**LETTER**

**p63**\(^+\)Krt5\(^+\) distal airway stem cells are essential for lung regeneration

Wei Zuo\(^1\), Ting Zhang\(^1\), Daniel Zheng’An Wu\(^1\), Shou Ping Guan\(^1\), Audrey-Ann Liew\(^1\), Yusuke Yamamoto\(^2\), Xia Wang\(^2\), Siew Joo Lim\(^1\), Matthew Vincent\(^3\), Mark Lessard\(^4\), Christopher P. Crum\(^1\), Wa Xin\(^1,5,6,7\) & Frank McKeon\(^1,2,6\)

Lung diseases such as chronic obstructive pulmonary disease\(^1\) and pulmonary fibrosis\(^2\) involve the progressive and inexorable destruction of oxygen exchange surfaces and airways, and have emerged as a leading cause of death worldwide. Mitigating therapies, aside from impractical organ transplantation, remain limited and the possibility of regenerative medicine has lacked empirical support. However, it is clinically known that patients who survive sudden, massive loss of lung tissue from necrotizing pneumonia\(^3,4\) or acute respiratory distress syndrome\(^6\) often recover full pulmonary function within six months. Correspondingly, we recently demonstrated lung regeneration in mice following H1N1 influenza virus infection, and linked distal airway stem cells expressing Trp63 (p63) and keratin 5, called DASC\(^{p63/Krt5}\), to this process\(^7\). Here we show that pre-existing, intrinsically committed DASC\(^{p63/Krt5}\) undergo a proliferative expansion in response to influenza-induced lung damage, and assemble into nascent alveoli at sites of interstitial lung inflammation. We also show that the selective ablation of DASC\(^{p63/Krt5}\) in vivo prevents this regeneration, leading to pre-fibrotic lesions and deficient oxygen exchange. Finally, we demonstrate that single DASC\(^{p63/Krt5}\)-derived pedigrees differentiate to type I and type II pneumocytes as well as bronchiolar secretory cells following transplantation to infected lung and also minimize the structural consequences of endogenous stem cell loss on this process. The ability to propagate these cells in culture while maintaining their intrinsic lineage commitment suggests their potential in stem cell–based therapies for acute and chronic lung diseases.

H1N1 influenza virus infection of murine lung triggers a process of leukocyte infiltration and lung damage similar to that of acute respiratory distress syndrome (ARDS)\(^8-10\) (Fig. 1a). Damaged regions are marked by densely packed, CD45\(^+\) neutrophils and macrophages\(^11\) and an absence of markers for type I (Pdpn\(^+\)) and type II (SPC\(^+\)) pneumocytes (Fig. 1a; Extended Data Fig. 1a). Despite the local destruction of alveoli, these same regions harbour discrete clusters of p63\(^+\)Krt5\(^+\) epithelial cells proposed to be the early stages of de novo alveoli formation (Fig. 1a)\(^7\). Three dimensional reconstruction of serial sections of lungs at 15 days post-infection (dpi) reveal a broad distribution of Krt5\(^+\) cells along the axis of the bronchioles (Fig. 1b; Supplementary Video 1).

To decipher the origin of these p63\(^+\)Krt5\(^+\) cells appearing in response to lung damage, we performed genetic lineage-tracing of Krt5\(^+\) cells starting before infection through the cycle of lung damage and resolution. Mice expressing a tamoxifen-dependent lacZ gene under the control of the Krt5 promoter\(^12\) (Tg(KRT5-Cre\(^\text{ERT2}\)-ROSA26-lsl-lacZ))\(^13\) were treated with tamoxifen before intratracheal delivery of H1N1 influenza virus (Fig. 1c; Extended Data Fig. 1b). At 0 dpi, lacZ activity was not detectable in whole-mount lung and yet Krt5\(^+\) cells were evident in distal lung as clusters of peribronchiolar Krt5\(^+\) cells were observed in one of three to four consecutive sections of lung. Approximately 50% of these cells expressed Escherichia coli–specific β-galactosidase (7 clusters of p63\(^+\) cells were observed in 25 slides with a total of 39 p63\(^+\) cells, of which 19 were β-galactosidase positive; Fig. 1c). No labelling of other cell types in the lung was observed. In addition, colonies of distal airway stem cells (DASC) with long-term self-renewal (passage 4) were generated\(^14,15\) from three 0 dpi mice and stained with antibodies to Krt5

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1. Genome Institute of Singapore, A-STAR, 138672 Singapore. 2. The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut 06032, USA. 3. Advanced Cell Technologies, Marlborough, Massachusetts 01752, USA. 4. The Jackson Laboratory, Bar Harbor, Maine 04609, USA. 5. Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA. 6. Department of Medicine, National University Health System, 119228 Singapore. 7. Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, Connecticut 06030, USA.

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Figure 1 | Lineage tracing of Krt5\(^+\) cells following viral infection. a. Left, mouse lung before and after viral infection. Right, immunofluorescence images of infected lung of anti-Krt5 (red), anti-Pdpn (green) with DNA counterstain (DAPI, blue). Scale bar, 1 mm. Insets, high magnification of indicated regions. n = 10 mice. Scale bars, 100 μm. b. Three-dimensional reconstruction of serial sections of lungs at 15 days post-infection (dpi) reveal a broad distribution of Krt5\(^+\) cells along the axis of the bronchioles (Fig. 1b; Supplementary Video 1).

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and β-galactosidase. All 256 colonies stained with anti-Krt5 antibodies and 132 co-expressed β-galactosidase (Fig. 1c; Extended data Fig. 1c). At 9 dpi, lacZ activity was subtle and restricted to the airways (Fig. 1c), consistent with our previous observations that at 9 dpi, p63+ Krt5+ cells had accumulated within mouse bronchioles with no evidence of migration to interstitial regions7. By 15 dpi, however, the lacZ signal was significantly more robust and included broader regions of interstitial lung (Fig. 1c; Extended Data Fig. 1d). At 60 dpi, the lacZ signal was distributed along the conducting airways and the surrounding interstitial lung (Fig. 1c; Extended Data Fig. 1d). At 30 dpi, the lacZ signal was distributed along the conducting airways and the surrounding interstitial lung (Fig. 1c; Extended Data Fig. 1d). At 60 dpi, the lacZ signal was distributed along the conducting airways and the surrounding interstitial lung (Fig. 1c; Extended Data Fig. 1d). Of 1,051 lineage-labelled cells expressed type I (1H8+ and Pdpn+) or type II (SPC+) pneumocyte markers with the remainder being secretory cells in the bronchioles (n = 3 mice; Fig. 1d; Extended Data Fig. 1d, f).

To generate a mouse model in which DASCp63/Krt5 could be conditionally ablated, we engineered the human diphtheria toxin receptor (DTR)6 into the Krt6a (Krt6) locus (Krt6–DTR; Fig. 2a; Extended Data Fig. 2a, b) as the Krt6 gene becomes activated specifically in DASCs at approximately 8–10 dpi, rather than the p63 or Krt5 gene that are expressed in stem cells of many stratified epithelia17. Consistently, DASCp63/Krt5 from the Krt6a–DTR mice were found to co-express DTR and Krt6a in vivo and in vitro (Extended Data Fig. 2c, d). To test this ablation model in vivo, we infected the Krt6–DTR mice with influenza virus and injected diphtheria toxin at 8 dpi (Fig. 2a). By 15 dpi, exposure to toxin resulted in a rapid loss of interstitial clusters of Krt5+ Krt6+ cells (Fig. 2b, c), indicating that we had generated a highly efficient ablation model. Compared to wild-type controls, Krt6–DTR mice lost 90% of Krt5+ cells and over 99% of Krt6+ cells following diphtheria toxin treatment (Fig. 2c).

We next evaluated the effect of ablating DASCp63/Krt5+ on the process of lung regeneration itself. Focal infiltrates of leukocytes appearing as haematoxylin–eosin staining densities form at about 10–15 dpi in wild-type mice and are typically resolved over the next 15–45 days (Fig. 2d, e). However, in diphtheria-toxin-treated Krt6–DTR mice, these densities fail to resolve over time (Fig. 2d, e). The persistent damage in DASCp63/Krt5+ ablated lungs is also evident by whole-lung genome expression analyses (Extended Data Fig. 3a, false discovery rate (FDR) q < 0.001). Histological comparisons of the persistent densities at 30 dpi revealed the possible basis for this difference. Although wild-type lungs at 30 dpi still display some lung densities, nearly all of these densities were negative.

**Figure 2 | Conditional ablation of activated DASCp63/Krt5.**

- **a.** Modified Krt6a locus driving the human diphtheria toxin receptor and experimental scheme. DTox, diphtheria toxin. **b.** Immunofluorescence images of distal lung at 15 dpi with indicated diphtheria toxin (+/−DTox) condition. c. Quantification of Krt5+ and Krt6+ cells in DTox-treated mice. n = 2 mice per group, 6 sections covering the whole lung for each mouse. d. Lung sections from indicated mice following influenza virus infection and treatment with DTox. e. Morphometric analysis of interstitial densities in sections of lung (n = 3 mice per condition and time). Error bars, s.e.m. f. Anti-Pdpn (red) immunofluorescence of lung densities to reveal type I pneumocytes counterstained with DAPI (blue). Scale bar, 100 μm. H&E, haematoxylin and eosin. g. Peripheral capillary oxygen saturation (SpO2) values obtained by pulse oximetry (WT, n = 3, DTR + DTox, n = 4) at indicated times. Error bars, s.e.m. ***P < 0.01 for −DTox versus +DTox. h. Gene ontology classes and fold-change DASCp63/Krt5+ ablation versus normal mouse lung at 30 dpi. Below, heat map of differentially expressed (P < 0.05) genes involved in pre-fibrosis, alveolar structure and vasculature.
for the CD45 leukocyte marker and instead possess unusual networks of Pdpn$^+$ type I pneumocytes (Fig. 2f; Extended Data Fig. 3b). These networks of type I pneumocytes lacked markers of type II pneumocytes (Extended Data Fig. 4). Interestingly, these networks stain positive for other type I pneumocyte markers (Aqp5) but not all (for example, Hopx; Extended Data Fig. 4), suggesting the possibility that the type I pneumocytes in these networks are undergoing a maturation process. Remarkably, similar alveoli-like structures formed by type I pneumocytes lacking type II pneumocytes in anatomical analyses of mice recovering from infection by the NWS influenza A virus were reported nearly 40 years ago$^{18}$. In contrast to the type I pneumocytes networks in persistent densities of wild-type mice, DASCp63/Krt5-ablated mice showed no such alveolar networks at 30 dpi (Fig. 2f) and instead maintained persistent infiltration of leukocytes evidenced by anti-CD45 staining (Extended Data Fig. 3b).

We next asked if the loss of DASCp63/Krt5 also affected aspects of pulmonary function in these mice. Using pulse oximetry$^{19}$ to assess peripheral capillary oxygen saturation (SpO$_2$), we found that the normal pulmonary function in these mice showed the presence of pre-fibrosis gene signature$^{21,22}$ including vimetin, FSP1 and collagen genes (Fig. 2h). Consistently, with this apparent decline in pulmonary function, peri-bronchiolar clusters seen in one of every three to four sections of distal lung (Fig. 3a, arrows). Consistently, 100-fold fewer DASCp63/Krt5-ablated mice only reached SpO$_2$ values approaching 75% saturation (Fig. 2g. Consistent with this apparent decline in pulmonary function, the persistent densities in the 30 dpi Krt6–DTR lung showed staining for smooth muscle actin ($\alpha$-SMA; Extended Data Fig. 5), a marker of myofibroblasts known to be associated with a pre-fibrotic state of the lung$^{20}$. These same interstitial regions showed weak but detectable staining with Masson’s trichrome blue, a marker of fibrosis (Extended Data Fig. 5). Correspondingly, whole-genome expression profiles of wild-type and DASCp63/Krt5-ablated lungs indicated the persistence of inflammatory gene expression and a relative decrease in gene expression linked to vasculature development in the DASCp63/Krt5-ablated lungs at 30 dpi (Fig. 2i; Extended Data Fig. 5). Moreover, the DASCp63/Krt5-ablated mice showed the presence of pre-fibrosis gene signature$^{21,22}$ including vimetin, FSP1 and collagen genes (Fig. 2h). Together these data suggest that the ablation of DASCp63/Krt5 arising during acute injury results in a failure of the regenerative process with structural and functional consequences for the lung.

Whereas p63$^+$ Krt5$^+$ cells are prominent features of the proximal lung, their presence in distal lung has been less clear$^9$. This is reflected in the abundant Krt5$^+$ cells in proximal lung (Fig. 3a) and the intermittent, peri-bronchiolar clusters seen in one of every three to four sections of distal lung (Fig. 3a, arrows). Consistently, 100-fold fewer DASCp63/Krt5 colonies arise from cell suspensions of distal lung than tracheobronchiolar stem cell (TBSCp63/Krt5) colonies from proximal lung (Extended data Fig. 6a). Regardless, both DASCp63/Krt5$^+$ and TBSCp63/Krt5$^+$ can be cloned and propagated as single-cell-derived pedigrees$^7$ that show very different fates upon differentiation. In air–liquid interface (ALI) cultures$^{24}$, TBSCs yield a stratified epithelium with Krt5$^+$ basal cells and apical ciliated and secretory cells typical of proximal airway, while DASCs yield a monolayer of differentiated cells expressing Pdpn (Fig. 3b; Extended Data Fig. 6b). In three-dimensional Matrigel cultures, DASCs form unilaminar, alveolar-like spheres composed of cells expressing type I (Pdpn and Aqp5) and type II (SPC) pneumocyte markers (Fig. 3c).

The very minor variation in gene expression between TBSCs and DASCs...
(less than 1% with fold change > 1.5, P < 0.05; Fig. 3d) transforms to major differences between TBSCs differentiated in air–liquid interface (TBSC-ALI) with DASCs in Matrigel (DASC-MAT), consistent with their divergent fates (Fig. 3e). To further probe the differential fates of TBSC-ALI and DASC-MAT, we used laser-capture microdissection (LCM) to generate gene expression profiles of normal tracheal and alveolar epithelium and compared with those of TBSC-ALI and DASC-MAT (Fig. 3f).

Gene-set enrichment analysis revealed a strong coincidence in gene expression patterns between in vitro-differentiated TBSCs and DASCs and their in vivo counterparts (FDR q value < 0.001), including many genes not previously identified as differential markers though confirmed by publically available antibody data sets24 (Fig. 3g).

To determine if cloned lung stem cells could incorporate into damaged lung, we first generated single-cell-derived pedigrees of DASClacZ and TBSClacZ from murine lung (Fig. 4a; Extended data Fig. 7a). We delivered one million TBSClacZ or DASClacZ to syngeneic mice five days after influenza virus infection (Fig. 4a). At 40 dpi (35 days post-transplantation), DASClacZ were distributed in interstitial regions emanating from major differences between TBSCs differentiated in air–liquid interface (less than 1% with fold change)

cell-derived DASClacZ to syngeneic mice five days after influenza virus infection (Fig. 4a). At 40 dpi (35 days post-transplantation), DASClacZ were distributed in interstitial regions emanating from airspace (Fig. 4b). At 90 dpi, DASClacZ showed a more homogenous pattern in interstitial spaces compared to 40 dpi (Fig. 4b). Significantly, mock-infected lungs, or mock-transplanted, infected lungs, showed no incorporation of DASClacZ at 35 days post-transplantation (Fig. 4b: Extended data Fig. 7b). As with the lineage-tracing experiments, we used E. coli–specific β-galactosidase antibodies to mark the transplanted cells in 90 dpi lungs and observed that at least 40% of the cells in alveolar region express pneumocytes markers (Pdpn, IHP and SPC) and at least 80% of the β-galactosidase-positive cells in bronchial region express secretory cell marker CC10 (mouse number n = 3; Fig. 4c; Extended data Fig. 7c). Gene expression analysis of the lacZ-positive regions of these lungs using laser-capture microdissection revealed a typical alveoli gene signature very different from that of immature DASCs or of damaged lung (Fig. 4d).

Together these findings demonstrate that single-cell-derived pedigrees of DASCs can readily incorporate into damaged lung during the process of lung regeneration and give rise to multiple epithelial cell types of bronchioles and alveoli. In contrast, transplanted TBSClacZ appeared confined to major airways (Fig. 4e). Parallel transplantations with green fluorescent protein (GFP)-labelled DASC (Fig. 4f) yielded similar patterns of co-blabelling of lineage and type I and type II pneumocyte markers at 40 dpi seen with transplanted DASClacZ (Fig. 4f).

Significantly, a fraction of these transplanted DASCGFP or their progeny continue to express the proliferation marker Ki67 even up to 60 dpi (Extended data Fig. 8), suggesting their high viability and extended contribution to the regenerative process. Lastly, morphometric analyses of diphtheria-toxin-treated, virally infected Krt6–DTR mice indicate that transplantation of DASCs results in a significant reduction of interstitial densities at 40 dpi (Extended data Fig. 9).

In the present work, we highlight the remarkable regenerative capacity of the lung following large-scale, acute lung damage and the function of a very discrete, pre-existing population of lung stem cells in this process. In addition, we demonstrate that upon transplantation, single-cell-derived DASCGFP and Krt5 pedigrees contribute multiple epithelial lineages, including bronchial secretory cells as well as alveolar type I and type II pneumocytes, to regenerating distal lung. Thus DASCGFP and Krt5 act in an emergent, conditional manner that is generally distinct from that of type II pneumocytes25–29, progenitor cells of limited self-renewal capacity that participate in highly focal, homeostatic lung repair. Our findings provide a mechanistic framework for the still emerging concept of lung regeneration30 and underscore potential therapeutic strategies exploiting this process.

**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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**Figure 4 | Transplantation of TBSClacZ and DASClacZ.** 

a. Schematic of pedigree generation and transplantation. 4OH-Tmox, 4-hydroxy-tamoxifen; ITD, intratracheal delivery. b. β-galactosidase activity in whole lung following DASClacZ transplantation. c. Comparison between β-galactosidase-positive (left panels) and -negative (right panels) regions of transplanted lung and markers of type I (IHP) and type II (SPC) cells. Scale bar, 50 μm. d. Heat map of selected, differentially expressed genes (P < 0.05) comparing immature DASClacZ before transplantation with laser-capture microdissected of lacZ-positive cells from transplanted lungs at 90 dpi. e. β-galactosidase activity in whole lung following TBSClacZ transplantation. f. From left, DASClacZ colony in culture; middle, cryosection of lung following DASClacZ transplantation. Scale bar, 50 μm. Right, immunofluorescence of anti-GFP, anti-Pdpn and anti-SPC in 40 dpi transplanted lung. Scale bar, 20 μm.
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Author Contributions Experiments were performed by W.Z., D.Z.W., S.P.G. and A.-A.L. Experimental design and conception were done by W.Z., M.V., C.P.C., W.X. and F.M.; T.Z., W.Z., X.W., S.J.L. and Y.Y. performed microarrays and computational analysis, and provided methodological advice. M.L. and W.Z. performed the serial reconstructions of infected lung. Author Information Datasets generated for this study have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under superseries GSE60849. 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 F.M. (mckeon.xian@gmail.com) or W.A. (waxian@jax.org).
METHODS
Influenza virus infection. All mouse experiments were conducted under IACUC guidelines and approved protocols. Influenza A (H1N1) mouse-adapted PR/8/34 (VR-95, ATCC, USA) was used for all viral infections. The virus stock was amplified by V. Chow (Department of Microbiology, National University of Singapore) in chicken eggs. Virus dilutions were made in DMEM medium containing 1 μg ml⁻¹ TPCK trypsin (Sigma-Aldrich, USA) on ice, aliquoted and stored at −80 °C. The viral titre is measured by plaque assay on Madin Darby canine kidney cells (MDCK, ATCC, USA). Virus was further diluted to final concentration in PBS on ice and use freshly. The infection of mice by H1N1 influenza virus was performed in an Animal Biosafety Level 2 (ABSL-2) facility. Adult mice (>6 weeks old) were anaesthetized with intraperitoneal injection of ketamine (150 mg per kg body weight) + xylazine (10 mg per kg body weight). The anaesthetized mouse was rested on a stand with its front teeth hung over a suture. This causes the mouse airway to be relaxed and accessible. Using a flat forceps the tongue of the animal was drawn out of its mouth so that the anatomy can be easily visualized. Intratracheal delivery was performed by pipetting 50 μl virus directly into the larynx/trachea. Sterile PBS was administered to the control animals. The tongue of mouse was held throughout the procedure so that the virus was aspirated into the lungs.

Tissue histology. At appropriate time points, mice were euthanized by CO₂ asphyxiation followed by exsanguination, and the diaphragm was carefully cut open without touching the lungs. A small incision was made in the proximal region of the trachea and lung was inflated with 4% formaldehyde using a 30G needle. The inflated lungs were dissected and fixed with 4% formaldehyde before whole mount imaging, paraffin section or cryosection. For whole mount imaging, lungs were dehydrated in graded ethanol series and sunk in BABB (benzyl alcohol/ benzyl benzoate 1:2 ratio) at 4 °C overnight. For paraffin section, lungs were processed in a automatic tissue processor (Leica Microsystems, Germany) and embedded into paraffin blocks. The blocks were cut using microtome (Leica Microsystems, Germany) to 5–7 μm thickness at distinct planes. The sections were placed on poly-lysine coated glass slides and stored at room temperature until further use. For cryosection, lungs were embedded within Tissue-Tek O.C.T. compound, solidified on dry ice and cut using a cryotome (Leica Microsystems, Germany) of 10 μm thickness.

Haematoxylin and eosin (H&E) staining was performed using standard procedures. To analyse lung damage level, interstitial densities were assessed by H&E staining backed up by type I (anti-Pdln) and type II (anti-SPC) pneumocytes staining. A minimum of 8 axial lung interval sections (typically 400 mm²) covering >2 mm tissue depth were cut, each was stained, scored for densities, and quantified for percentage of total lung area by Zeiss AxioVision (Carl Zeiss, Germany) morphometric software. In addition, random histological sections are scored based on the general pathological morphology by blinded expert to confirm the conclusion. Stitching scanning of H&E slides were performed in histopathology lab of IMCB, A-STAR, Singapore. Masson Trichrome staining of lung fibrosis was performed using the Trichrome Staining Kit (Sigma-Aldrich, USA). The kit involves sequential staining of the sections with Biebrich Scarlet- Acid Fuchsin, PTA/PA and Aniline Blue. After staining, sections were dehydrated and mounted using Vectamount and visualized under a light microscope (Imager Z1, Carl Zeiss, Germany).

Immunofluorescence staining. For immunofluorescence staining, paraffin-embedded tissue slides were subjected to antigen retrieval in citrate buffer (pH 6, Sigma-Aldrich, USA) at 120 °C for 20 min with the exception of CD45 staining. Antibodies used for immunofluorescence included stem cell markers: Krt5 (1:200, EP1601Y, Thermo), Krt6 (1:100, T-18, Santa Cruz and Ab24646, Abcam), p63 (1:200, A-1132, Life Technologies and 93631, Abcam) or X-gal staining. For X-gal staining, lungs were briedly fixed on ice for 30 min and subjected to X-gal (Invitrogen, USA) whole-mount staining overnight using standard protocol. After staining, lungs were washed and fixed again in 4% formaldehyde before whole-mount visualization or paraffin sectioning. For whole-mount visualization, lungs were made transparent by BABB as described before and images were taken using dissection microscope (Leica Microsystems, Germany). For paraffin section, 5–7 μm sections were cut and immunofluorescence staining is performed following standard protocol and visualized under light microscope (Imager Z1, Carl Zeiss, Germany).

Generation of the Kr5Δ6–DTR mouse. The complementary DNA of diphtheria toxin receptor (DTR), which is also known as human heparin-binding epidermal growth factor-like growth factor (HB-EGF), and a neomycin resistance selection cassette flanked with loxPs (Floxed Neo) with an introduced Pacl restriction endonuclease site, were introduced to replace the first 4 exons of Krt6a in a modified bacterial artificial chromosome (mBAC). Retrieval and linearization of a selected section of this mBAC resulted in the targeting construct, which was electroporated into V6.4 B6.129 hybrid embryonic stem cells to be selected with G418. Single colonies screened by Southern blot analysis of Pacl digests detected by a 5' external probe (hybridizing unmodified Krt6a genomic DNA) revealed fragment size differences due to the introduced Pacl site. Wild-type alleles with endogenous Pacl sites returned 35.9 kb fragments, while a recombination event returned a shorter 14.7 kb. Floxed Neo probes provided further verification for a single specific insertion. Successfully engineered embryonic stem cells were micro-injected into blastocysts to generate chimaeras, which were similarly tested for germline transmission via backcrosses to C57BL/6. Progeny from crosses with FVB/N-Tg(Actb-cre)2Mrt/J (Jackson Laboratory, USA) were screened for stable transmission of Krt6a–DTR alleles Cre-mediated excision of Floxed Neo as indicated by a reduced 12.9 kb Pacl digest fragment.

Oxygen saturation measurements. Peripheral capillary oxygen saturation (SpO₂) was measured using MouseOx Plus pulse oximeter (Starr Life Sciences, USA). An S-size CollarClip sensor was applied to depilated regions of the back neck skin and mice were rested for one hour before measuring SpO₂. Ten minutes before measurement, mice were anaesthetized by ketamine (150 mg per kg body weight) + xylazine (10 mg per kg body weight) intraperitoneal injection. After SpO₂ readings were stable, data collection was started and SpO₂ readings were recorded every second for one minute to calculate an average value.

Cloning of TBSC and DASC and in vitro differentiation. To isolate airway stem cells, trachea and lung were collected from adult mice and immersed in cold wash buffer (F12 medium, 1% Pen/Strep, 5% FBS). The trachea and two main bronchi were cut from the lungs and the lobes were cut with a sterile surgical blade into small pieces and digested with dissociation buffer (F12/DMEM, 1mg ml⁻¹ protease, 0.0035% trypsin and 10ng ml⁻¹ DNase I) overnight with gentle rocking. The dissociated cells were washed with wash buffer, passed through a 40-μm cell strainer, counted and plated onto irradiated 3T3 feeder cells as described. After 4 consecutively passages, single cell colonies were picked up by cloning ring and expanded. Colonies were characterized by immunofluorescence staining (E-cadherin “Krt5” p63 “Pdln” “CC10” “SPC”). These colonies have been passed up to 12 months with no observable phenotypic or chromosome count changes. To compare gene expression in immature TBSCs and DASCs, whole genome expression microarrays were carried out on passages 2-3 of the cell lines. Unsupervised comparison of the TBSC and DASC whole transcriptome data. Gene names are indicted for outliers (fold change > 3, P value <0.001) typically reported as markers or well known for other biology and for which we validated expression by quantitative PCR. To activate lacZ in TBSCs and DASCs cloned from ROSA26-lsl-lacZ; Krt5-CreERT2 mice, we exposed colonies in vitro to 4-OH-tamoxifen (1 μg ml⁻¹; Sigma-Aldrich) for 5 days at which point >70% cells express β-galactosidase and all cells were Krt5 p63 “SPC” “CC10” “Pdln”. GPP labelling was performed by retrotransduction of pmX-GFP (Cell Biolab, USA) followed by manual sorting of single GFP+ DASCs to 96-well plates.

Matriptatin differentiation assays were performed as previously described7. FGFI10 (50 ng ml⁻¹) was included in medium to favour distal airway differentiation. Under this condition, DASCs clustered and grew into sphere-like structures marked by unilaminar epithelia surrounding a clear lumen. TBSCs were grown on air–liquid interface (ALI) cultures for differentiation. ALI differentiation was performed as

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One million cells were diluted in 50 µl of DMEM/F12 medium for transplantation into each mouse. Adult mice (>6 weeks old) were anaesthetized with intraperitoneal injection of ketamine (150 mg per kg body weight) + xylazine (10 mg per kg body weight) and rested on a stand gesture. Intratracheal aspiration was performed by pipetting the virus directly into trachea via mouth. For rescue of Krt6–DTR phenotype by DASC transplantation, 3 x 10^5 cells were transplanted on 8 dpi (the same day DTox was given) and control mice received medium alone.

**Microarray and bioinformatics.** RNAs obtained from LCM, cell colonies or whole lobe of mouse lungs were used for microarray after being amplified using WT-Ovation Pico RNA Amplification System (NuGEN, USA) for 1 min then dehydrated in 50%, 75%, 100% ethanol for 30 s each and xylene for 5 min. Slides were allowed to air dry and LCM was performed immediately using an inverted microscope and PALM Robo software (Carl Zeiss, Germany). Cut elements were catapulted onto an Adhesive Cap-500 tube (Carl Zeiss) and subsequently transferred into a PCR tube with 50 µl of l Arcturus PicoPure RNA Isolation kit (Life Technologies, USA). For the comparison between gene expression profiles of in situ alveoli and tracheal epithelium with those of in vitro differentiated TBSCs and DASCs (see Fig. 3f, g), gene set enrichment analyses were performed using web-based tools developed by the Broad Institute. 605 genes highly expressed in tracheal epithelium (trachea versus alveoli, 4-fold higher, P value < 0.05) and 914 genes highly expressed in alveolar epithelium (trachea versus alveoli, 4-fold lower, P value < 0.05) were used as the queried gene sets (signature), respectively. Enrichment scores were determined after 1,000 permutations, and the permutation type was configured to the gene sets. Therefore the whole genome expression data of DASC-MAT versus TBSC-ALI were applied to GSEA program to evaluate enrichment of signature in the fold-change ordered list. Results with normalized enrichment score > 1.4 and FDR q value < 0.001 were considered significant. The comparison between gene expression profiles of normal alveoli and damaged interstitium with those of lung tissue with or without DASC ablation (see Extended Data Fig. 3a) was performed in similar manner.
Extended Data Figure 1 | Lineage tracing of Krt5<sup>+</sup> cells. a, Left, immunofluorescence images of sections of 15 dpi lung with staining patterns of antibodies to pan-leukocyte marker CD45 and the type I pneumocyte marker Pdpn with DNA counterstained with DAPI. Right, immunofluorescence images of pan-leukocyte marker CD45 and the type II pneumocyte marker SPC. Scale bar, 150 μm. b, X-gal staining (blue) to reveal lacZ-dependent β-galactosidase activity in whole lungs after 15 and 40 days post infection following long time gaps between induction of lacZ labelling by tamoxifen and influenza infection-induced lung damage. The similarity of this long gap labelling and the short gap labelling presented in Fig. 1 argues against prolonged actions of tamoxifen in these lineage-labelling protocols. Tamoxifen is given at indicated times before infection and no-tamoxifen control is included. c, Immunofluorescence images of colonies of DASCs derived from tamoxifen-treated ROSA26-lsl-lacZ, Krt5-Cre<sup>ERT2</sup> mice stained with antibodies to keratin 5 (Krt5) or Krt5 and E. coli-specific β-galactosidase. d, Histological section of lung at 15 dpi stained with E. coli-specific β-galactosidase antibody and markers of secretory cells (CC10<sup>+</sup>) and expanded stem cells (Krt5<sup>+</sup>). Scale bar, 50 μm. e, Whole-mount image of X-gal developed, uninfected lung from ROSA26-lsl-lacZ, Krt5-Cre<sup>ERT2</sup> which received tamoxifen treatments at −69, −66 and −63 days before dissection. f, Histological section of 60 dpi lung stained with E. coli-specific β-galactosidase antibody and markers of type I pneumocytes (Pdpn<sup>+</sup>) and type II pneumocytes (SPC<sup>+</sup>).
Extended Data Figure 2 | Conditional DASC63/Krt5 ablation mouse model.

a, Schematic of Krt6a locus, the targeting vector constructed to introduce the human diphtheria toxin receptor (DTR).
b, The structure of the modified Krt6a locus in embryonic stem cells screened by Southern blot.
c, Co-expression of Krt6 and DTR in Krt5+ pods in 15 dpi lung.
d, Histogram showing resistance of wild-type, 12 dpi DASC63+Krt5+ to diphtheria toxin (DTox) and the sensitivity of DASC63-Krt5+ to diphtheria toxin. n = 3 mice per group. Error bars, s.e.m.
Extended Data Figure 3 | Persistent damage in DASC6.5/Krt5-ablated lungs.

a, Gene set enrichment analysis (GSEA) showing the overrepresentation of normal alveolar signature gene sets in WT rather than Krt6–DTR mouse lungs (whole lobes). For normal alveolar signature build up, laser capture microdissection of frozen sections was used to dissect normal alveoli region from 0 dpi lung and damaged interstitial infiltrated region from 15 dpi lung for microarray analysis. Differentially expressed genes (fold change > 5, \( P < 0.01 \)) were used to develop normal alveolar gene expression signatures. b, Top panel, histological analysis of lung densities using anti-Pdpn antibodies (red) and anti-CD45 (green) to reveal type I pneumocytes and leukocyte infiltration, respectively. Left, wild-type mice showing apparently normal lung region adjacent to interstitial density having Pdpn\(^{-}\) network but lacking CD45\(^{+}\) infiltrates. Right, Krt6–DTR lung showing apparently normal region adjacent to zone of damaged interstitial lung lacking Pdpn\(^{-}\) network but having CD45\(^{+}\) infiltrates. Bottom panel, H&E staining of the same histological region. Scale bar, 100 µm.
Extended Data Figure 4 | Networks of type I pneumocytes in 30 dpi mouse lung. a. Histological analysis of lung densities using anti-Pdpn antibodies (red) and anti-SPC (green) to reveal type I and type II pneumocytes respectively. Left, wild-type mice showing apparently normal lung region adjacent to interstitial density having Pdpn⁺ network but lacking SPC⁺ cells. Right, Krt6–DTR lung showing normal region adjacent to zone of damaged interstitial lung lacking both pneumocytes. Scale bar, 100 μm. b. Top panel, histological analysis of wild-type lung densities using anti-Pdpn antibodies (red) and anti-Aqp5 (green) type I pneumocyte markers showing the interstitial density having Pdpn/Aqp5 double-positive network. Bottom panel, wild-type mice show apparently normal lung region adjacent to interstitial density having Pdpn⁺ network but the density lack expression of another type I pneumocyte marker, Hopx. Scale bar, 100 μm.
Extended Data Figure 5 | Failure of regeneration in DASC\textsuperscript{K63/K65}-ablated lungs. a, Histological section through 30 dpi DASC-ablated lung (Krt6–DTR +DTox) showing normal region (Pdpn\textsuperscript{+}) adjacent to interstitial density positive for α-SMA and weakly positive for Masson’s trichrome (MT) staining for fibrosis. Scale bar, 100 μm. b, Histological section through 30 dpi control lung showing normal region (Pdpn\textsuperscript{+}) and interstitial density (Pdpn\textsuperscript{−}) which are both negative for α-SMA. c, Expression heat map of selected, differentially expressed genes (\(P < 0.05\)) comparing wild-type mouse lungs with DASC-ablated mouse lungs at 30 dpi. Scale bar, 100 μm.
Extended Data Figure 6 | Cloning and in vitro differentiation of TBSC<sup>p63<sup>+/+<sup>/Krt5<sup>−/−</sup> and DASC<sup>p63<sup>+/+<sup>/Krt5<sup>−/−</sup>. a, Histogram of cloning efficiency of TBSCs and DASCs on irradiated 3T3-J2 cells per 1 million tracheal or distal airway cells derived from respective tissues of adult mice. Tissues derived from 3 mice. Error bars, s.e.m. b, Immunofluorescence images of sections of TBSC and DASC air–liquid interface cultures using an antibody to the type I pneumocyte marker Pdpn (green). Sections were counterstained with DAPI (blue).
Extended Data Figure 7 | Transplantation of DASClacZ.

a. Immunofluorescence characterization of DASCs isolated from Krt5-CreERT2;ROSA26-Isl-lacZ mice following Cre activation with 4OH-tamoxifen. From left, colony stained with antibodies to p63 (green) and Krt5 (red), p63 (green) and E. coli β-galactosidase (red), Krt5 (red) and CC10 (green), and Krt5 (red) and SPC (green).

b. Whole mount image of lung 90 days after infection without stem cell transplantation.

c. Left, bright field/immunofluorescence image of section of lung at 90 dpi following transplantation of DASClacZ stained with antibodies to β-galactosidase (red). Right panels, immunofluorescence images of co-staining of transplanted DASClacZ with antibodies to Pdpn, SPC, or CC10 at high magnification.
Extended Data Figure 8 | Persistent proliferation of transplanted DASC.
Co-staining of antibodies to GFP (green) with the cell proliferation marker Ki67 (red) in sections of lung transplanted with DASC$^{GFP}$ at 12 dpi lung (7 days post transplantation) and 60 dpi lung (55 days post transplantation). Top left, immunofluorescence image of lung following transplantation of DASC$^{GFP}$ (7 days post-transplantation;12 dpi) stained with anti-GFP (green) and the cell cycle marker Ki67 (red, in nucleus). Top right, bronchiole co-stained with antibodies to GFP and Ki67 from 7 days post-transplantation lung. Bottom, staining of interstitial lung transplanted 55 days prior with DASC$^{GFP}$ with antibodies to GFP and Ki67. Arrows indicate cells co-expressing GFP and Ki67.
Extended Data Figure 9 | Stem cell transplantation reduces interstitial densities in DASC<sup>63/p63</sup>-ablated lungs. a, Histological sections through entire lobe of Krt6–DTR mice with (left) and without (right) diphtheria toxin treatment forty days post-influenza infection. b, Histogram of morphometric quantification of lung densities following 40 day influenza virus infection of Krt6–DTR mice without diphtheria toxin (−DTox, mouse number n = 3), with diphtheria toxin (+DTox, n = 4), or with diphtheria toxin and transplanted DASCs (+DTox+DASC, n = 4). Error bars indicate s.e.m. and # indicates P value = 0.029 by Wilcoxon rank-sum test.