Alternative Progenitor Lineages Regenerate the Adult Lung Depleted of Alveolar Epithelial Type 2 Cells

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Abstract

An aberrant oxygen environment at birth increases the severity of respiratory viral infections later in life through poorly understood mechanisms. Here, we show that alveolar epithelial cell (AEC) 2 cells (AEC2s), progenitors for AEC1 cells, are depleted in adult mice exposed to neonatal hypoxia or hyperoxia. Airway cells expressing surfactant protein (SP)-C and ATP binding cassette subfamily A member 3, alveolar pod cells expressing keratin (KRT) 5, and pulmonary fibrosis were observed when these mice were infected with a sublethal dose of HKx31, H3N2 influenza A virus. This was not seen in infected siblings birthed into room air. Genetic lineage tracing studies in mice exposed to neonatal hypoxia or hyperoxia revealed pre-existing secretoglobin 1a1⁺ cells produced airway cells expressing SP-C and ATP binding cassette subfamily A member 3. Pre-existing Kr5⁺ progenitor cells produced squamous alveolar cells expressing receptor for advanced glycation endproducts, aquaporin 5, and T1 α in alveoli devoid of AEC2s. They were not the source of KRT5⁺ alveolar pod cells. These oxygen-dependent changes in epithelial cell regeneration and fibrosis could be recapitulated by conditionally depleting AEC2s in mice using diphtheria A toxin and then infecting with influenza A virus. Likewise, airway cells expressing SP-C and alveolar cells expressing KRT5 were observed in

human idiopathic pulmonary fibrosis. These findings suggest that alternative progenitor lineages are mobilized to regenerate the alveolar epithelium when AEC2s are severely injured or depleted by previous insults, such as an adverse oxygen environment at birth. Because these lineages regenerate AECs in spatially distinct compartments of a lung undergoing fibrosis, they may not be sufficient to prevent disease.

Keywords: genetic lineage mapping; influenza A virus; mice; neonatal oxygen; stem cells

Clinical Relevance

It is unclear why exposure to low or high amounts of oxygen at birth increases risk of severe respiratory viral infections later in life. This study uses mice to show how alternative progenitor lineages are recruited to repair the respiratory epithelium of infected mice, the alveolar type II cells of which are depleted by neonatal hypoxia or hyperoxia. Because these lineages were seen in fibrotic lungs, they may not be sufficient to properly regenerate the injured lung and prevent respiratory morbidity.

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Respiratory viral infections can cause significant pulmonary morbidity and mortality in susceptible individuals, including children and the elderly. Growing evidence indicates that children born preterm are especially vulnerable to pulmonary infections (1, 2). Preterm infants face a 3.6-fold increase in their risk of being rehospitalized during the first year of life when infected with respiratory syncytial virus (RSV) (3). Although RSV infections are the primary cause of readmission (4), increased morbidity has also been reported for rhinovirus, human bocavirus, metapneumovirus, and parainfluenza virus (5, 6). Children born with cyanotic congenital heart failure or at high altitude also have an increased risk of severe respiratory morbidity after a viral infection. For example, the odds ratio of being readmitted for RSV-specific infections increases 25% among infants younger than 1 year and 50% among children between ages 1 and 4 years who were living above 2,500 m (1.5 miles) (7). Because children born preterm are often exposed to excess oxygen and children born at high altitude are exposed to less oxygen, an aberrant oxygen environment at birth may influence the severity of pulmonary morbidity after a respiratory viral infection later in life.

We have been using mice to understand how high oxygen (hyperoxia) exposure at birth alters lung epithelial development and changes the response to influenza A virus (IAV) later in life. In this model, newborn mice are exposed to room air or hyperoxia (100% oxygen) between Postnatal Days (PNDs) 0 and 4. This time period of oxygen exposure represents the saccular phase of lung development in mice, which parallels the developmental stage when many preterm humans are born. At 8 weeks of age, mild airway hyperreactivity and alveolar simplification is seen in mice exposed to neonatal hyperoxia (8–10). Persistent inflammation and fibrotic lung disease is seen after these mice are infected with a sublethal dose of IAV (HKx31, H3N2) (11, 12). Because this is not seen in infected siblings exposed to room air at birth, neonatal hyperoxia may have disrupted or altered stem cells needed to properly regenerate the injured lung. Consistent with this hypothesis, adult mice exposed to neonatal hyperoxia have significantly fewer alveolar epithelial cell (AEC) 2 cells (AEC2s) than their siblings that were birthed into room air (9). This

prompted us to investigate how the oxygen environment at birth influences postnatal expansion of AEC2s. By varying the amount of oxygen at birth, we discovered that exposure to 12% oxygen (hypoxia) or 60% or greater oxygen (hyperoxia) at birth stimulates expansion of AEC2s through both self-renewal and differentiation of secretoglobin (*Scgb*) 1a1⁺ progenitor cells (13). AEC2s expanded during hyperoxia are slowly depleted when mice are returned to room air (14). Whether AEC2s expanded during hypoxia are also depleted during recovery in room air remains to be determined.

A reduced number of AEC2s in mice exposed to an aberrant oxygen environment at birth could affect how the lung responds to viral infection or other agents that damage the alveolar epithelium. AEC2s express surfactant phospholipids and proteins that reduce surface tension along the alveolar surface. They defend against bacterial and viral infections by expressing the collectins, surfactant protein (SP)-A and SP-D, eosinophil-associated RNase 1, and many other host defense molecules, including IFN- γ , several CXCL chemokines, and IL-29 (15–18). AEC2s also function as adult stem cells capable of both self-renewal and differentiation into AEC1s (19). However, AEC1s and AEC2s are also targets of IAV, and may die during infection (20, 21). This raises the question of how AECs are replenished when AEC2s are depleted or excessively injured, such as when infected by virus. Genetic lineage studies using *Scgb1a1-rtTA* or *Scgb1a1-CreER* driver mice indicate that airway Club cells can serve as progenitors for AEC2s after infection with IAV or exposure to bleomycin (19, 22, 23). AECs may also derive from a small number of epithelial cells expressing the laminin receptor $\alpha 6\beta 4$ (24). Epithelial cells expressing p63 and keratin (KRT) 5 have been detected in alveolar pod structures of mice infected with a highly pathogenic strain of IAV (PR8, H1N1) (25). Single-cell transplantation studies suggest that Krt5⁺ progenitors can regenerate AEC1s and AEC2s (26). On the other hand, genetic lineage tracings suggest that KRT5⁺ cells derive from a lineage-negative epithelial progenitor cell that rarely produces mature epithelium unless Notch signaling is inhibited (27, 28). Hence, there is great interest in identifying the source of KRT5⁺ cells and their role in regenerating the injured lung.

Here, we use genetic lineage labeling and cell depletion studies to investigate how the oxygen-dependent loss of AEC2s influences lung regeneration after infection with IAV. We chose to use HKx31, H3N2 because it is a weak strain of IAV that does not cause significant morbidity or mortality in mice exposed to room air (21, 29). We provide evidence that alternative progenitor lineages regenerate the alveolar epithelium when AEC2s are depleted by early-life oxygen exposures. However, they unexpectedly fail to restore normal airway and alveolar epithelial architecture, implying that this alternative form of epithelial regeneration is not wholly effective in preventing fibrotic lung disease. Our findings help explain how AEC2 injury, or depletion by an early-life insult, such as an adverse oxygen environment at birth, might increase the severity of respiratory viral infections.

Materials and Methods

Additional details for these procedures may be found in the online supplement.

Mice

Sftpc-CreER mice (23) were supplied by B. Hogan (Duke University, Durham, NC), and *Scgb1a1-CreER* (30), *Rosa26R-mT/mG* (31), *Rosa26R-StopDTA* (32), and *Krt5-CreER* (33) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All studies with mice were performed under Institutional Animal Care and Use Committee guidelines that were reviewed and approved by the University Committee on Animal Resources at the University of Rochester (Rochester, NY).

Oxygen Exposures

Newborn mice were exposed to 12% oxygen (hypoxia) or 100% oxygen (hyperoxia) as previously described (8, 13). Littermates that were maintained in 21% oxygen (room air) served as controls for all experiments.

IAV Infection and Administration of Bleomycin

Adult mice were infected intranasally with 120 hemagglutinating units of IAV (strain HKx31, H3N2) in 25 μ l saline (11, 34). Bleomycin (2.5 U/kg) in 50 μ l of PBS was administered to adult mice by tracheal instillation (35). Lungs were removed before treatment and on Post-treatment

Days 5, 14, 21, or 28. The right lobes were fixed and processed for histology. The left lobe was used to assess total soluble collagen levels using a Sircol collagen assay kit (Bicolor, Belfast, UK).

Conditional Activation of Genes Using Tamoxifen

Corn oil (Sigma-Aldrich, St. Louis, MO) was sterilized using a steriflip vacuum-assisted filter unit (Millipore, Billerica, MA). Tamoxifen (T5648; Sigma-Aldrich) was dissolved in filter-sterilized corn oil to make solutions of 20 mg/ml.

Histology and Immunohistochemistry

Lung tissues were inflation fixed overnight in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained as described previously (8, 9). Sections were stained with antibodies against proSP-C or ATP binding cassette subfamily A member 3 (ABCA3) (Seven Hills Bioreagents, Cincinnati, OH), T1 α (Developmental Studies Hybridoma Bank, Iowa City, IA), enhanced green fluorescence protein (EGFP), KRT5, and aquaporin (AQP5) (Abcam, Cambridge, MA), Scgb1a1 (Millipore and B. Stripp, Cedars-Sinai Medical Center, Los Angeles, CA), receptor for advanced glycation endproducts (R&D Systems, Minneapolis, MN), and α -smooth muscle actin (α -SMA; Sigma-Aldrich). Sections were incubated with fluorescently labeled secondary antibody (Jackson Immune Research, West Grove, PA), stained with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Waltham, MA), and positive cells quantified as previously described (9). Collagen was detected using Gomori's trichrome stain kit (Thermo Scientific, Waltham, MA). Sections were examined and photographed using a Nikon E800 fluorescence microscope (Microvideo Instruments, Avon, MA) and a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Ultrastructural Studies

Lungs were inflation fixed overnight at 4°C using 0.1 M sodium cacodylate-buffered 2.5% glutaraldehyde/4% paraformaldehyde, postfixed in 1% osmium tetroxide, and embedded in EPON/Araldite epoxy resin. Sections were stained with Toluidine blue to identify terminal airway, and then stained with aqueous uranyl acetate and lead citrate before examining on a Hitachi 7,650 Transmission Electron Microscope (Hitachi High-Technologies Group,

Clarksburg, MD) with an attached Gatan Erlangshen 11-megapixel digital camera (Gatan, Inc., Pleasanton, CA).

Human Lung Tissues

Paraffin-embedded human lung tissue sections were stained after histopathological confirmation that the tissue was healthy or was consistent with usual interstitial pneumonia, the pathology associated with idiopathic pulmonary fibrosis (IPF). The medical records were reviewed to exclude secondary causes of fibrosis. Biopsies were reviewed and approved by the University of Rochester's Office for Human Subject Protection.

Statistical Analysis

Data were analyzed using JMP11 software (SAS Institute, Cary, NC) and graphed as means ($\pm$ SEM). A two-way ANOVA was used to determine overall significance, followed by Tukey-Kramer honest significance test.

Results

The Oxygen Environment at Birth Alters Regeneration of the Adult Lung

Adult mice exposed to neonatal hyperoxia between PNDs 0 and 4 develop pulmonary fibrosis when infected with IAV (HKx31, H3N2), whereas infected siblings exposed to room air do not (11). We investigated whether birth into low oxygen also promotes fibrotic lung disease after infection. Adult mice exposed to 12% (hypoxia), 21% (room air), or 100% (hyperoxia) oxygen between PNDs 0 and 4 were infected with IAV (Figure 1A). Pulmonary fibrosis was not seen in any mice before infection (data not shown). It was also not seen in room air mice infected with IAV. In contrast, increased soluble collagen and fibrotic lung disease were observed on Postinfection Day 14 in lungs of adult mice exposed to neonatal hypoxia or hyperoxia (Figures 1B and 1C).

KRT5⁺ cells are found in alveolar pods of mice infected with IAV (PR8, H1N1) (25–27). We investigated whether they were also present in mice infected with IAV (HKx31, H3N2). KRT5⁺ cells were not seen in distal lungs of any mice before infection (data not shown). They were also not seen after room air mice were infected with IAV. In contrast, KRT5⁺ cells were detected on Postinfection Day 14 in distal airway and alveolar pod cells of adult mice exposed to

neonatal hypoxia or hyperoxia (Figure 1D). Alveolar KRT5⁺ cells were often found adjacent to α -SMA⁺ myofibroblasts. KRT5⁺ cells were initially detected around Postinfection Day 5, peaking in abundance around Postinfection Day 14, and declining by Postinfection Day 28 (see Figure E1 in the online supplement). We also observed clusters of SP-C⁺ and ABCA3⁺ cells in the distal airways of these mice (Figure 1E). Approximately 60% of airways within the lung lobe contained these cells, and they comprised 30% of cells seen in that region. SP-C⁺ airways were often found adjacent to or terminating in alveoli that were hypercellular and laden with myofibroblasts. Airway cells containing lipid-laden intracellular inclusion bodies, similar to lamellar bodies of AEC2s, were also detected by transmission electron microscopy (Figure 1F). Airway cells expressing SP-C or ABCA3 were not seen in infected mice exposed to room air at birth.

To determine whether these changes in epithelial regeneration were specific for IAV, mice were administered bleomycin, because it is a profibrotic chemotherapeutic and because neonatal hyperoxia enhances its profibrotic effects in the lung (35). Relative to room air, neonatal hypoxia and hyperoxia increased bleomycin-induced lung fibrosis and levels of soluble collagen (Figure E2). Although airway cells expressing SP-C (data not shown) and KRT5⁺ cells were detected adjacent to α -SMA⁺ myofibroblasts in mice administered bleomycin (Figure E2C), they were more prevalent in mice exposed to neonatal hypoxia or hyperoxia. Taken together, these results using IAV and bleomycin show that neonatal hypoxia or hyperoxia disrupts lung regeneration, as defined by the presence of airway AEC2-like cells containing lamellar bodies and expressing SP-C and ABCA3, presence of KRT5⁺ cells underlying distal airway epithelium and in alveolar pod cells, and the development of fibrotic lung disease.

Conditional Depletion of AEC2s Alters Alveolar Regeneration in the Adult Lung

Neonatal hyperoxia stimulates proliferation of AEC2s that are slowly depleted when the mice are returned to room air (14). Because neonatal hypoxia also stimulates proliferation of AEC2s (13), we investigated whether excess AEC2s were also depleted during recovery in room air. The

Figure 1. The oxygen environment at birth alters alveolar and distal airway epithelial cells of the adult lung infected with influenza A virus (IAV). (A) Cartoon showing the experimental model of IAV infection in adult mice exposed to 12%, 21% (room air [RA]), or 100% oxygen between birth and Postnatal Day (PND) 4. Lungs were harvested on Postinfection Day 14. (B) Lungs were stained with Gomori's trichrome to visualize collagen and tissue pathology. (C) Total collagen protein was measured from infected lungs. Values were graphed as mean ($\pm$ SEM) of six mice per group and represent data from three independent experiments with similar results. $^*P < 0.05$ and $^{***}P < 0.001$ compared with room air. (D) Lungs were stained for α -smooth muscle actin (α -SMA; green), keratin (KRT) 5 (red), and 4',6-diamidino-2-phenylindole (DAPI; blue). Inset boxes are enlarged below the image. (E) On Postinfection Day 14, lungs were stained for secretoglobin (SCGB) 1A1 (green), surfactant protein (SP)-C or ATP binding cassette subfamily A member3 (ABCA3) (red), and DAPI (blue). Arrows point to airway cell expressing SP-C or ABCA3. (F) Representative ultrastructural image of distal lung airway of infected mice exposed to 100% oxygen at birth. Inset boxes containing lamellar bodies (arrows) are enlarged in the right panels. Scale bars, 100 μ m (B and D), 25 μ m (E), 2 μ m (left panel) and 0.5 μ m (right panels) (F). Data are representative of five independent experiments with $n = 15$ mice per group per experiment.

proportion of SP-C⁺ to all DAPI⁺ cells was quantified in lungs of adult mice exposed to 12%, 21% (room air), or 100% oxygen at birth (Figure 2). Adult mice exposed to neonatal hypoxia or hyperoxia had roughly 50% fewer SP-C⁺ alveolar cells than siblings exposed to room air (Figures 2B and 2C). This reflects a loss of AEC2s for several reasons. First, flow cytometry confirmed that the percent of EGFP⁺ cells present in *Sftpc-EGFP* transgenic mice was reduced by neonatal hyperoxia (data not shown). Second, neonatal hyperoxia does not increase the number of immune cells recruited to the lung before infection (11).

Third, genetic lineage studies confirmed that adult mice exposed to neonatal hyperoxia have fewer AEC2s than siblings exposed to room air (13, 14). Hence, the number of AEC2s present in adult lung is influenced by a U-shaped or hormesis response to oxygen levels at birth.

To determine how the oxygen-dependent depletion of AEC2s affects adult lung regeneration after IAV infection, an equivalent number of AEC2s was depleted by overexpressing the diphtheria A toxin gene under conditional control of the *Sftpc* promoter. Adult *Sftpc-CreER*; *Rosa26R-StopDTA* mice were administered

tamoxifen once daily for 1, 2, 3, or 4 days (Figure 2D). Lungs were harvested on the seventh day and the proportion of SPC⁺ to DAPI⁺ cells was determined by immunohistochemistry. When compared with control mice exposed to room air, the number of SP-C⁺ AEC2s was modestly diminished by one dose of tamoxifen and approximately 50% by two doses (Figures 2E and 2F). Three doses of tamoxifen reduced the number of alveolar SP-C⁺ cells by roughly 70%; however, SP-C⁺ cells were now detected in the distal airway (Figure 2F). Airway cells expressing SP-C also expressed ABCA3, but not

Figure 2. Oxygen-dependent depletion of alveolar epithelial 2 cells (AEC2s) can be recapitulated by conditionally expressing diphtheria A toxin in AEC2s. (A) Cartoon showing when the number of AEC2s was determined in adult mice exposed to 12%, 21% (room air), or 100% oxygen between PND 0 and 4. (B) The percent SP-C⁺/DAPI⁺ cells were quantified in adult mice exposed to 12%, 21% (room air), or 100% oxygen between PNDs 0 and 4. Values were graphed as mean (±SEM) of six mice per group. **P < 0.01 relative to room air. (C) Images of adult lungs exposed to 12%, 21% (room air), or 100% oxygen between PNDs 0 and 4 stained for SP-C (red), SCGB1A1 (green), and DAPI (blue). (D) Cartoon showing when the number of AEC2s was determined in adult *Sftpc-CreER; Rosa26R-StopDTA* mice administered tamoxifen once per day for one, two, or three consecutive days. (E) The numbers of SP-C⁺ and DAPI⁺ cells were quantified in adult *Sftpc-CreER; Rosa26R-StopDTA* mice administered one, two, or three doses of tamoxifen. Values were graphed as mean (±SEM) of six mice per group and represent data from three independent experiments with similar results. **P < 0.01 and ***P < 0.001 relative to one dose of tamoxifen. (F) Representative images of adult lungs administered one, two, or three doses of tamoxifen stained for SP-C, SCGB1A1, and DAPI. Inset boxes (F) containing airways are enlarged in the images defined by arrows. Scale bars, 100 μm (C and F).

KRT5 (data not shown). Four doses of tamoxifen were often fatal. Hence, two doses of tamoxifen were sufficient to reduce the number of alveolar AEC2s similar to levels seen in adult mice exposed to neonatal hypoxia or hyperoxia.

To investigate how the loss of AEC2s influences the response to IAV, adult *Sftpc-CreER; Rosa26R-StopDTA* mice were administered tamoxifen once a day for two consecutive days or corn oil vehicle as control (Figure 3A); 5 days later, lungs were harvested from some mice and the rest were infected with IAV. Before infection (Day 0), soluble levels of collagen were low and not different between mice administered two doses of tamoxifen or corn oil (Figure 3B). In contrast, increased collagen was detected on Postinfection Day 14 in mice administered tamoxifen. Collagen levels were not significantly

increased in infected mice administered corn oil vehicle (Figures 3A and 3B). KRT5⁺ cells were also detected in airway and alveoli of infected mice administered two or three doses of tamoxifen, but not in mice administered one dose of tamoxifen or corn oil vehicle (Figure 3C). Although airway SP-C⁺ cells were not seen with two doses of tamoxifen, they were now seen after mice administered two doses of tamoxifen were infected with IAV (Figure 3D). Some of these cells expressed SCGB1A1.

As a control, we investigated whether depletion of airway Club cells affected distal lung regeneration after IAV infection. *Scgb1a1-CreER; Rosa26R-StopDTA* mice were administered two doses of tamoxifen or corn oil vehicle control, followed by 5 days of recovery (Figure E3). As expected, airway cells expressing SCGB1A1 were

partially depleted in mice administered tamoxifen, but not vehicle control. Increased collagen and lung fibrosis was not seen in mice before or after infection with IAV.

SP-C⁺ Airway Cells Derive from the *Scgb1a1*⁺ Lineage

Because airway SCGB1A1⁺ progenitor cells can self-renew and differentiate into AEC2s (22, 23, 36, 37), we investigated whether they were progenitors of airway AEC2-like cells found in infected mice exposed to neonatal hypoxia or hyperoxia. *Scgb1a1-CreER; Rosa26R-mT/mG* mice were exposed to 12%, 21% (room air), or 100% oxygen at birth and then administered four doses of tamoxifen, followed by 3 days of recovery as adults (Figure 4A). We first confirmed that the oxygen environment at birth did not influence the labeling index of

Figure 3. Depletion of AEC2s alters repair of the adult lung infected with IAV. (A) Cartoon showing how adult *Sftpc-CreER; Rosa26R-StopDTA* mice were administered tamoxifen and infected with IAV. (B) Total collagen protein in Preinfection Day 0 and Postinfection Day 14 lung was measured by Sircol assay and graphed as mean ($\pm$ SEM) of six mice per group and represent data from three independent experiments with similar results. $^{**}P < 0.01$ compared with no tamoxifen plus virus, and $^{***}P < 0.001$ compared with no virus. (C) Lungs harvested on Preinfection Day 0 or Postinfection Day 14 were stained with Gomori's trichrome to visualize collagen and tissue pathology. (D) *Sftpc-CreER; Rosa26R-StopDTA* mice were administered tamoxifen or vehicle (0 dose) daily for one, two, or three consecutive days. Mice were infected with IAV 5 days after the last injection. Lungs harvested on Postinfection Day 14 were stained for KRT5 (red) and DAPI (blue) or SP-C (red), SCGB1A1 (green), and DAPI (blue). Inset boxes (D) containing airways are enlarged in the images defined by arrows. Scale bars, 100 μ m (C), 50 μ m (D, upper panel), and 10 μ m (D, lower panel). Tam, tamoxifen.

Figure 4. Airway *Scgb1a1* lineage produces airway SP-C⁺ cells, but not KRT5⁺ cells in mice infected with IAV. (A) Cartoon showing the experimental model of infecting *Scgb1a1-CreER; Rosa26R-mT/mG* mice that were administered tamoxifen as adults and exposed to 12%, 21% (room air), or 100% oxygen as neonates. (B–D) Lungs were harvested on Postinfection Day 14 and stained for enhanced green fluorescence protein (EGFP) (green, conditionally expressed by the *Scgb1a1*⁺ lineage) and SCGB1A1 (red) (B), SP-C (red) (C), or KRT5 (red) (D), followed by counterstaining with DAPI (blue). Thin arrows point to EGFP overlap with SCGB1A1 (B) or SP-C (C). Thick arrows point to airway SCGB1A1⁺ cells that do not express EGFP. Scale bars, 50 μ m (B–D). Data are representative of three independent experiments, with $n = 15$ mice per group per experiment.

SCGB1A1⁺ cells by staining and counting the percent of cells expressing EGFP and SCGB1A1 (Figure E4A). We also confirmed that EGFP derived from the *Scgb1a1*⁺ lineage was detected in a small number of SP-C⁺ cells present at the bronchoalveolar junction, consistent with bronchoalveolar stem cells (BASCs). Additional mice were then infected with IAV. On Postinfection Day 14, EGFP was detected in most airway cells of mice exposed to room air and approximately 50% of airways of mice exposed to neonatal hypoxia or hyperoxia

(Figure 4B). EGFP was also detected in airway SP-C⁺ cells of infected mice exposed to neonatal hypoxia or hyperoxia, suggesting that airway AEC2-like cells derive from pre-existing *Scgb1a1*⁺ progenitors (Figure 4C). Airway cells expressing EGFP did not express KRT5 (Figure 4D). Interestingly, EGFP⁺ cells that expressed SCGB1A1 were also seen in infected mice exposed to neonatal hypoxia or hyperoxia (Figure 4B). These cells did not express SP-C or KRT, implying that they are Club cells derived from a

non-lineage-labeled progenitor cell that survived infection.

Genetic Lineage Studies Were also Used to Identify Progeny of AEC2s

Sftpc-CreER; Rosa26R-mT/mG mice were exposed to 12%, 21% (room air), or 100% oxygen at birth and then administered four doses of tamoxifen, followed by 3 days of recovery as adults (Figure 5A). We confirmed that neonatal hypoxia and hyperoxia does not alter the high labeling index of AEC2s by counting the proportion of EGFP⁺ cells expressing SP-C (Figure E4B). However, BASCs were not labeled, because we were unable to detect EGFP in SCGB1A1⁺ cells at the bronchoalveolar junction. Additional mice were then infected with IAV. On Postinfection Day 14, EGFP derived from the SP-C⁺ lineage was detected in cuboidal SP-C⁺ alveolar cells of all mice (Figure 5B). It was also detected in thin squamous cells that express T1 α , with the greatest amount seen in infected mice exposed to neonatal hypoxia or hyperoxia (Figure 5C). EGFP derived from the *Sftpc*⁺ lineage was never detected in KRT5⁺ cells present in infected mice exposed to neonatal hypoxia or hyperoxia (Figure 5D). It was also never detected in airway SP-C⁺ cells (Figure E5A). However, SP-C⁺ airway cells could be effectively labeled with EGFP by administering tamoxifen on Postinfection Days 9–12 when airway cells express SP-C (Figure E5B).

Krt5⁺ Cells Regenerate AEC1 in Alveoli Devoid of AEC2s

Because pre-existing *Scgb1a1*⁺ and SP-C⁺ cells do not generate KRT5⁺ cells, genetic lineage studies were used to determine if Krt5⁺ progenitor cells generate SCGB1A1⁺ or SP-C⁺ cells. *Krt5-CreER; Rosa26R-mT/mG* mice were exposed to 12%, 21% (room air), or 100% oxygen at birth and then administered four doses of tamoxifen, followed by 3 days of recovery as adults (Figure 6A). Before infection, EGFP derived from the *Krt5*⁺ lineage was detected uniformly in KRT5⁺ basal cells of all mice, regardless of oxygen exposure (Figure E6A). Although EGFP was not detected in alveolar cells before infection, it was readily detected after infection in squamous cells of mice exposed to neonatal hypoxia or hyperoxia (Figure E6B). Interestingly, EGFP was not detected in alveolar pod cells expressing KRT5 (Figure E6C). Instead,

Figure 5. SP-C lineage regenerates AEC1s, but not KRT5⁺ cells, in mice infected with IAV. (A) Cartoon showing the experimental model of infecting *Sftpc-CreER; Rosa26R-mT/mG* mice that were administered tamoxifen as adults and exposed to 12%, 21% (room air), or 100% oxygen as neonates. (B–D) Lungs were harvested on Postinfection Day 14 and stained for (B) EGFP (green, conditionally expressed by the *Sftpc*⁺ lineage) and SP-C (red), (C) T1α (red), (D) or KRT5 (red), followed by counterstaining with DAPI (blue). Thin arrows point to cuboidal EGFP⁺ alveolar cells and thick arrows point to EGFP⁺ squamous cells that also express T1α. Inset boxes in C are magnified in upper row of D. Scale bars, 50 μm (B–D). Data are representative of three independent experiments, with *n* = 15 mice per group per experiment.

EGFP was detected in alveolar cells expressing RAGE, AQP5, and T1α, but not SP-C (Figures 6B–6E). Close examination revealed that EGFP derived from Krt5⁺ lineage was predominantly seen in alveoli severely deficient or completely devoid of SP-C⁺ AEC2s.

Airway SP-C⁺ and KRT5⁺ Cells Are Present in Human Idiopathic Lung Fibrosis

Because human idiopathic and familial forms of pulmonary fibrosis have been linked to AEC2 dysfunction (38), we investigated whether aspects of the altered

epithelial regeneration observed in mice born into an aberrant oxygen environment were evident in humans with IPF. Analogous to the findings in mice, KRT5⁺ cells were found in IPF, but not healthy lung near fibroblast-rich regions expressing α-SMA (Figure E7). Likewise, SP-C⁺ cells were found lining the airspace of IPF samples, with an occasional positive cell present in underlying fibroblast-rich regions. These regions represent remodeled airway, because SP-C⁺ cells were found among SCGB1A1⁺ cells.

Discussion

Mechanisms by which exposure to hypoxia or hyperoxia at birth increase the risk of severe respiratory morbidity and hospitalization of children infected with a respiratory virus are not well understood. Here, we show that neonatal hypoxia or hyperoxia at birth depletes AEC2s by approximately 50% in adult mice (Figure 7). Although the oxygen-dependent depletion of these cells by itself was not sufficient to cause significant pulmonary disease, it sensitized the mice to develop fibrotic lung disease after infection with IAV or bleomycin. Genetic lineage mapping and conditional depletion studies revealed how alternative progenitor lineages were recruited to replace the lost regenerative functions of AEC2s. *Scgb1a1*⁺ progenitors, presumably pre-existing airway Club cells, regenerated SP-C⁺ AEC2-like cells in the distal airway. A Krt5⁺ progenitor regenerated squamous AEC1-like cells expressing RAGE, AQP5, and T1α in alveoli that were relatively devoid of SP-C⁺ cells. Interestingly, a nonlabeled progenitor produced alveolar KRT5⁺ pod cells. We found no evidence that the Krt5⁺ lineage contributes to airway or alveolar cells expressing SP-C. Presumably, generation of alveoli devoid of AEC2s causes alveolar collapse and progression to fibrotic lung disease. Supporting this idea, airway SP-C⁺ and alveolar KRT5⁺ cells were seen in lung biopsies of human IPF. Collectively, these findings suggest that alternative progenitor lineages for AEC2s can effectively restore AECs in the short term, but not a normal healthy lung in the long term. Our findings help explain how an aberrant oxygen environment at birth might increase the severity of respiratory viral infections later in life.

Figure 6. Krt5 lineage regenerates AEC1s, but not AEC2s, in mice infected with IAV. (A) Cartoon showing the experimental model of infecting *Krt5-CreER; Rosa26R-mT/mG* mice that were administered tamoxifen as adults and exposed to 12%, 21% (room air), or 100% oxygen as neonates. (B–E) Lungs were harvested on Postinfection Day 14 and stained for (B) EGFP (green, conditionally expressed by the *Krt5*⁺ lineage) and SP-C (red), (C) aquaporin (AQP) 5 (red), (D) RAGE (red), or (E) SP-C (red, left panel) or T1α (red, right panel), followed by counterstaining with DAPI (blue). Inset boxes (E) containing alveolar cells are enlarged in the lower images defined by arrows. Scale bars, 20 μm (B–D), 100 μm (E, upper row), 25 μm (E, lower row). Data are representative of two independent experiments, with *n* = 15–20 mice per group. RAGE, advanced glycosylation end products.

Growing evidence indicates that the lung contains region-specific stem and progenitor cell niches that serve to protect and maintain proper lung function (39). Genetic lineage tracing and ³H-thymidine labeling studies indicate that AEC2s are long-lived adult stem cells with a half-life of 1 month or more (19, 40). This low rate of turnover suggests that the total number of AEC2s present in the adult lung may be modified by fetal and postnatal influences on the developing lung. We recently showed how exposure to neonatal hypoxia or hyperoxia at birth stimulates expansion of AEC2s through both self-renewal and differentiation of the airway *Scgb1a1*⁺ lineage (13). AEC2s expanded during neonatal hyperoxia were slowly depleted when mice were returned to room air, resulting in an adult lung containing approximately 50% fewer AEC2s than normal (9, 13). Although mild alveolar simplification and increased compliance was seen in these mice, it was not severe enough by itself to perturb surfactant biophysical activity, or cause respiratory distress or lung disease (8). In the current study, we provide evidence that adult mice exposed to neonatal hypoxia (12% oxygen) also had approximately 50% fewer AEC2s than siblings exposed to room air. Although mild alveolar simplification was again seen, interestingly, a 50% reduction in the number of AEC2s still did not cause obvious lung disease. Likewise, significant lung disease was not seen when the number of SP-C⁺ AEC2s was reduced by 50% using *Sftpc-CreER; Rosa26R-StopDTA* mice.

Increasing the dose of tamoxifen caused greater depletion of AEC2s and mortality of mice, but still did not cause significant lung scarring. Other investigators using the same mice and similar doses of tamoxifen also reported minimal changes in lung architecture at 2, 4, 7 and 21 days after treatment (19). In contrast, pulmonary fibrosis was seen when transgenic mice expressing the diphtheria toxin receptor in AEC2s were administered diphtheria toxin protein (41). Pulmonary fibrosis was also seen when gancyclovir was administered to mice expressing thymidine kinase in AEC2s (42). Why some models of AEC2 depletion cause lung pathology and others do not is presently unknown.

Our findings demonstrate that pre-existing *Scgb1a1*⁺ cells produce AEC2-like

Figure 7. Cartoon model of progenitor cells involved in distal lung epithelial regeneration. Airway and AECs die (cells with dotted lines) when infected by IAV or damaged by the immune response to infection. Surviving airway Club cells regenerate new Club cells, whereas AEC2s regenerate both new AEC2s and AEC1s (black arrows). Alternative progenitors that serve to replace the progenitor functions of AEC2s are recruited when AEC2s are excessively depleted below 50% (red arrows). Airway AEC2-like cells are now regenerated from an airway *Scgb1a1*⁺ lineage, whereas AEC1-like cells are regenerated by a *Krt5*⁺ lineage of progenitors that do not produce alveolar *Krt5*⁺ pod cells. Hypothetically, alveolar collapse and myofibroblast expansion occurs when cellularity of distal airway and alveoli is not properly restored.

airway cells when alveolar AEC2s are injured or depleted. AEC2-like airway cells expressing SP-C or ABCA3 are not detected when AEC2s are depleted by approximately 50% using neonatal hypoxia and hyperoxia or conditional depletion of diphtheria toxin fragment A. They are detected when mice exposed to neonatal hypoxia or hyperoxia are infected with IAV or when AEC2s are depleted below 50% using diphtheria A toxin. Because AEC2s can be infected by IAV and may die during infection (43, 44), neonatal hypoxia and hyperoxia may reduce the number of AEC2s close to a threshold where further loss by the virus activates *Scgb1a1*⁺ progenitors to produce airway AEC2-like cells. Whether airway AEC2-like cells are derived from *Scgb1a1*⁺ Club cells or *Scgb1a1*⁺ BASCs remains to be determined. BASCs are progenitor cells found at the bronchoalveolar junction that express both SCGB1A1 and SP-C (37). Although we were able to label BASCs using *Scgb1a1-CreER* mice, we were not able to label them with *Sftpc-CreER* mice. However, we speculate that airway AEC2-like cells are not derived from BASCs, because they do not reside solely near the bronchoalveolar junction. The mouse models used in our study could not be used to determine if airway AEC2-like cells migrate and replenish AEC2s in the

alveolar space. The *Sftpc-CreER* mouse would not be able to distinguish between resident alveolar SP-C⁺ cells from those that migrated from the airway. The *Scgb1a1-CreER* mouse would also not be able to distinguish between the two types of SP-C⁺ cells, because it expresses reporter genes in approximately 10% of AEC2s before infection (30). Regardless, airway SP-C⁺ cells may represent a transient population that eventually dies, differentiates into another cell type, or migrates out of the airway, because they are not easily seen 6 months after infection even though lung scarring is still evident (data not shown).

Our research also found that *KRT5*⁺ cells present in the distal lung after the number of AEC2s falls below 50%. *KRT5*⁺ cells were only seen in the distal lung of infected mice, the AEC2s of which were depleted by 50% using neonatal hypoxia or hyperoxia, or by conditional depletion with diphtheria A toxin. They were also seen in uninfected mice, but only after AEC2s were severely depleted below 50%. Together, this implies alveolar *KRT5*⁺ cells are present when the severity of injury to AEC2s causes more than 50% loss. *KRT5*⁺ cells have been detected in pod-like structures of mice infected with a highly pathogenic strain of IAV (PR8, H1N1) (25),

and can produce some AEC1s and AEC2s when expanded *ex vivo* and transplanted in mice that are then infected (26). It is unclear why PR8 is so much more pathogenic than HKx31. It is widely accepted that PR8 infects distal alveolar cells, whereas HKx31 primarily infects upper airways (29, 45–48). PR8 also does not infect or produce virus from macrophages, whereas HKx31 can infect macrophages; however, this is not a productive infection. Hypothetically, oxygen-dependent depletion of AEC2s makes HKx31 act more like PR8, as defined by presence of *Krt5*⁺ alveolar pod structures and regeneration of AEC1s via a *Krt5*⁺ lineage. Our studies did not address how *Krt5*⁺ cells are recruited to the injured lung. However, it is interesting to note that EGFP derived from the *Krt5*⁺ lineage labeled before infection overlapped strongly with squamous alveolar cells expressing RAGE, AQP5, and T1α, but not *KRT5*. This suggests that *KRT5*⁺ progenitor cells, perhaps from basal cells of the trachea, home to the alveolar space during infection, where they differentiate into AEC1s. This conclusion supports prior findings that distal airway *Krt5*⁺ progenitors can regenerate AECs when transplanted into infected lungs (26). On the other hand, we found that alveolar *KRT5*⁺ pod cells present in the distal lung are not derived from a lineage-labeled *Krt5*⁺ progenitor. This conclusion is consistent with another study showing that the *KRT5*⁺ pod cells present in the distal lung derive from a currently unknown lineage-negative progenitor cell (27, 28). In these studies, inhibition of Notch signaling was required for lineage-labeled *Krt5*⁺ cells to regenerate mature epithelium. Our findings, therefore, suggest that the loss of AEC2s mobilizes pre-existing *Krt5*⁺ cells to regenerate some AEC1s, but not AEC2s, whereas a non-lineage-labeled progenitor produces *KRT5*⁺ alveolar pod cells, the functions of which remain to be determined.

The idea that fibrotic lung disease is caused by epithelial injury and dysregulated repair by AEC2s was first proposed in 1979 by Haschek and Witschi (49). Numerous studies since then have demonstrated the importance of AEC2s in maintaining distal lung homeostasis, in part by serving as an adult stem cell for itself and AEC1s. However, how dysregulated epithelial

repair was influenced by excessive injury to AEC2s is not known. Here, we took advantage of a weak strain of IAV that causes minimal distal lung disease to show how alternative progenitor lineages used to replace injured AECs were activated when AEC2s were depleted by an aberrant oxygen environment at birth or expression of diphtheria A toxin gene. Although these lineages successfully regenerated AEC1- and AEC2-like cells, they occurred in spatially distinct regions of the lung and were unable to prevent progression to fibrotic lung disease. Recruitment of alternative lineages seen in neonatal oxygen

and viral infections may have broader implications to the pathogenesis of lung fibrosis, because similar findings were seen when oxygen-exposed mice were administered bleomycin. Likewise, SP-C⁺ airway cells and alveolar KRT5⁺ cells among α -SMA⁺ myofibroblasts were seen in human IPF. Preserving AEC2s against excessive injury or early-life insults, such as an adverse oxygen environment at birth, may therefore be more effective at limiting respiratory morbidity than relying on the recruitment of alternative progenitor cell lineages to repair the injured lung. ■

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