4− population might be restricted to a smaller subset. Immunostaining revealed multicilia in 78% of this population, whereas ΔNp63+ cells were less than 1% (Extended Data Fig. 5b). To address this heterogeneity, we performed single-cell RNA sequencing (RNA-seq) on CC10− β4− cells and on rare Krt5-CreERT2-labelled cells, a subset of this population (Fig. 2i). The ΔNp63 transcript was detected in several cells in the CC10− β4− population (Fig. 2b, red circle, far left) as well as the Krt5-traced cells (Fig. 2b, green triangle). Analysis of variance (ANOVA) comparison between putative LNEPs (Krt5-traced cells combined with all p63-expressing cells) and the remaining cells revealed enrichment of ~900 genes (>2-fold change, >1 FPKM, P ≤ 0.05) in the LNEP group (Supplementary Data 1). We note enrichment for pluripotency-associated transcription factors (Myc and Klf4) in the LNEP group (Fig. 2b), while many genes enriched in the remaining cells (Fig. 2b, top row, Supplementary Data 2) are known markers of ciliated cells10. Surprisingly, ΔNp63+ CC10− β4− cells most closely related to the Krt5-traced cells also expressed cilia-associated genes (Fig. 2b, denoted by asterisk). Cytospins of CC10− β4− cells revealed primary cilia on ΔNp63+ cells and additional cells without discernible ΔNp63 (Fig. 2c, right), indicating that the LNEP profile extends to a larger fraction of ΔNp63-low or -negative cells.

To assess the potential of LNEPs in vivo, we devised a transplantation assay by which ~105 fluorescent CC10− β4− cells were delivered orthotopically into influenza-injured mice (Fig. 2d). Seeded LNEPs developed into multicellular structures in two patterns seemingly dependent on location: areas of type II cells that were virtually indistinguishable.
from surrounding endogenous type II cells (Extended Data Fig. 6a, b, h), and engraftments expressing Krt5 (Extended Data Fig. 6a, c) and CC10 (Extended Data Fig. 6g) near endogenous Krt5<sup>−</sup> CC10<sup>−</sup> structures, β4<sup>−</sup> type II cells engrafted infrequently in small clusters (≤8 cells), and expressed only alveolar markers (Extended Data Fig. 6i). CC10<sup>−</sup> cells could engraft but exhibited scant differentiation, even losing CC10 expression (Extended Data Fig. 6j, k). Transplantation of multi-ciliated cells resulted in only occasional persistence of single cells without structures (Extended Data Fig. 6l), consistent with their lack of progenitor properties<sup>11,12</sup>.

Transplantation of mixed enhanced green fluorescent protein (eGFP)-labelled and tdTomato-expressing LNEPs demonstrated engraftments to be largely non-overlapping (Fig. 2e) and highly proliferative (Extended Data Fig. 6e), arguing for near-clonal expansion. Although mature type II cells do not express integrin β4 (ref. 13), clones derived from donor LNEPs exhibited β4 and SPC co-expression 5 days after transplant (Extended Data Fig. 6e), confirming their LNEP origin. These data demonstrate multipotency of LNEPs as well as the viability of orthotopic cell transplantation as a functional tool.

We interrogated the RNA-seq analysis and identified enrichment for CD14 in ΔNp63<sup>+</sup> CC10<sup>−</sup> β4<sup>−</sup> cells (Fig. 2b, asterisk). In combination with CD200, which further selects against multi-ciliated cells (Extended Data Fig. 5e), CD14<sup>−</sup> cells were isolated and transplanted. β4<sup>−</sup> CD200<sup>−</sup> CD14<sup>−</sup> cells (~3,000) (Fig. 2f) phenocopied the larger (150,000) CC10<sup>−</sup> β4<sup>−</sup> population (Fig. 2g, h and Extended Data Fig. 7a–c), validating this small population as the active LNEPs. Using a complementary approach, distal Krt5-CreERT2-labelled ΔNp63<sup>+</sup> cells within the LNEP fraction were transplanted (1,000 cells per mouse) (Fig. 2i). Multipotency was again observed, although we noted many fewer SPC-expressing cells (Fig. 2j, k and Extended Data Fig. 7d, e). We posited that isolation using Krt5-driven Cre enriches for LNEPs that have undergone partial commitment to the Krt5 program, whereas surface-marker-based selection represents a less biased approach. This is consistent with lineage analysis (Fig. 1h) indicating Krt5-CreERT2-traced cells can only account for a small fraction of the Krt5<sup>−</sup> expansion.

Accordingly, LNEPs cultured<sup>ex vivo</sup> did not express Krt5 even when treated with various trophic/morphogenic factors (Supplementary Table 2). However, bronchoalveolar lavage fluid (BALF) from injured mice induced marked proliferation and Krt5 expression. A total of 77 ± 13% (mean ± s.d.) of colonies treated with the BALF stained positive for Krt5 (Fig. 3a–d), whereas type II cells treated with the same BALF did not respond.

Although the active principle(s) in injury BALF is uncertain, a screen of pathway inhibitors implicated a critical role of Notch. The γ-secretase inhibitor DAPT in conjunction with active BALF attenuated intensity to < 21-day chase (Fig. 3e, f). Notch activity was further validated using a Notch reporter mouse (Cp-eGFP) (Extended Data Fig. 8a). When DAPT was administered to mice after influenza, the fraction of lung area bearing Krt5<sup>−</sup> cells by day 11 was markedly reduced (Fig. 3g and Extended Data Fig. 7h).

During development, Notch signalling is known to suppress alveolar differentiation in both the lung and the mammary gland<sup>14,15</sup>. When LNEPs were cultured in the presence of γ-secretase inhibitors, we observed strong induction of SPC expression, further promoted by 3-isobutyl-1-methylxanthine (IBMX)<sup>16</sup> (Fig. 3h, i). Therefore, persistent Notch signalling prevents alveolar differentiation, whereas removal of this signal promotes maturation towards type II cells. This result proved...
relevant to the long-term outcome of regeneration in the influenza injury model.

Although regions of relatively normal histology bearing the Krt5-CreERT2 trace develop after resolution of bleomycin injury (Extended Data Fig. 3e), we were surprised to find few traced SPC+ type II cells after influenza (Extended Data Fig. 8e). Instead, large regions of Krt5-CreERT2-traced cells (triangles) (columns). Listed genes (rows) were selected from >1,200 differentially expressed genes identified by ANOVA. Immunofluorescent staining for ΔNp63 in uninjured lungs from Krt5-CreERT2 (tdTomato+) mice. Single cells from cytopsins of the CC10− β4+ population demonstrate primary cilium (green) in a subset of non-multiciliated cells (right).

Figure 2 | Isolation and transplantation of a lineage-negative distal epithelial population. a, FACS segregation of epithelial (EpCAM+) cells by β4 expression and a CC10-CreERT2 lineage tag (GFP), demonstrating a β4+ population distinct from club cells. b, Hierarchical clustering/heat map of RNA-seq transcriptomes from single CC10− β4+ cells (circles) and distal Krt5-CreERT2-traced cells (triangles) (columns). Listed genes (rows) were selected from >1,200 differentially expressed genes identified by ANOVA. Immunofluorescent staining for ΔNp63 in uninjured lungs from Krt5-CreERT2-labelled LNEPs also differentiate into SPC+ (g) and Krt5+ (h) cells, representative of n = 3 transplants. i–k, FACS isolation and transplantation of Krt5-CreERT2-labelled LNEPs also differentiate into SPC+ (j) and Krt5+ (k) cells, representative of n = 2 transplants. Scale bars, 20 μm (c, left, g, h, j, k), 10 μm (c, right) and 100 μm (e). Notch antagonism in vivo via intranasal delivery of dibenzazepine (DBZ; in conjunction with dexamethasone and IBMX) resulted in a significant increase in the number of cyst-derived SPC+ cells (12.3% versus 1.6%) (Fig. 3j–l).

Persistent cysts bear a strong resemblance to micro-honeycombing in the lungs of patients with idiopathic pulmonary fibrosis (IPF) (Fig. 4d, k). These lungs (n = 10) showed almost all cystic epithelia were comprised of KRT5+ cells surrounded by either additional metaplastic KRT5+ cells or pseudostratified epithelium with ectopic but otherwise typical basal cells17. Distinct foci of hyperplastic SPC+ cells were also present. Notch activity correlated with KRT5+ cysts but was absent in most hyperplastic SPC+ cells (Fig. 4d–f and Extended Data Fig. 9a–d) and in normal alveolar regions (Extended Data Fig. 9k).

In lungs from patients with scleroderma (n = 7), fibrotic areas displayed the IPF pattern of persistent KRT5+ and HESt-1 cystic structures (Extended Data Fig. 9e, g, h). However, in three less-fibrotic specimens, we observed extensive double-positive KRT5+ and SPC+ cells lining...
alveoli (Fig. 4g and Extended Data Fig. 9i). Although the origin of this KRT5+ expansion is uncertain in humans, we note ΔNp63− KRT5+ cells in normal terminal airways (Extended Data Fig. 9i), analogous to LNEPs in mice.

These experiments identify a rare, undifferentiated epithelial population that is the major responder in distal lung after severe damage (Extended Data Fig. 10a). Notch signalling modulates the quiescence, activation and differentiation state of murine LNEPs (Extended Data Fig. 10b), providing a signalling model to frame the dynamic aspects of LNEP function. The persistently abnormal parenchymal structures that derive from LNEPs after influenza infection represent a failed regenerative process, promoted at least in part by ongoing Notch activity. The striking parallels to the currently inexplicable micro-honeycombing

Figure 4 | Persistent Notch activity promotes cystic honeycombing in both mouse and human. a, Krt5-CreERT2-traced (tdTomato+) cells develop into cystic structures at late time points after influenza. b, Cyst cells demonstrate nuclear expression of Hes1 indicative of persistent Notch signalling. c, Lung from patient with IPF bearing honeycomb cysts with mutually exclusive KRT5+ and SPC+ cells. d, IPF honeycomb cysts with nuclear HES1 in KRT5+ cells and surrounding epithelium, similar to mouse (b). e, SPC+ type II cells in hyperplastic foci infrequently express HES1, quantified in f (n = 8 patients, mean ± s.d.). g, Scleroderma lung demonstrating sub-pleural KRT5+ and SPC+ cell expansion with many KRT5+ SPC+ double-positive cells (right). Scale bars, 20 μm (a, d, e, g) and 100 μm (c).

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions A.E.V. and H.A.C. designed the study, analysed the data, and wrote the manuscript. A.E.V. performed lineage tracing, flow cytometry purification, and characterization of lung cells; J.E.G. titred PR8 virus and initiated all infections; A.N.B. isolated lung cell suspensions, assisted with flow cytometry, and designed and performed most of the immunostaining; Y.X. assisted with biochemistry, RNA analysis, and immunostaining; K.T. and V.T. managed the mouse genotyping and performed in vivo mouse experiments; V.T. isolated lung cells and designed quantification methods; F.C.L. and M.R.L. performed lung transplantations; M.M. procured and screened human lungs; D.G.B. and B.T. synthesized libraries and provided initial data analysis for RNA-seq experiments J.R.R. provided key reagents and assisted with study design.

Author Information The FPKM files from single-cell RNA-seq experiments have been deposited in Gene Expression Omnibus (GEO) under accession GSE61300. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.E.V. (andrew.vaughan@ucsf.edu) or H.A.C. (hal.chapman@ucsf.edu).
METHODS

Animals. SPC-CreERT2 (5FpTm1(cml cre)ERT2;Hap), Kr5-CreERT2 (Kr51 homozygous), CC10-CreERT2 (Sgob1 homozygous), FoxJ1-CreERT2 (Tg(FosFX-crel cre)ERT2;Bb), and Cp-eGFP (Tg(Cp-EFGP)25Gaia) mice are previously described1,18–20. All of these strains were bred to either mTmG (Gt(Rosa)26Sec14(CAG-tdTomato)Fuke) or Ai14-tdTomato (Gt(Rosa)26Sec14(CAG-tdTomato)Fuke) mice to generate mice expressing a fluorophore in Cre-expressing cells. SPC-CreERT2, Kr5-CreERT2 and CC10-CreERT2 mice were developed in a 129 background and backcrossed to C57BL6 for at least three generations. For transplant experiments, mTmG and/or Ub-GFP (Tg(UBC-GFP)36Scha) were used for donor cells. For all experiments, 6–8-week-old animals of both sexes were used in equal proportions. Investigators were not blinded to mouse identity. All studies were approved by University of California, San Francisco (UCSF) Institutional Animal Care and Use Committees (IACUC), protocol AN088356-03. All animal studies used a minimum of three mice per group with the exception of DBZ (see below).

For lineage analysis the cell of origin of Kr5+ cells, mice were administered three doses (Kr5-CreERT2) or five doses (SPC-CreERT2 and CC10-CreERT2) of 0.25 mg 1 body weight tamoxifen in 50 μl corn oil. A chase period of >21 days was used to insure the absence of residual tamoxifen before injury.

Injury (influenza, bleomycin). Mice were administered 200 focus-forming units (FFU) of influenza A/H1N1/Puerto Rico/8/34 (PR8) intranasally. PR8 virus dissolved in 30 μl PBS was pipetted onto the nostrils of heavily anaesthetized mice (visual confirmation of agonal breathing), whereupon mice aspirated the fluid directly into their lungs. The mice were allowed to recover and weighed twice a week. For experiments analysing the lineage fate of Krt5+ cells, a single dose of 0.125 mg 1 body weight tamoxifen was administered at day 10 after PR8 infection.

Infective viral particles were assayed by inoculation of either stock virus or homogenate (in 1 ml PBS) of left lung, spleen and brain onto 96-well plates of confluent MDCK cells. After 1 h, samples were decanted and replaced with serum-free media containing modified trypsin (L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated) at 100 μg ml -1 sodium borohydride (PFA) and fixed for 1 h at room temperature and subsequently embedded in OCT. Sections (7 μm) were cut on a cryostat, with fresh-frozen tissue immediately fixed for 5 min in 4% paraformaldehyde (PFA) and fixed for 1 h at room temperature and subsequently embedded in OCT. Sections (7 μm) were cut on a cryostat, with fresh-frozen tissue immediately fixed for 5 min in 4% paraformaldehyde at room temperature. All sections were subsequently incubated for 3 × 10-3 min intervals with 1 μg ml -1 sodium borohydride (Sigma) in PBS to reduce aldehyde-induced background fluorescence. Slides were subsequently blocked ≥1 h in PBS plus 1% bovine serum albumin (Affymetrix), 5% nonimmune serum (UCSF Cell Culture Facility), 0.1% Triton X-100 (Sigma) and 0.02% sodium azide (Sigma). Slides were incubated overnight in primary antibodies listed below, diluted in block solution. Slides were washed three times with PBS plus 0.1% Tween 20, and incubated with secondary antibodies (typically Alexa Fluor conjugates, Life Sciences) at a 1:2,000 dilution ≥1 h. Finally, slides were again washed, incubated with 1 μM DAPI for 5 min, and mounted using Prolong Gold (Life Sciences).

The following antibodies were used: rabbit anti-proSPC (1:3,000; Millipore, AB3786), goat anti-proSPC (1:2,000; Santa Cruz, M-20), goat anti-CC10 (1:10,000, a gift from B. Stripp), rabbit anti-Krt5 (1:1,000; Covance, PRB-160P), chicken anti-Krt5 (1:1,000; Covance, SIG-3475), rabbit anti-ANP3d (1:100; Biolegend, POLY6190), rat anti-CD45 (1:200, BD 30-F11), sheep anti-eGFP (1:500; Pierce, 10396164), rabbit anti-phospho histone H3 (1:500; Millipore, 06-570), rabbit anti-Hes1 (1:1,000; Cell Signaling, D6P2U), rabbit anti-activated Notch1 (1:1,000; Abcam, ab8925), mouse anti-acetylated tubulin (1:500, Sigma, 6-11B-1).

Quantification of lineage tracing. Samples were prepared for immunofluorescence staining. Quantification at day 11 after influenza is the result of counting >2,900 cells (CC10 trace), >4,000 cells (SPC trace), or >1,300 (Krt5 trace) from at least three mice per genotype. Cells were counted from over five sections per mouse and included at least three individual lobes. Mutual exclusivity of CC10-traced and Krt5+ cells at days 7–8 was determined with a smaller sample size, n = 2 mice, 12 Krt5+ airways, >500 cells examined. Only mice possessing the appropriate genotype were included in studies.

Epithelial cell isolation and flow cytometry. Lung epithelial cells were isolated in previously described1, with the following modifications. After installation with agarose and subsequent hardening by a brief incubation on ice, each lobe was cut away from the mainstem bronchi. The proximal-most quarter of each lobe surrounding the bronchi was then cut away to minimize the inclusion of basal cells in the cell preparation, and the previous protocol was followed from this point on. For FACS analysis, single-cell preparations were incubated for 30–45 min at 4 °C with the following primary antibodies: phycoerythrin (PE), Alexa Fluor 488, or BV421-conjugated rat anti-mouse EpCAM (1:500; Biolegend, G8.8), Alexa Fluor 647 or PE-conjugated rat anti-mouse integrin β1 (1:75; BD, 450-9D), Alexa 647-conjugated CD200 (1:100, Biolegend, Ox-90), and PE/Cy7-conjugated CD14 (1:100, Biolegend, Sa14-2). Antibody incubations were done in DMEM (without phenol red) plus 2% FBS, and cells were washed twice with PBS after antibody incubations. Sorting and analysis was performed on BD FACS Aria cytometers.

Orthotopic cell transplantation. Recipient C57BL/6 mice were infected with PR8 (see Animals). At 9 days after infection, donor cells were sorted from mTmG or Ub-GFP mice (Animals) and reseeded in 50 μl sterile PBS. Recipient mice received cell solution intranasally as described above for influenza administration. The total number of β+ cells ranged from 150,000 to 350,000 per transplant (n = 6), and equivalent numbers of β+ cells were always transplanted into injured littermates for comparison. For transplantation of Kr5-CreERT2-labelled cells, 1,000 cells were transplanted per recipient (n = 2). For β-CD14+ 200 cell transplants, 3,000–10,000 cells were transplanted per mouse (n = 3). FoxJ1-CreERT2-labelled or CC10-CreERT2-labelled cell transplants were performed in n = 3 or 4 mice each, respectively (1 × 103 to 3 × 103 cells per mouse). Endpoint analysis was performed at day 21 after infection unless otherwise noted. For analysis of proliferation, recipient mice were administered 50 mg kg -1 body weight Edu (Santa Cruz) in PBS daily. Edu was detected with Click-iT Edu Alexa Fluor 488 Imaging Kit (Invitrogen).

Primary culture. Isolated primary lung epithelial cells were plated and cultured on Matrigel as follows. Eight-well chamber slides were coated with 150 μl Matrigel per well, allowed to solidify at 37 °C, and then equilibrated with SABM (Lonza) for at least 30 min before cell plating. A total of 15,000–40,000 cells were plated in each well of and maintained in ‘baseline’ media consisting of SAGM (Lonza) supplemented with 5% charcoal-stripped FBS and 10 ng ml -1 KGF (FGF-7, Peprotech). Other growth factors were included in the media only when indicated and are summarized in Supplementary Table 6.

BALF was collected from injured animals for cell culture as follows. Euthanized mice were intratracheally intubated before cardiac perfusion and 1 ml of baseline media was lavaged. The lungs were repeatedly lavaged with the media at least three times. BALF was then centrifuged three times for 5-min spins at 1,500g to remove the cells and other debris. Clarified BALF was then filtered through a 0.25-μm Spin-X filter (Sigma) to remove any additional debris and to ensure a cell-free preparation. BALF prepared in this way was either added to cells immediately or frozen in aliquots at −80 °C and added to cultured cells without dilution.

Long-term cell culture. Cells isolated as above were maintained in SAGM as above, with the addition of 10 μM Y-27632 (Sigma) and 50 ng ml -1 murine noggin (PeproTech). Cells were passaged every 7–10 days by initial incubation with 25 μl ml -1 dispase at 37 °C for 20 min to allow colonies. Single-cell dissociation was performed by additional 10-min incubation with 2 μM EDTA in PBS in combination with mechanical disaggregation by pipetting.

γ-secretase treatment of LNEPs in vitro. LNEPs maintained as above were dissociated and re-plated directly into SAGM baseline media with added DAPT or GSI-X (Calbiochem) at 40 or 20 μM concentrations (unless otherwise indicated).
For SPC induction experiments, IBMX was added when indicated. LNEPs were cultured for 7–10 days and then analysed by immunofluorescent staining.

**Immunofluorescence analysis of cultured cells.** Cells grown on matrigel were fixed for 5–10 min in IHC Zinc Fixative (BD) and subsequently stained as indicated above, except that all staining solutions were prepared with TBS as the zinc fixative and run on an Eppendorf Realplex2 thermocycler. Primer sequences are as listed:

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reverse 5'-GAGAATGGAAAA-3'
forward, 5'-ACTTTGAAGTA-3'
reverse, 5'-ATAACCAGACTCT-3'
forward, 5'-SPC (also known as Jag2), forward, 5'-Krt5-CreERT2/tdTomato mice were administered 280 FFU of 1 KGF during imaging with the addition of 500nM hydro-
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**Live slice imaging.** Krt5-CreERT2/tdTomato mice were administered 280 FFU PR8 (as above) and received a single 0.25 mg·kg⁻¹ dose of tamoxifen 24 h before culling at the indicated time points. Injured mice were euthanized and perfused and lavaged with PBS. Lungs were instilled with 2% low-melting point agarose and ~300-μm slices were prepared on a vibratome. Lung slices were maintained in SAGM plus 10 ng ml⁻¹ KGF during imaging with the addition of 500nM hydroxytamoxifen (Sigma) to induce recombination in all Krt5-expressing cells. Slices were imaged continuously for 12 h in a 37°C chamber on an inverted stage with a Leica SP5 confocal microscope. Images obtained were deconvoluted with Bitplane Imaris for presentation.

**Quantitative reverse transcriptase PCR.** RNA was isolated from sorted cells using the Promega RNA Rliprep kit. cDNA was synthesized and amplified using the Ovation PicoSL WTA V2 kit (NuGen). Reverse transcription PCR (RT–PCR) reactions were performed using Faststart Universal SYBR green Master Mix (Roche) and variance was analysed at the time of manuscript but is available upon request. No statistical method was used to pre-
determine sample size.

For calculations involving single cell RNA-seq, Fluidigm Singular software running in R was used. All other statistical calculations were performed using Graphpad Prism. P values were calculated from two-tailed t-tests (paired or unpaired depending on experimental design) or ANOVA for multivariate comparisons. Variance was analysed at the time of t-test analysis. This data are not included in the manuscript but is available upon request. No statistical method was used to pre-
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Extended Data Figure 1 | Characterization of influenza-induced Krt5\(^{+}\) cells.  

**a–c**, Alveolar (a, b) and airway (c) Krt5\(^{+}\) cells strongly express \(\beta_4\) after influenza injury.  

**d**, FACS plot of epithelial (EpCAM\(^{+}\)) cells from tamoxifen-treated Krt5-CreERT2/tdTomato mice at day 15 after influenza, demonstrating \(\beta_4\) expression in nearly all traced (tdTomato\(^{+}\)) cells.  

**e, f**, Most Krt5\(^{+}\) cells co-express \(\Delta N p63\) (e) and Krt14 (f).  

**g, h**, Expanded Krt5\(^{+}\) cells are invariably associated with abundant CD45\(^{+}\) inflammatory cells (g) and few if any remaining normal E-cadherin\(^{+}\) epithelial cells other than the Krt5\(^{+}\) cells themselves (h).  

**i**, Krt5\(^{+}\) cells are unlabelled in SPC-CreERT2/mTmG mice. Inset in i demonstrates appropriate labelling of type II cells in an uninjured region of the same lung.  

**j, k**, Krt5\(^{+}\) cells are not fluorescent after trachea transplantation from tdTomato donor. Basal cells in transplanted section of trachea retained fluorescence (j, inset in k). Scale bars, 100 \(\mu\)m (a) and 20 \(\mu\)m (b, c, e–k).
Extended Data Figure 2 | Influenza-induced Krt5\textsuperscript{+} cells arise in both airways and alveoli and migrate across, around and through airway and parenchymal tissue. a, b, Krt5\textsuperscript{+} cells are detected in alveoli as early as day 5 and are found in larger clusters over time. c, d, Krt5\textsuperscript{+} cells similarly arise in airways in greater abundance with time. e, Distinct alveolar and airway expansion is apparent 11 days after infection. f, Freeze-frames of live imaging from a Krt5-CreERT2/tdTomato mouse 11 days after influenza, in which tdTomato\textsuperscript{+} cells migrate from their original location (white box) outward. See Supplementary Video 1. g, Freeze-frames from a small airway in the same mouse; arrow denotes a single cell crossing the basement membrane. See Supplementary Video 2. Scale bars, 20 μm (a, b, g) and 100 μm (c, d, f).
Extended Data Figure 3 | Characterization of bleomycin-induced Krt5<sup>+</sup> cells. a–c, Krt5<sup>+</sup> cells also arise after bleomycin injury and express ΔNp63 (b, c). d, Western blotting demonstrating more pronounced and reproducible Krt5 induction after influenza injury at day 11 than after bleomycin injury at day 17. Each lane was loaded with whole-lung lysate from a single mouse; average percentage lung area corresponding to a band in influenza-injured mice is 3.6 ± 0.5% (n = 13 mice quantified, see Fig. 3g as an example). e, Lineage tracing of bleomycin-injured Krt5-CreERT2 mice reveal traced (tdTomato<sup>+</sup>) type II cells expressing SPC and cells morphologically resembling type I cells. In total, 31% of Krt5-CreERT2 traced cells express SPC by day 50 after bleomycin (n = 3 mice, 264 Krt5-CreERT2-labelled cells counted). Scale bars, 100 μm (a) and 20 μm (b, c, e). Full western blot scan in d is available as Supplementary Fig. 1.
Extended Data Figure 4  | Krt5^{+} cells do not arise from CC10-expressing progenitors but rather upregulate CC10 during expansion.  

a, Krt5^{+} cells express detectable levels of CC10 (top) compared to isotype control (bottom) in alveolar clusters (a).  
b, Representative image of CC10-CreERT2 lineage trace in which waiting only 7 days after tamoxifen administration before influenza injury results in significant labelling of Krt5^{+} cells (quantified in Fig. 1d).  
c, Strong CC10 expression in Krt5-CreERT2-traced (tdTomato^{+}) cells by day 22 after influenza. For comparison, see single channel images (c, right and bottom) of the same region. Scale bars, 20 μm.
Extended Data Figure 5 | Heterogeneity of the LNEP-containing CC10−β4+ population. a, Rare Krt5-CreERT2-traced (tdTomato+) cells were observed in uninjured distal lung airways that lacked Krt5 staining compared to trachea basal cells (inset) in the same section. All distal tdTomato+ cells express ΔNp63 but most ΔNp63+ cells are untraced (see Fig. 2c). b, Cytospins of sorted CC10−β4+ cells reveal the presence of abundant multiciliated cells (green, acetylated tubulin+) and a small fraction of ΔNp63+ cells (red). c, Quantitative reverse transcriptase PCR (qRT−PCR) analysis of mature lineage genes and genes of interest in all populations. n = 3 biological replicates; data are mean ± s.d. d, Principal component analysis plot of cells sequenced in Fig. 2b, demonstrating that p63+ cells in the CC10−β4+ population (outlined, asterisk) cluster with multi-ciliated cells. e, CD200 is not expressed by FoxJ1-CreERT2-labelled multi-ciliated cells, highlighting its use in excluding such cells. f, Cytospin of Foxj1-CreERT2-labelled β4+ cells demonstrating reliable selection for multi-ciliated cells (198 cells quantified). g, Gating on CD14 expression within the EpCAM+β4+CD200− population excludes CC10-expressing club cells. Scale bars, 20 μm.
Extended Data Figure 6 | Orthotopic transplantation of LNEPs reveals their multipotency and differentiation appropriate to the local microenvironment. a, Several distinct areas of LNEP engraftment (red) reflect differentiation in response to location. Left dashed box demonstrates SPC expression in engrafted cells with nearby endogenous SPC-expressing cells (white); far right dashed box demonstrates Krt5 expression in engrafted cells and nearby endogenous Krt5-expressing cells (green). b, c, Cells in regions of SPC\(^+\) differentiation (b) lack Hes1 expression (right), whereas those in areas of Krt5\(^+\) differentiation (c) strongly express Hes1 (right). d, Distinct areas of LNEP engraftment demonstrate an inverse relationship between SPC expression (left) and Hes1 expression (right) in probable single clones. e, Examination of transplanted cells 5 days after engraftment demonstrate abundant Edu incorporation (see Methods) indicative of proliferation. At this time point cells can be identified co-expressing \(\beta_4\) and SPC (right, circled). f, g, Krt5\(^+\) cells and CC10\(^+\) cells were often found clustered in single regions of engraftment. h, Many engrafted cells in Fig. 2e are also SPC positive. i, \(\beta_4\) type II cells engraft in small clusters and only express SPC. j, k, CC10\(^+\) cells engraft but do not express SPC, CC10 or Krt5. l, Multi-ciliated cells engraft but only persist as isolated single cells, losing acetylated tubulin expression. Scale bars, 100 \(\mu\)m (a) and 20 \(\mu\)m (b–l).
Extended Data Figure 7 | Transplantation of β4⁺ CD14⁺ CD200⁺ and Krt5-CreERT2-traced cells recapitulates multipotency of the heterogenous CC10⁺ β4⁺ population. a, Single channels images from Fig. 2h demonstrate Krt5 expression in transplanted β4⁺ CD14⁺ CD200⁺ cells. b, c, Transplanted β4⁺ CD14⁺ CD200⁺ can also differentiate towards type II cells (b) and club cells (c). d, e, Transplantation of rare Krt5-CreERT2-traced cells from uninjured mice resulting in donor-derived Krt5⁺ cell expansion indistinguishable from endogenous expansion. Images in d and e are representative images from four attempted transplants, two of which exhibited engraftment in two or four individual lobes. Scale bars, 20 μm.
Extended Data Figure 8 | Notch activity in normal and injured lung.

a, Uninjured Notch reporter mice (Cp-eGFP) show dim GFP in small airways and no detectable GFP in alveoli. b, Krt5+ cells arising in distal airways express GFP in Notch reporter mice 7 days after influenza infection. c, d, Some Krt5+ cells persist within Krt5-CreERT2-labelled (tdTomato+) cysts (d) long-term (day 88) after influenza injury, and many traced cells express CC10 (c).

e, Cysts rarely contain SPC+ type II cells (arrows). f, g, Hes1 expression is maintained in Krt5-CreERT2-traced (GFP+) cyst cells 98 days after influenza (f) but is absent in normal alveolar parenchyma from the same mice (g).

h, Representative images of Krt5+ cell expansion in vehicle- (left) or DAPT- (right) treated mice at day 11 after influenza, quantified in Fig. 3g. Scale bars, 20 μm (a–g) and 100 μm (h).
Extended Data Figure 9 | IPF and scleroderma lungs both contain HES1$$^+$$ honeycomb cysts, but scleroderma lungs also possess SPC and KRT5 co-expressing cells. Normal human lungs contain putative LNEPs and lack HES1 in alveoli. a–d, Honeycomb cysts in several IPF lungs; many KRT5$$^+$$ cells as well as surrounding cystic epithelium demonstrate strong nuclear HES1 signal. e, Region of scleroderma honeycombing similar to IPF lung. f, Scleroderma subpleural alveolar region with type II cell hyperplasia demonstrating cells co-expressing SPC and KRT5. g, h, Cystic epithelium in scleroderma lungs expresses HES1 as in IPF. i, KRT5$$^+$$ ΔNp63$$^+$$ cells (white outlines) distinct from KRT5$$^+$$ ΔNp63$$^-$$ basal cells (red outlines) are present in distal airways. j, k, HES1 staining is apparent in small airways of normal lung (j) but very low in alveolar parenchyma (k). All images are from patient samples in addition to those shown in Fig. 4. Scale bars, 20 μm (a–d, g–k) and 100 μm (e, f).
Extended Data Figure 10 | Hierarchical cellular responses to injury severity and Notch-regulated LNEP dynamics. a, Distinct epithelial cell types contribute to regeneration depending on the severity of parenchymal injury. Examples of each are referenced. b, Notch signalling regulates the activation, expansion and differentiation of LNEPs. Notch is required for activation and maintenance of LNEPs. Alveolar differentiation requires subsequent loss of Notch activity, whereas persistent Notch results in either airway differentiation or abnormal cystic honeycombing.