In Vivo Imaging of Tracheal Epithelial Cells in Mice during Airway Regeneration

Jun Ki Kim[1,2*], Vladimir Vinarsky[3*], John Wain[3], Rui Zhao[3], Keehoon Jung[1], Jinwoo Choi[1], Adam Lam[3], Ana Pardo-Saganta[3], Sylvie Breton[4], Jayaraj Rajagopal[3,5], and Seok Hyun Yun[1,2,6]

[1]Harvard Medical School and Wellman Center for Photomedicine, and [3]Center for Regenerative Medicine, Massachusetts General Hospital, Boston, Massachusetts; [2]WCU Graduate School of Nanoscience and Technology, Korea Advanced Institute of Science and Technology, Daejeon, Korea; [4]Center for Systems Biology, Program in Membrane Biology/Nephrology Division, Massachusetts General Hospital, Boston, Massachusetts; [5]Harvard Stem Cell Institute, Holyoke Center, Cambridge, Massachusetts; and [6]Harvard–Massachusetts Institute of Technology Health Sciences and Technology, Cambridge, Massachusetts

Many human lung diseases, such as asthma, chronic obstructive pulmonary disease, bronchiolitis obliterans, and cystic fibrosis, are characterized by changes in the cellular composition and architecture of the airway epithelium. Intravital fluorescence microscopy has emerged as a powerful approach in mechanistic studies of diseases, but it has been difficult to apply this tool for *in vivo* respiratory cell biology in animals in a minimally invasive manner. Here, we describe a novel miniature side-view confocal probe capable of visualizing the epithelium in the mouse trachea *in vivo* at a single-cell resolution. We performed serial real-time endotracheal fluorescence microscopy in live transgenic reporter mice to view the three major cell types of the large airways, namely, basal cells, Clara cells, and ciliated cells. As a proof-of-concept demonstration, we monitored the regeneration of Clara cells over 18 days after a sulfur dioxide injury. Our results show that *in vivo* tracheal microscopy offers a new approach in the study of altered, regenerating, or metaplastic airways in animal models of lung diseases.

Keywords: *in vivo* fluorescence microscopy; mouse imaging; epithelial regeneration

CLINICAL RELEVANCE

The work presented here represents an initial step towards high-resolution live microscopy in respiratory cell biology, which impacts basic research in genetically tractable model organisms. Furthermore, mechanistic studies of airway regeneration and metaplasias using these novel techniques can influence our understanding of airway disease pathogenesis of recovery from a smoke inhalation, chemical pneumonitis from aspiration, and a variety of viral infections.

The airway epithelium has the capacity to heal rapidly after epithelial injury (1). With various tools for genetic manipulation available, mice are an attractive model system to study the mechanism of airway diseases and epithelial regeneration after injury. The mouse trachea has a similar cellular organization as the human conducting airways, the primary site of many airway diseases. Basal cells, which have been identified as the stem cells in the human airway epithelium, are present in the mouse trachea, and have been shown to possess the same ability to self-renew and generate transit-amplifying Clara cells and differentiated ciliated cells (2). The traditional method of analyzing these cells in the mouse trachea is by histology of fixed tissue. However, this static method is inherently suboptimal for understanding dynamic cellular processes in epithelial regeneration, which might be best appreciated by direct visualization and time-lapse monitoring. Although sophisticated *in vitro* culture systems (2, 3) allow *in situ* imaging, these models cannot fully replicate the natural environment *in vivo*, because many elements that influence the behavior of cells, such as physical forces, complex cell–cell interactions, innervation, and the immune system, are difficult to reproduce reliably *in vitro*. By contrast, *in vivo* imaging of the epithelial cells and their interactions in live mice has the potential to provide valuable information and insights that cannot be easily obtained from *in vitro* assays and histological analysis of fixed tissue.

Several studies have reported *in vivo* imaging of the mouse respiratory system. Kimura and colleagues (4) performed intravital fluorescence imaging of the proliferation of a cancer cell line in the mouse lung using open chest surgery. Looney and colleagues (5) demonstrated two-photon fluorescence microscopy of immune cells in the mouse lung through a surgically implanted glass window. Cortez-Retamozo and colleagues (6) showed fluorescence fiberoptic bronchoscopy for imaging the eosinophil-mediated matrix metalloproteinase activity in the mouse lung in a model of asthma. In addition, fiberoptic confocal reflectance and fluorescence microscopy have been used in humans to analyze normal and pathologic mucosa of the airways (7). Despite these technical advances, current studies of respiratory epithelial regeneration in mouse models have been hampered by the difficulty of visualizing the tracheal epithelium minimally invasively *in vivo* at a single-cell resolution.

Here, we report on the first endotracheal confocal fluorescence imaging of basal stem cells, Clara cells, and ciliated cells in live mice A custom-made, small-diameter optical probe allowed us to perform repeated *in vivo* endotracheal imaging experiments over a period of 3 weeks. For proof of concept, we monitored the changes in a number of these cells after a chemical injury and during epithelial regeneration. The ability to monitor proliferative and differentiating phases of airway regeneration after injury will be useful for mouse models of asthma and cancer using fluorescent epithelial reporters.

MATERIALS AND METHODS

Optical Probe and Imaging System

The imaging probe was fabricated using three graded-index (GRIN) lenses (NSG America, Somerset, NJ): a proximal 0.4-numerical

(*Received in original form May 3, 2012 and in final form September 12, 2012*)

* These authors contributed equally to this work.

This work was supported by National Institutes of Health grants RC2DK088661, U54CA143837, R01AI081734, P41EB015903, P30HL101287, HD040793, DK085715, and T32HL007874, National Research Foundation of Korea grants R31-2008-000-10071-0 and 2009-352-C00042, and by the Harvard Stem Cell Institute.

Correspondence and requests for reprints should be addressed to Seok Hyun Yun, Ph.D., 65 Lansdowne St., UP-5, Cambridge, MA 02139. E-mail: syun@hms.harvard.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 47, Iss. 6, pp 864–868, Dec 2012

Originally Published in Press as DOI: 10.1165/rcmb.2012-0164OC on September 13, 2012
Internet address: www.atsjournals.org

aperture (NA) coupling lens with 0.25 pitch (ILW-035-025), a 0.1-NA relay lens, 60 mm in length (four pitches, SRL-035-400), and a distal 0.4-NA focusing lens with 0.14–0.17 pitch (reduced from standard 0.25 pitch by abrasive polishing). All of the lenses had an outside diameter (OD) of 350 μm. For side-view imaging, a micro-prism mirror was attached to the distal end of the focusing lens. A stainless metal sheath (OD, 610 μm; inside diameter [ID], 370 μm) was sleeved over the probe for mechanical protection. The imaging probe was integrated into a laser-scanning confocal microscope using a rotational mount (Thorlabs, Newton, NJ) (8, 9).

In Vivo Endotracheal Imaging

Mice were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (9 mg/kg) and placed on the heated plate of a three-dimensional translational stage (Sutter MP285 motorized micromanipulator; Sutter, Novato, CA). An endotracheal intubation cannula with a Y-adapter (OD, 1.0 mm; ID, 0.7 mm; Harvard Apparatus, Holliston, MA) was inserted into the trachea. Once the cannula was in place, the oxygen supply was connected, and the optical probe was inserted into the tube through a paraffin film (Parafilm; Pechiney Plastic Packaging Co., Chicago, IL) that had been applied to seal the tube. The mouse was carefully positioned to ensure a smooth insertion of the probe. The ventilator was set at 250 breaths per minute at a tidal volume of 300 μl. Fluorescence images were acquired at 15 to 30 frames per second and displayed in real time on a computer screen at the video rate. Each frame consists of 512 × 512 pixels, with a resolution of 2 pixels/μm (4 megapixels/mm^2). In postprocessing, frame averaging (typically 90–120 frames) was applied to reduce the noise level of acquired frames, and an image registration algorithm (10) was applied to compensate for the effects of respiration-induced motion. At the completion of every imaging procedure (~40 min) the optical probe and the cannula were gently removed from the trachea, and the mouse was allowed to recover with spontaneous breathing.

Sulfur Dioxide Injury

To study airway regeneration, adult mice were placed in individual compartments of a custom-built polystyrene chamber (Lab Products, Inc., Seaford, DE) and exposed to 500 ppm sulfur dioxide (SO_2) in air for 3.5 hours (11). After a single exposure to SO_2, the mice were allowed to recover in standard housing cages (2). The mice appeared mildly dehydrated on Day 1 after exposure, but recovered fully by the following day, and did not require any additional treatment. The earliest *in vivo* imaging experiments were performed 24 hours after SO_2 exposure. All animal experiments were performed in compliance with institutional guidelines and approved by the subcommittee on research animal care at Massachusetts General Hospital (Boston, MA).

Mice, Immunohistochemistry, and Tracheal Epithelial Cell Quantitation

Please see the online supplement for detailed descriptions of mouse strains, the immunohistochemistry protocol, and the tracheal epithelial cell quantitation used in the studies.

RESULTS

B1–Enhanced Green Fluorescent Protein Mouse Is a Tracheal Clara Cell Reporter

To find a fluorescent reporter for the Clara cells (Figure 1A), we examined the B1–enhanced green fluorescent protein (EGFP) mouse strain in which the expression of EGFP is driven by the promoter of the B1 subunit of vacuolar H(+)-ATPase (V-ATPase). It has been shown that this mouse strain expresses GFP in the nonciliated cells of the small airways in the lung (12). However, the GFP expression pattern in the trachea has not been reported. Immunohistochemistry of tracheal sections from adult B1-EGFP mice showed that nearly all of the GFP-positive cells colocalized with the Clara cell–specific protein (CCSP)–positive Clara cells, but not with the acetylated tubulin–positive ciliated cells or cytokeratin 5 (CK5)–positive basal stem cells (Figure 1B). In fact, 486 of 522 (93.1%) GFP-positive cells were positive for

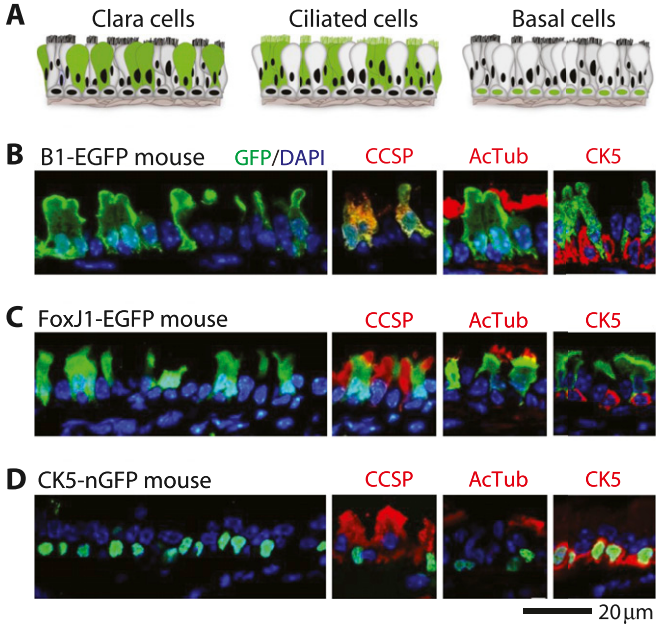

Figure 1. *Ex vivo* characterization of GFP-expressing tracheal epithelial cells. (*A*) Cartoon depictions of ciliated, basal and Clara cells, each labeled by green fluorescence. (*B–D*) Immunofluorescence images of tissue sections, labeled by GFP (*green*), nucleus-staining 4′-6-diamidino-2-phenylindole (DAPI; *blue*), and other molecular markers, such as Clara cell–specific protein (CCSP), acetylated tubulin (AcTub), and cytokeratin 5 (CK5; *red*). (*B*) B1–enhanced green fluorescent protein (EGFP) mouse, where only the Clara cells express GFP. (*C*) forkhead box protein J1 (FoxJ1)–EGFP mouse, where only the ciliated cells are GFP positive. (*D*) CK5-nGFP mouse after administration of doxycycline in drinking water for 10 days, where GFP is expressed in the nuclei of basal cells. *Scale bar*, 20 μm.

CCSP, the cell type–defining marker of Clara Cells. Only 8 of 524 (1.5%) CK5-positive basal cells and 22 of 512 (4.3%) ciliated cells were GFP positive. These results indicated that, in the B1-EGFP mouse, the fluorescent reporter is both highly specific (93%) and highly sensitive (97%) for Clara cells. In a B1-EGFP mouse tracheal epithelium, there is a 96% positive predictive value that a green signal seen by *in vivo* imaging is a CCSP-positive Clara cell.

Reporter Mice for Tracking Ciliated Cells and Basal Stem Cells

Aside from the Clara cells described previously here, the other two major cell types of the tracheal epithelium—ciliated cells and basal stem cells—were also visualized by live fluorescent cell type–specific GFP reporters. The ciliated cells are identified by the transcription factor, forkhead box protein J1 (FoxJ1), and by the cilia at the apical surface of the cell. The FoxJ1-EGFP mouse strain was previously shown to express GFP in the ciliated cells of an adult tracheal epithelium by immunohistochemical detection of GFP and visualization of cilia (13). We confirmed the published findings by immunofluorescence of tracheal sections using the following antibody markers: CCSP for Clara cells, CK5 for basal cells, and acetylated tubulin for ciliated cells (Figure 1C). In fact, 522 of 537 (97.2%) GFP-positive cells colocalize with FoxJ1, 8 of 512 (1.5%) GFP-positive cells colocalize with CK5, and 14 of 507 (2.8%) GFF-positive cells colocalize with CCSP. As expected, the GFP signal in the FoxJ1-EGFP mouse is both highly sensitive (99%) and specific (98%) for Ciliated cells in the mouse trachea, with a positive predictive value of 97% that a GFP-positive cell is, in fact, a ciliated cell.

The basal stem cells of the airway are characterized by the expression of several molecular markers, including CK5 and the transcription factor p63. We constructed a double-transgenic mouse strain by mating a CK5-driven reverse tetracycline-controlled transactivator (CK5-rtTA) driver to a tet(O)H2BGFP reporter, in which the expression of GFP in the nuclei of the CK5-expressing basal cells can be activated by administration of doxycycline in the drinking water ("CK5-nGFP" mice). We found that, in the tissue sections of the CK5-nGFP mice, after 2 weeks of doxycycline induction, 557 of 582 (95.7%) of the GFP-positive nuclei colocalized with the CK5 antibody stain (Figure 1D). The GFP expression was only found in 7 of 503 (1.4%) FoxJ1-positive cells and 16 of 515 (3.1%) CCSP-positive cells, confirming that this strategy labels almost exclusively the basal stem cells of the mouse trachea. Thus, the GFP signal in the CK5-nGFP mouse is both highly sensitive (94%) and specific (98%) for basal cells, with a positive predictive value of 96% that a GFP-positive nucleus seen by *in* vivo imaging is, in fact, a basal cell.

Development of an Endotracheal Imaging System

We have previously imaged dendritic cells in the upper trachea using a side-view GRIN optical probe with a diameter of 1.25 mm (8). However, the size of the probe necessitated tracheostomy and precluded longitudinal time-lapse imaging. To solve this problem, we fabricated a new probe with an OD of 0.61 mm, using GRIN lenses with diameters of only 0.35 mm. This thin, 62-mm-long, semirigid probe was inserted into the mouse trachea through a standard intubation cannula (OD, 1.0 mm; ID, 0.7 mm) without blocking the ventilated airflow (Figure 2A). The imaging plane or focal plane of the optical probe was adjusted remotely by moving the objective lens mounted on a microtranslational stage in the confocal microscope (14) (Figure 2B). The field of view of the optical probe was approximately90 μm in diameter. To visualize different locations along the trachea, the mouse was moved parallel to the tube using a translational stage. To scan along the circumferential axis, the probe was rotated using a rotational stage (Figure 2C).

Using this setup and procedure, we imaged the three strains of mice described previously here and identified the Clara cells in B1-EGFP mouse, the ciliated cells in FoxJ1-EGFP mouse, and the basal cells in CK5-nGFP mouse after doxycycline treatment (Figure 2D). The image resolution was sufficient to discern individual cells. The confinement of GFP in the nucleus of the basal cells is appreciated by the size of each bright area. Real-time video-rate imaging revealed significant tissue motion due to breathing and heartbeat. For frame averaging (typically 90–120 frames; 3–4 s), a rigid registration (10) was used to align the movie frames to minimize motion-induced artifacts. However, motion blur and distortion are apparent in some of the images.

Longitudinal Imaging of Basal Cells via Airway Transgene Activation

To demonstrate the possibility of longitudinal imaging, we performed endotracheal imaging of a CK5-nGFP mouse multiple times over the course of 10 days during a doxycycline treatment. Before the administration of doxycycline (at Day 0), *in vivo* imaging showed no GFP-positive cells (Figure 3A). Imaging at Days 2, 7, and 10 in the same mouse showed an increasing number of GFP-expressing basal cells with time (Figure 3A). At Day 10, the number of detected cells reached ~4,000 basal cells per 1 mm^2 (Figure 3B). Histology on fixed tissue qualitatively validated our *in vivo* observations (Figure 3C).

In Vivo Imaging of Regenerated Clara Cells after Injury

SO_2 inhalation causes rapid sloughing of suprabasal epithelial cells in the mouse trachea, and is a widely used model to study epithelial regeneration (15). We applied our imaging technique to visualizing the epithelial cells over the course of injury and regeneration. *In vivo* imaging of a B1-EGFP mouse showed the normal epithelium with a uniformly dense distribution of Clara cells (Figure 4A). At 24 hours after the SO_2 inhalation injury, we

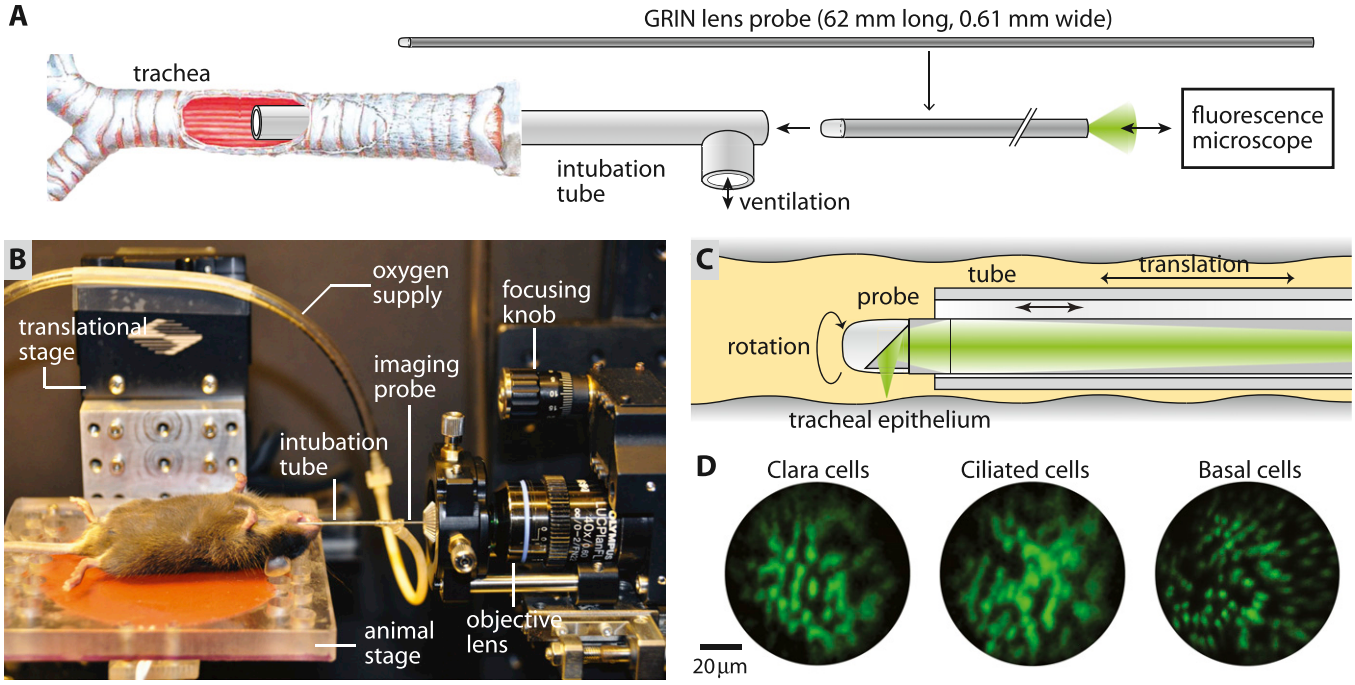

Figure 2. *In vivo* endotracheal fluorescence microscopy. (*A*) Illustration of the assembly of a graded-index (GRIN) lens imaging probe and an intubation tube. (*B*) Imaging setup. (*C*) Illustration of tracheal imaging. (*D*) Fluorescence images of the tracheal epithelium *in vivo*, showing Clara cells in a B1-EGFP mouse, ciliated cells in a FoxJ1-EGFP mouse, and basal cells in a CK5-nGFP mouse after doxycycline treatment. *Scale bar*, 20 μm.

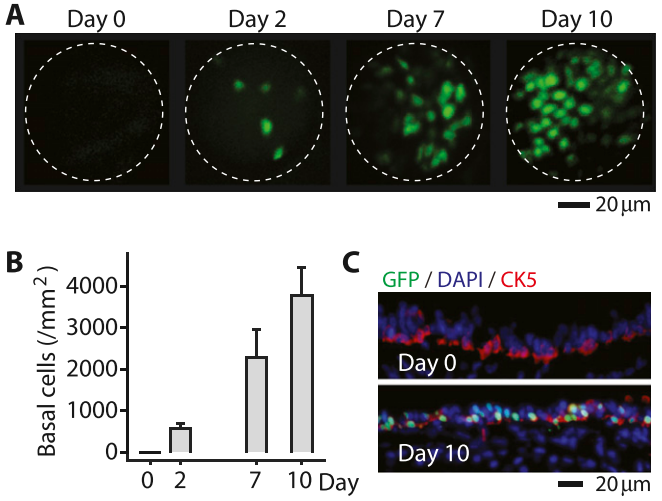

Figure 3. Time-lapse imaging of transgene activation *in vivo*. (*A*) Fluorescence images of a CK5-nGFP mouse obtained at Days 0, 2, 7, and 10 during the induction by doxycycline. (*B*) The measured number of GFP-expressing cells per unit area (1 mm^2). Error bars, SD. (*C*) Immunofluorescence images of tissue sections obtained at two different time points with costaining for GFP (*green*), DAPI (*blue*), and CK5 (*red*). At Day 0, no GFP-positive cells are detected; at Day 10, most CK5-positive cells express GFP in their nuclei. *Scale bars*, 20 μm.

found that B1-EGFP–positive Clara cells have been completely depleted (Figure 4B). Immunofluorescence of tissue sections harvested at 24 hours after the injury confirmed the absence of Clara cells and ciliated cells, whereas the basal stem cells remain intact (Figure 4C) (16). *In vivo* images at 9 days showed the regeneration of GFP-expressing Clara cells, and the population of Clara cells increased at Day 18 (Figure 4D). We found some differences between animals (even among littermates) in terms of the speed of regeneration of Clara cells (Figures 4E and 4F).

DISCUSSION

In vivo trachea imaging in genetically tractable organisms, such as mice, offers a new and exciting window into the study of cell biology of airway diseases. Such imaging studies require cell type–specific fluorescent reporters. We used immunofluorescence to establish the relationship of the GFP signal in relation to the three major epithelial cell types in the mouse trachea (basal, Clara, and ciliated cells) in three separate strains of reporter mice (CK5-nGFP, B1-EGFP, and FoxJ1-EGFP). Although CK5rtTA and FoxJ1-EGFP were known to express transgenes in basal and ciliated cells, respectively, our results establish the B1-EGFP mouse as a novel fluorescent reporter strain for visualizing Clara cells *in vivo* in the mouse trachea. It is also worth noting that basal cells are known to give rise to Clara cells and ciliated cells based on the established cellular lineage relationships of the mouse tracheal epithelium (2). Therefore, in homeostasis, we expect to identify a few nonbasal cells with GFP-positive nuclei in the CK5rtTA–tet(O)H2BGFP mouse (CK5-nGFP). However, these nonbasal cells represent a relatively small fraction of the GFP-positive cells (as seen by the high specificity of the GFP signal for CK5-positive cells in the CK5-nGFP mouse), indicating that the vast majority of the GFP-positive nuclei in the CK5-nGFP mouse visualized by *in vivo* imaging are in the basal cells.

After validating three tracheal epithelial cell type–specific fluorescent reporters, we demonstrated the first visualization of three different types of epithelial cells in the mouse trachea *in vivo* and over time by minimally invasive endotracheal imaging.

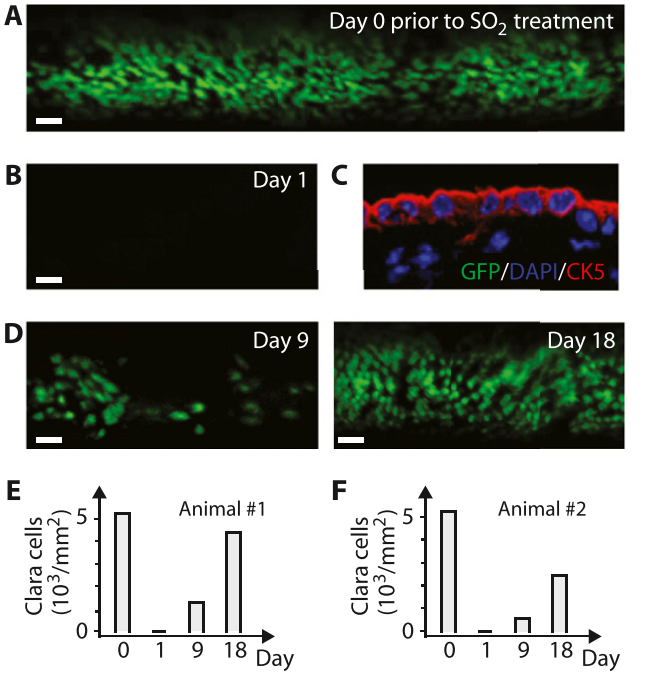

Figure 4. Longitudinal monitoring of Clara cells after sulfur dioxide (SO_2) injury. (*A*) A mosaic image of the normal epithelium in a B1-EGFP mouse. (*B*) The same mouse at Day 1 after the exposure to SO_2, revealing a complete loss of Clara cells. (*C*) Immunofluorescence of a section obtained at Day 1 after the injury with staining for GFP (*green*), DAPI (*blue*), and CK5 (*red*). (*D*) *In vivo* images taken at Days 9 and 18. *Scale bars*, 20 μm. (*E–F*) The density of Clara cells measured in two different mice.

The resolution of the sub–millimeter-diameter confocal imaging probe made of GRIN lenses was sufficient to resolve individual cells in the epithelium. The constant movement inherent to the function of the respiratory system defines one of the challenges of imaging a mouse airway at a single-cell resolution. However, cellular-level imaging was made possible by the side-view design, which results in a close proximity between the epithelium and the imaging window of the probe for high resolution, as opposed to the typical straight-view bronchoscopes (6, 7).

The imaging system, combined with the B1-EGFP mouse, enabled us to view the loss of Clara cells after epithelial injury and their regeneration in a live mouse over time in a quantitative manner. Although the measurement of cell population can also be done *ex vivo* by killing animals at different stages of injury and regeneration, *in vivo* imaging offers a number of distinct advantages. First, longitudinal monitoring can reduce the statistical sampling errors originating from animal-to-animal variability. Second, it generally requires a much smaller number of animals than histological analysis to achieve the same level of accuracy, especially with respect to the variability of injury in different animal models (15, 16), and rapidly and dynamically evolving processes, such as epithelial regeneration. Third, more importantly, the ability to comprehensively image the entire epithelium over a long segment of trachea, as well as for esophagus and descending colon (8), can provide information about the spatial distribution and population of cells and their proliferation and migration, the information that is often difficult to appreciate in tissue sections.

Across many research fields, from immunology to neuroscience, intravital imaging (17) has proven to be a powerful tool by visualizing various biological processes at the cellular and molecular level, such as gene expression, cell activation, trafficking, and interactions between cells, in real time in the natural environment *in vivo*. The endotracheal intravital fluorescence microscopy

presented here represents a synergy between the use of a novel, high-resolution, live imaging technique and newly developed transgenic mouse fluorescent epithelial cell type–specific reporters, and paves the way for further *in vivo* imaging studies in the field of respiratory cell biology.

Our work represents an initial step toward a myriad of applications of live microscopy in respiratory cell biology. Epithelial regeneration in the airway is central to recovery from a smoke inhalation, chemical pneumonitis from aspiration, and a variety of respiratory viruses, such as influenza and respiratory syncytial virus. Furthermore, failed epithelial regeneration may be central to the pathogenesis of airway fibrosis. *In vivo* imaging is expected to be useful for understanding the mechanisms of these disorders, especially as more epithelial cell subtype–specific fluorescent reporters become available and sophisticated animal models employing fluorescent tracers and reporters are developed. In addition to epithelial regeneration, the imaging technique presented here should be applicable to animal models of various airway problems, such as mucous metaplasia and squamous cell cancer, as well as infectious diseases.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Barry Stipp for providing the goat anti–Clara cell–specific protein antibody and Dr. Adam Glick for providing the CK5-rtTA mouse.

References

1. Rock JR, Randell SH, Hogan BL. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. *Dis Model Mech* 2010;3:545–556.
2. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BL. Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci USA* 2009;106:12771–12775.
3. You Y, Richer EJ, Huang T, Brody SL. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1315–L1321.
4. Kimura H, Hayashi K, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, Kishimoto H, Bouvet M, Hoffman RM. Real-time imaging of single cancer-cell dynamics of lung metastasis. *J Cell Biochem* 2010; 109:58–64.
5. Looney MR, Thornton EE, Sen D, Lamm WJ, Glenny RW, Krummel MF. Stabilized imaging of immune surveillance in the mouse lung. *Nat Methods* 2010;8:91–96.
6. Cortez-Retamozo V, Swirski FK, Waterman P, Yuan H, Figueiredo JL, Newton AP, Upadhyay R, Vinegoni C, Kohler R, Blois J, *et al*, Real-time assessment of inflammation and treatment response in a mouse model of allergic airway inflammation. *J Clin Invest* 2008;118:4058–4066.
7. Thiberville L, Moreno-Swirc S, Vercauteren T, Peltier E, Cavé C, Bourg Heckly G. *In vivo* imaging of the bronchial wall microstructure using fibered confocal fluorescence microscopy. *Am J Respir Crit Care Med* 2007;175:22–31.
8. Kim P, Chung E, Yamashita H, Hung KE, Mizoguchi A, Kucherlapati R, Fukumura D, Jain RK, Yun SH. *In vivo* wide-area cellular imaging by side-view endomicroscopy. *Nat Methods* 2010;7:303–305.
9. Kim J, Lee WM, Kim P, Choi M, Jung K, Kim S, Yun SH. Fabrication and operation of GRIN probes for *in vivo* fluorescence cellular imaging of internal organs in small animals. *Nat Protoc* 2012;7:1456–1469.
10. Barbara Zitová JF. Image registration methods: a survey. *Image Vis Comput* 2003;21:977–1000.
11. Becker C, Fantini MC, Neurath MF. High resolution colonoscopy in live mice. *Nat Protoc* 2006;1:2900–2904.
12. Miller RL, Zhang P, Smith M, Beaulieu V, Paunescu TG, Brown D, Breton S, Nelson RD. V-ATPase B1-subunit promoter drives expression of EGFP in intercalated cells of kidney, clear cells of epididymis and airway cells of lung in transgenic mice. *Am J Physiol Cell Physiol* 2005;288:C1134–C1144.
13. Ostrowski LE, Hutchins JR, Zakel K, O'Neal WK. Targeting expression of a transgene to the airway surface epithelium using a ciliated cell-specific promoter. *Mol Ther* 2003;8:637–645.
14. Kim P, Puoris'haag M, Cote D, Lin CP, Yun SH. *In vivo* confocal and multiphoton microendoscopy. *J Biomed Opt* 2008;13:010515.
15. Kavet RI, Brain JD. Reaction of the lung to air pollutant exposure. *Life Sci* 1974;15:849–861.
16. Rawlins EL, Okubo T, Xue Y, Brass DM, Auten RL, Hasegawa H, Wang F, Hogan BLM. The role of Scgb1a1^{+} Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. *Cell Stem Cell* 2009;4:525–534.
17. Pittet MJ, Weissleder R. Intravital imaging. *Cell* 2011;147:983–991.