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A subset of mouse tracheal epithelial basal cells generates large colonies in vitro

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Schoch, Kelly G., Adriana Lori, Kimberlie A. Burns, Tracy Eldred, John C. Olsen, and Scott H. Randell. A subset of mouse tracheal epithelial basal cells generates large colonies in vitro. Am J Physiol Lung Cell Mol Physiol 286: L631–L642, 2004. First published September 5, 2003; 10.1152/ajplung.00112.2003.—Airway epithelial stem cells are not well characterized. To examine clonal growth potential, we diluted single, viable B6.129S7-Otrosa26 (Rosa26) mouse tracheal epithelial cells that constitutively express β-galactosidase into non-Rosa26 cells in an air-liquid interface cell culture model; 1.7% of the cells formed colonies of varying size, and, on average, 0.1% of the cells formed large colonies. Thus only a small subset of cells displayed progenitorial capacity suggestive of stem or early transient amplifying cells. Prior studies identified cells with high keratin 5 (K5) promoter activity in specific niches in the mouse trachea and these cells corresponded to the location of bromodeoxyuridine label-retaining cells, thought to be stem cells (Borthwick DW, Shahbazian M, Todd KQ, Dorin JR, and Randell SH, Am J Respir Cell Mol Biol; 24: 662–670, 2001). To explore the hypothesis that stem cells were present in the K5-expressing compartment, we created transgenic mice in which enhanced green fluorescent protein (EGFP) was driven by the K5 promoter. These mice expressed EGFP in most basal cells of the body including a subset of tracheal basal cells apparently located in positions similar to previously identified stem cell niches. Flow cytometrically purified EGFP-positive cells had an overall colony-forming efficiency 4.5-fold greater than EGFP-negative cells, but the ability to generate large colonies was 12-fold greater. Thus adult mouse tracheal epithelial cells with progenitorial capacity sufficient to generate large colonies reside in the basal cell compartment. These studies are a first step toward purification and characterization of airway epithelial stem cells.

stem cell; cell lineage

THE AIRWAY EPITHELIUM OCCUPIES a critical interface between organism and environment, and its normal physiological function is essential for host well being. It is central to the pathogenesis of asthma, chronic bronchitis, cystic fibrosis, well-known and emerging infectious diseases, lung cancer, and posttransplant lung allograft rejection and is a prime target for gene therapy. Understanding basic cellular processes such as progenitor-progeny relationships and regulation of proliferation and differentiation is of fundamental importance. However, stem cells of this tissue and patterns of cell renewal and migration, at steady state, after injury, or in disease have not yet been identified with certainty.

Adult multipotent stem cells are defined by self-renewal and the ability to generate all the cell types within the tissue compartment. The long-held model of progenitor-progeny relationships within a tissue (for review see Ref. 19) consists of a linear, irreversible progression in which adult multipotent stem cells are usually held in reserve but generate highly proliferative transiently amplifying cells by asymmetric cell division as needed to fulfill the cell renewal needs of the tissue. Committed transiently amplifying progenitors replicate but ultimately undergo terminal differentiation. More recently, evidence for stem cell plasticity has challenged this paradigm, suggesting graded and potentially reversible changes in cell proliferation and differentiation capacity (reviewed in Ref. 2). Transdifferentiation may enable stem cells of one organ to repopulate another organ, as exemplified by bone marrow stem cell reconstitution of multiple distal organs (22). Recent reports suggest homing of bone marrow-derived cells to the airway and lung where they acquire expression of epithelium-specific proteins (14, 20, 22, 33, 34). Fusion of bone marrow stromal cells with small airway epithelial cells has been demonstrated in vitro (31). However, the overall physiological role of bone marrow-derived progenitors in vivo remains controversial (35). Regardless of stem cell origin, most cell turnover within a tissue is likely accomplished by tissue-specific, transiently amplifying cells periodically recruited from the stem compartment. In well-understood organs such as the skin and gut, there are specific spatial and temporal relationships between stem cells and their differentiated progeny (19). A “niche” environment is critical for maintaining stem cells and promoting appropriate cell fate and migration decisions (30, 36). However, we are only just beginning to understand progenitor-progeny relationships in the airway (25). In the pseudostratified mouse tracheal epithelium, bromodeoxyuridine (BrDU) label-retaining cells (LRC), thought to represent the stem cell compartment, have been localized to gland ducts in the upper trachea and to systematically arrayed foci in the lower trachea (3). In the columnar epithelium of the bronchioles, a subset of xenobiotic-resistant Clara cells within pulmonary neuroendocrine bodies likely represents a reserve population (16), whereas a different population of Clara progenitor cells exists at the bronchoalveolar duct junction (13). Yet there is still much to learn regarding airway epithelial stem cell niches and patterns of cell proliferation and migration. Furthermore, there is little information on the gene expression profile of airway stem cells, as is being established in other systems (17, 24, 26, 32). Thus airway epithelial stem cells remain poorly understood.

Classic studies of the epidermis reveal a spectrum of growth and differentiation potential among keratinocytes (1), and this approach was vital toward localizing stem cells to a specific portion of the pilosebaceous unit (21, 28). There are few
systematic studies of the clonal growth potential of airway epithelial cells in models supporting organ-specific differentiation. However, airway epithelial cells have been separated into subpopulations and examined in a tracheal graft model. Studies of this type give conflicting results, some suggesting that both basal and secretory cells can regenerate a complete mucociliary epithelium (23, 27) and others suggesting that only basal (11) or only secretory (18) cells are multipotent progenitors. The single study of clonal growth of the airway epithelium to date predicts a subset of surface epithelial cells pluripotent to generate glands (10).

The initial goal of the current investigation was to establish a model to examine the clonal growth potential of mouse tracheal epithelial cells. To accomplish this, single, viable B6.129S7-Gtrosa26 (Rosa26) tracheal epithelial cells that constitutively express β-galactosidase (βgal) were added at several dilutions into non-Rosa26 cells and seeded in a recently developed air-liquid interface (ALI) cell culture system supporting secretory and ciliated cell differentiation (37). This model consistently revealed the colony-forming efficiency (CFE) of the dissociated cells and suggested a diverse capacity for growth among tracheal epithelial cells.

As noted above, LRCs, thought to represent the stem cell compartment, localize to specific niches in the mouse trachea (3). An initial hint leading to identification of these cells was high-level keratin 5 (K5) promoter activity in specific locations, and the majority of the LRCs in the surface of the lower trachea were basal cells that are typically enriched with K5, -14, and -15 (12). To determine whether colony-forming cells were present in the K5-expressing basal cell compartment, we created transgenic mice expressing enhanced green fluorescent protein (EGFP) driven by the K5 promoter. K5EGFP mice were bred to Rosa26 mice, creating bigenic mice with EGFP in a subset of airway basal cells and βgal in all cells. EGFP-positive and -negative tracheal epithelial cells from bigenic mice were sorted by flow cytometry and were tested in the aforementioned CFE assay. Our results suggest that adult mouse tracheal surface epithelial cells with progenitorial capacity sufficient to generate large colonies in vitro reside in the basal cell compartment.

MATERIALS AND METHODS

Mice. All animals were handled under Institutional Animal Care and Use Committee-approved protocols. Rosa26 homozygote mice were from Jackson Labs (Bar Harbor, ME) and were bred to B6C3 F1 hybrid mice (Taconic Labs, Germantown, NY) to generate Rosa26 heterozygote mice. Mouse genotype was assessed by staining toes for βgal activity as previously described (6). We generated a novel transgenic mouse strain in which 6,000 bp of the basal cell-specific human K5 promoter drives expression of EGFP. The creation and initial characterization of K5EGFP mice will be reported elsewhere (Bruen K., personal communication). Briefly, a 6,000-bp fragment of the human K5 promoter (a kind gift from Dr. Elaine Fuchs, Rockefeller University) was cloned into a DNA expression vector coding for EGFP (BD Biosciences Clontech, Palo Alto, CA) with an intron and polyadenylation site from the pCI mammalian expression vector (Promega, Madison, WI). Promolecular injection of B6C3 F1 hybrid eggs with the appropriate DNA fragment was performed, and embryos were implanted, resulting in 51 potential founders. PCR and Southern blot confirmed EGFP gene sequences in seven mice, and fluorescence was visible in frozen sections of tail skin in six mice. The single transgenic with the strongest and most consistent EGFP expression in basal keratinocytes was propagated by breeding to B6C3 F1 hybrid mice. Genotype was routinely confirmed by visual inspection of excised toes under fluorescent illumination and by PCR for EGFP. Bigenic, Rosa26-K5EGFP mice were generated by crossing Rosa26 and K5EGFP heterozygote mice. The genotype of the offspring was determined by examining toes for EGFP as described above and staining for βgal (6). Confocal microscopy of 4% paraformaldehyde (PFA)-fixed frozen sections and anti-EGFP immunostaining of 4% PFA-fixed paraffin sections (see below) was used to assess EGFP protein expression in the trachea.

Lectin staining and immunostaining. Grifonia simplicifolia isolecitin B4 (GSI) B4 binding sites in mouse trachea were detected using biotinylated lectin (20 μg/ml) as previously described (29). Affinity-purified polyclonal antibodies against the mouse homologs of human cytokeratin 14 and 18 were generated as reported (3). All affinity-purified or monoclonal antibodies were used at a dilution of 5 μg/ml except as indicated and dilutions of antisera are given below. Anti-β-tubulin 4 antibody (2 μg/ml) was from Sigma (St. Louis, MO), affinity-purified rabbit anti-EGFP antibody was from AbCam (Cambridge, UK), anti-mouse CD45 was from Pharmingen (San Diego, CA), rabbit anti-mouse K5 antisera (1:4,000) was from Research Diagnostics (Flanders, NJ), rabbit anti-mouse K15 antisera (1:2,000) was a kind gift from Dr. Elaine Fuchs (Rockefeller University), a frequent anti-mouse Clara cell secretory protein (CCSP) antisera (1:1,000) was kindly provided by Dr. Francesco DeMayo (University of North Carolina), and rabbit (1:15,000) and goat anti-mouse CCSP (1:4,500) antisera were kind gifts from Drs. Susan Reynolds and Barry Stripp (University of Pittsburgh). Anti-βgal IgG fraction was from Rockland Immunochemicals (Gilbertsville, PA), and an affinity-purified antibody was prepared using bacterial βgal (Sigma) conjugated to Aminolink Gel (Pierce, Rockford, IL) and custom affinity purification procedures. Immunostaining was performed on acetone-fixed frozen sections, 4% PFA-fixed paraffin sections of trachea, or air-dried spots of dissociated airway epithelial cells (see below) using biotin-labeled secondary antibody and streptavidin-conjugated horseradish peroxidase or alkaline phosphatase or fluorescent secondary antibodies (all secondary antibodies were from Jackson Immunoresearch, West Grove, PA).

Mouse tracheal epithelial cell preparations and cell culture. Mouse tracheal epithelial cells were cultured as reported by You et al. (37) with slight modifications as given below. Briefly, following CO2 asphyxiation and external washing in 70% ethanol, tracheas were excised, freed from extratracheal tissues, placed in cold Ham’s F-12 medium with penicillin and streptomycin, slit longitudinally, and incubated in the same media containing 1.5 mg/ml pronase (Roche Molecular Biochemicals, Indianapolis, IN) at 4°C on a rocker for 18 h. FBS (Sigma) was added to a concentration of 10%, and tracheas were inverted 12 times, transferred to another tube of F-12 medium with 10% FBS, and inverted again. The process was repeated, the tracheas were discarded, and the three tubes were pooled and centrifuged at 400 g for 10 min at 4°C. Cells were resuspended in 200 μl/trachea of F-12 medium containing 0.5 mg/ml crude pancreatic DNase I (Sigma) and 10 mg/ml BSA, incubated on ice for 5 min, centrifuged at 400 g for 5 min at 4°C, and resuspended in mouse tracheal epithelial culture (MTEC) basic medium (see Media for mouse cultures) with 10% FBS. Cells were seeded in Primaria tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) for 3–4 h in 5% CO2 at 37°C to remove fibroblasts. Nonadherent cells were collected by centrifugation, resuspended in 100–200 μl/trachea of MTEC/Plas (see Media for mouse cultures), and counted. Cell yield averaged 0.52 ± 0.05 × 10⁶ cells per mouse (mean ± SEM from 79 mice in seven experiments using 8–14 mice per experiment) with viability always >95%. Very few cells were positive for the panleukocyte marker, CD45. 12-mm diameter Transwell Clear (0.4 μm pore size, Corning-Costar, Corning, NY) membranes were precoated with a filter-sterilized solution of 300 μg/ml type I rat tail collagen (Becton Dickinson) in 0.02 N acetic acid for 18 h at 25°C. The apical membrane surface was typically
seeded with 2.5 × 10^5 cells, and the upper and lower chambers were filled with MTEC/Plus medium (see Media for mouse cultures). Cultures were grown in 5% CO₂ at 37°C, and medium was changed every two days until visible confluence (between days 5 and 10) when the medium was removed from the upper chamber to establish an ALI. MTEC/NS medium (see Media for mouse cultures) was replaced in the lower chamber every two days, and cultures were grown for a total of 21 days. All cultures initiated for the experiments reported herein grew to confluence between days 5 and 10 and maintained an ALI until fixation and analysis on day 21.

**Media for mouse cultures.** All ingredients were from Sigma unless specified otherwise. MTEC Basic consisted of DMEM/Ham’s F-12 1:1, 15 mM HEPES, 3.6 mM sodium bicarbonate, 4 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone. MTEC/Plus consisted of MTEC Basic media plus 10 µg/ml insulin, 5 µg/ml transferrin, 0.1 µg/ml cholera toxin, 25 ng/ml

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**Fig. 1.** Clonal growth and colony forming efficiency (CFE) of single, viable Rosa26 mouse tracheal epithelial cells. **A:** polygons indicate gates used for flow cytometric cell sorting. Gating on forward (FSC) and side scatter (SSC) [region 1 (R1)] eliminated debris, gating on pulse width (R2) eliminated cell clusters, and propidium iodide (PI) exclusion (R3) was used to gate out dead cells. Cells were accepted if they were within the overlap of all the regions, R1, R2, and R3. **B:** enface micrographs of 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal)-stained cultures seeded with 100% Rosa26 cells, 100% non-Rosa26 cells, or a 1:100 mixture as indicated (original magnification ×2.5). **C:** enface micrographs of individual X-gal-stained colonies from chimeric cultures illustrating the range of observed colony sizes (original magnification ×20). **D:** CFE results from 3 consecutive experiments. No difference in CFE was observed using 1:100, 1:500, or 1:1,000 dilutions of Rosa26 cells, and the combined results are shown. The bars represent means ± SE of 6–9 wells.
epidermal growth factor (Becton-Dickinson), 30 μg/ml bovine pitui-
tary extract, 5% FBS, and freshly added 0.01 μM retinoic acid. MTEC/NS consisted of MTEC Basic media plus 2% NuSerum (Bec-
ton Dickinson) and freshly added 0.01 μM retinoic acid.

Flow cytometry and cultures of sorted cells. After pancreatic Dnase I treatment and centrifugation as described in Mouse tracheal epithelial cell preparations and cell culture, tracheal epithelial cells collected from Rosa26, KSEGFP, Rosa26/KSEGFP bigenic or litter-
mate control mice were resuspended in 1 ml of F-12 medium. An equal volume of declumping solution consisting of 2 mM EDTA, 0.5 mg/ml DTT, 0.25 mg/ml collagenase (Sigma), and 10 μg/ml Dnase was added, and, after 30 min on ice, FBS was added to 20%. Cells were centrifuged and resuspended in 1 ml PBS + 1% BSA, and 10 μl of propidium iodide (PI) solution (100 µg/ml, sterile filtered in PBS) were added ~15 min before analysis and sorting. Cells were run on a MoFlo flow cytometer (Cytomation, Fort Collins, CO), and single, viable cells from Rosa26 mice, and single, viable, EGFP-positive and -negative cells from KSEGFP or bigenic mice were obtained using gates as described in Results. After sorting, an aliquot of every preparation of EGFP-positive and -negative cells was rerun on the flow cytometer to assess their purity. Remaining sorted cells were resuspended in MTEC-Plus medium and manually counted in a hemacytometer, giving counts very close to the number obtained from cell enumeration. For CFE experiments with Rosa26 cells, a 1:100, 1:500, or 1:1,000 dilution of viable sorted cells was mixed with 250,000 negative cells (1:500) and cultured. For CFE experiments with EGFP-positive and -negative sorted cells were suspended in PBS at a concentration of 1 × 10^6 cells per ml, and 10-μl spots were placed within a hydrophobic circle (PAP Pen; The Binding Site, San Diego, CA) on microscope slides and air dried. Slides were stored at −80°C until staining with GSI lectin or antibodies as described in Lectin staining and immunostaining for tracheal sections. Slides were systematically scanned, and 500 cells per spot from three different sorts were scored for GSIb, lectin, tubulin, or CCSP staining, using the criteria of stronger staining than the negative control. The controls were inclu-
sion of 0.2 M galactopyranoside for lectin, an equal concentration of isotype-matched irrelevant mouse antibody for β-tubulin 4, and a twofold lower dilution of normal rabbit serum for CCSP. For electron microscopy (EM), ~0.5 × 10^6 EGFP-positive and -negative sorted cells were centrifuged and fixed in 2% formaldehyde, 2% glutaralde-
hyde and conventionally processed using osmium tetroxide postfix-
ation, epoxy embedding, and ultrathin sectioning and staining before viewing. Photomicrographs were prepared from representative cells.

Staining of cultures and scoring CFE. Control or chimeric cultures were stained for βgal activity (6). In each experiment, a membrane seeded with 100% Rosa26 cells and 100% non-Rosa26 cells was used as positive- and negative-staining controls, respectively. Colonies were scored in chimeric cultures under a Nikon stereo microscope at ×10. Small colonies had <20 blue cells, medium colonies 20–100 blue cells, and large colonies >100 blue cells. CFE was calculated as the number of βgal-positive colonies observed divided by the number

![Fig. 2. Expression of differentiation markers in colonies of varying size. Chimeric day 21 cultures (1:100 Rosa26 to B6C3) were simultaneously stained for β-galactosidase (βgal, the marker for a Rosa26-derived colony), Clara cell secretory protein (CCSP, Clara cell marker), and β-tubulin 4 (ciliated cell marker) and were viewed by laser scanning confocal microscopy. Colonies of different sizes are illustrated showing each fluorescent channel individually and a merged image as indicated. Although Clara cells were infrequent in these mature cultures, all large colonies, as well as approximately half of the medium-sized colonies and occasional small colonies, contained ciliated cells. Original magnification ×400.](http://ajplung.physiology.org/issue/286/4/A634/A636.html)
of βgal-positive cells seeded × 100. To determine the likelihood of colony derivation from more than a single cell, double chimeric cultures were created using both Rosa26 and C57BL/6-TGN(ActbEGFP)1Osb mice (the latter ubiquitously express EGFP driven by the chicken β-actin promoter, obtained from Jackson Labs) as described in an online data supplement (http://ajplung.physiology.org/cgi/content/full/00112.2003/DC1).

Evaluation of differentiation within colonies. Rosa26 chimeric (1:100 dilution), ALI cultures were fixed with 4% PFA in PBS for 10 min then processed for simultaneous localization of βgal, CCSP, and β-tubulin 4 using primary rabbit, goat, and mouse antibodies (as specified in Lectin staining and immunostaining), respectively. After being washed with PBS, fixed cultures were incubated, with gentle rocking, in blocking solution (5% normal donkey serum, 1% BSA, and 1% fish gelatin in PBS with 0.05% Tween 20) for 2 h, and all three primary antibodies were added in blocking solution overnight at 4°C. After being washed with diluent (a 1:3 dilution of blocking solution with PBS 0.05% Tween 20) three times for 15 min, Cy2-, TRITC-, and Cy3-conjugated donkey anti-rabbit, -goat, and -mouse antibodies (no species cross-reaction grade, Jackson Immunoresearch) in diluent were added at empirically optimized concentrations for 4 h. After extensive final washing, cultures were viewed under a Zeiss 510 Meta laser scanning confocal microscope. Chimeric cultures stained with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) were processed for cross-sectional histology to assess cell types within colonies.

RESULTS AND DISCUSSION
Establishment of a CFE assay for mouse tracheal epithelial cells. Ex vivo clonogenic assays are highly useful for identifying putative stem cells (1, 21, 28), but none has heretofore been...
described for the airway epithelium in a model supporting differentiation of basal, ciliated, and secretory cells. Our initial goal was to examine the CFE of cells capable of forming large colonies. We used new and improved methods for mouse tracheal cell isolation and culture as described by You et al. (37). Positive and negative control cultures were seeded with 100% Rosa26 or 100% non-Rosa26 cells, respectively. Single, viable Rosa26 cells were purified by flow cytometry with gating on forward and side scatter to exclude debris, pulse width to exclude clumps, and PI fluorescence to exclude dead cells. Chimeric cultures were seeded with a 1:100, 1:500, or 1:1,000 dilution of Rosa26 cells in non-Rosa26 cells (Fig. 1). At day 21 of culture, cells were fixed, stained for β-gal, and scored for small, medium, and large colonies. β-Gal-positive colonies of varying size were easily visible, and the results of three sequential experiments were highly consistent. The average total CFE was 1.73 ± 0.12% (mean ± SE, n = 3), and the CFE of cells capable of forming large colonies was 0.11 ± 0.07% (mean ± SE, n = 3). As described in the online data supplement (http://ajplung.physiology.org/cgi/content/full/00112-2003/DC1), double chimeric cultures using both Rosa26 and C57BL/6-TGN(ACTbEGFP)1Osb mice (EGFP driven by the chicken β-actin promoter) were created to assess the potential clonal error rate. The results suggested that <1% of the colonies were derived from more than one single cell.

In addition to self-replication and sufficient growth capacity to sustain cell numbers, an adult stem cell must generate each of the cell types constituting the tissue compartment. To evaluate differentiation capacity within clones, we simultaneously localized β-gal (the marker for a Rosa26 derived colony), CCSP (Clara cell marker), and β-tubulin 4 (ciliated cell marker) in day 21 chimeric cultures. As illustrated in Fig. 2, the CCSP stain worked well, as indicated by occasional strongly positive cells. CCSP expression was very scattered in day 21 cultures, and very few β-gal-positive clones contained CCSP-positive cells. This is consistent with the report of You et al. (37), who observed strong diminution in Clara cell number after day 14 in culture. In contrast, wide areas of day 21 cultures were highly ciliated. All large colonies, as well as approximately half of the medium-sized colonies and occasional small colonies, were observed to contain β-tubulin 4-positive ciliated cells. The confocal microscopic views shown in Fig. 2 represent x-y planes though the middle of the basal-to-apical colony axis, a compromise to show most of the cells in the colony, and were not optimal for colocalizing the β-tubulin 4-positive ciliated tufts. Because differentiation capacity is a key marker of stem cells, we analyzed day 21 triple-stained colonies by x-z confocal scanning and by histological analysis of X-gal-stained cross sections. As shown in Fig. 3, β-gal-positive ciliated cells were observed on both x-z confocal scans and histological cross sections of colonies. Cells generating large colonies consistently differentiated into polarized columnar cells, including ciliated cells, but small and medium colony formers also had the potential to generate ciliated cells. Because CCSP expression was lost in the mature cultures, we performed triple staining on earlier time points. As shown in Fig. 3, individual and small clusters of β-gal-positive cells visible in day 3 cultures stained for CCSP. By day 7 colonies were more diverse and CCSP staining was decreased, resulting in fewer CCSP/βgal dual-positive cells. In this set of cultures, CCSP was strongly reduced by day 14, and few CCSP-positive cells were present. Predictably, few β-tubulin 4-positive cells were visible on days 3 and 7, but by day 14 ciliated cells became apparent, and β-tubulin 4/βgal dual-positive cells were observed (not shown). In summary, cells destined to form colonies transiently expressed CCSP before colonial expansion and the development of ciliated cells. Large colonies contained both basally oriented and columnar β-gal-positive cells, indicating multilineage differentiation capacity.

The ability to generate a large differentiated colony may depend not only on the cells’ intrinsic properties but also on their environment. Thus we assessed whether the number of non-Rosa26 “stuffer” cells would affect CFE by keeping the number of Rosa26 cells constant and systematically manipulating the number of non-Rosa26 cells (Fig. 4). Despite the lower seeding density of stuffer cells in some wells, all cultures
grew to confluence within 10 days and maintained an ALI until day 21. The CFE was higher at high stuffer cell seeding densities, and average colony size was small. In wells with fewer stuffer cells, the CFE was lower, but colony size was somewhat larger. Our speculation is that at higher stuffer seeding densities, the dishes became confluent before the full proliferative capability of the cells was realized. At low stuffer seeding densities, cells may grow to their full potential, but cells with borderline growth potential may not be adequately supported by paracrine survival factors until the time of fixation and staining. Further studies are needed to define the mechanisms altering colony size and numbers at different seeding densities.

Are large colonies indicative of adult airway epithelial stem cells? Proliferative capacity sufficient to generate a large colony under the stress of in vitro culture coupled with consistent generation of differentiated columnar cells supports the concept that cells forming large colonies are stem or early transiently amplifying cells. While ciliated cells were consistently observed, few Clara cells were noted in the mature, day 21 cultures. Analysis of earlier time points in culture indicates that cells ultimately forming colonies transiently expressed CCSP. The reason for this pattern of CCSP expression is not totally clear. It is possible that the majority of tracheal cells capable extensive growth in the in vitro model, which likely originate mostly from the upper trachea (37), may not be programmed for sustained generation of Clara cells under the current culture conditions. Further studies are needed, including novel approaches to track the proliferation and differentiation of single cells and their progeny, to assess the full repertoire of colonial growth and whether a specific pattern is associated with airway epithelial stem cells. As illustrated in Fig. 4, CFE and colony size depend on growth conditions as well as intrinsic cellular properties. The key question is whether the culture conditions we used enabled successful recruitment of stem cell growth and differentiation potential. We assume that the compromise

**Fig. 5.** Characterization of enhanced green fluorescent protein (EGFP) expression in the trachea of K5EGFP mice. A and B: endogenous tracheal fluorescence of wild-type littermates of K5EGFP mice (wt) and K5EGFP hemizygous transgenic (\(\delta/\)) mice, respectively, as detected by confocal microscopy of paraformaldehyde-fixed frozen sections. Fluorescent basal cells (arrowheads) are visible just above the autofluorescent basal lamina and below columnar cells containing brightly autofluorescent granules/lysosomes in this view of a cross section from the vicinity of the cartilage-intercartilage junction. C–G: EGFP immunostaining of a longitudinal paraformaldehyde-fixed paraffin section of K5EGFP \(\delta/\) mouse trachea at low power (D), with high-power insets (C, E–G). EGFP-positive basal cells are a subset of all basal cells and are present within upper tracheal gland ducts (C, E), scattered foci in the upper tracheal surface (C, E, G), and above the cartilage-intercartilage junction in the middle to lower trachea (F). Wt K5EGFP mouse tracheas stained identically did not demonstrate any positive cells (not shown). H and I: *Griffonia simplicifolia* isoelectin B4 (GSIB4) lectin staining to reveal all basal cells in the upper (H) and lower (I) trachea for comparison to the pattern of EGFP-positive cells. Tracheal surface basal cells are contiguous with tracheal gland duct basal cells (H). Original magnification \(\times 400\) (A, B, \(\times 40\) (D), \(\times 500\) (C, E–G) and \(\times 200\) (H, I).
conditions we adopted for the bulk of our experiments enable stem cells or early transient amplifying cells to form large colonies that consistently differentiate to ciliated cells. However, formal demonstration that large colonies arise from adult airway epithelial stem cells will require additional proofs such as localization of LRC and subculture. Further studies are also necessary to determine whether small colonies originate from cells with limited proliferation potential or aborted colonies or whether they represent fragmentation of larger colonies. It is interesting to note that if large colony formers represent stem cells or early transient amplifying cells, and these cells grow efficiently in this model, then one would calculate on the order of 5,000–10,000 stem cells/early transient amplifying cells in the surface epithelium per mouse trachea. In summary, the CFE assay we describe is a consistent and useful method for quantitating the growth potential of tracheal epithelial cells. There is a broad range of progenitorial capacity among the epithelial cell population, and a relatively small proportion of cells has adequate growth capacity to generate large colonies.

**K5EGFP transgenic mouse.** Prior studies suggested that high-level K5 promoter activity in specific locations in the mouse trachea corresponded with the position of LRCs, thought to represent the stem cell compartment (3). Having established a useful model to study the CFE of tracheal epithelial cells, we next sought to determine whether progenitor cells were enriched in the K5-expressing basal cell compartment. To accomplish this, we created transgenic mice expressing EGFP driven by the K5 promoter. These mice consistently and faithfully express EGFP in most basal cells in the body, especially in epidermal keratinocytes (not shown). EGFP was present in tracheal epithelial basal cells (Fig. 5) but
was not as intense as in the epidermis. We used both direct fluorescence detection by confocal microscopy on PFA-fixed frozen sections and immunoperoxidase staining on PFA-fixed paraffin sections to determine the EGFP expression pattern. In our experience, the antibody detection method for EGFP was more sensitive and consistent than viewing direct fluorescence, which was apparently reduced by technical factors including fixation time, freezer storage time, and the "EGFP shelf life" on the slides after sectioning. We compared and contrasted EGFP expression to several other classical markers of airway epithelial cell type, including GSIB4 lectin, K14, K15, K18, β-tubulin 4, and CCSP. Serial sections stained for EGFP and K5 are illustrated in Fig. 6. We conclude that EGFP was expressed in a subset of airway epithelial basal cells. Interestingly, EGFP expression appeared to correspond generally to the pattern of BrdU LRCs noted in a prior study (3). Qualitatively, EGFP-positive cells were visible in tracheal gland ducts, in the surface epithelium near the gland duct opening in the upper trachea, and in systematically arrayed foci in the lower trachea centered over the cartilage-intercartilage junction. The precise reason for EGFP expression in a subset of basal cells in K5EGFP mice may relate to the specific line of transgenic mice, promoter activity in subsets of basal cells, cell migration patterns, and protein half-life. First, the level of EGFP expression was dependent on the transgenic mouse line. We detected seven transgenic lines among 51 potential founders and selected the single line with the most intense and consistent expression in epidermal basal keratinocytes for the present study. We have not seen evidence for chimerism or mosaicism in this line of mice. Lesser reporter gene expression in internal organs versus skin is similar to the pattern in another transgenic mouse using the same promoter (4, 5). EGFP expression in a subset of tracheal basal cells may reflect comparatively low levels of K5 expression in the airway versus in skin. It is likely that there is a subset of tracheal basal cells with relatively high K5 promoter activity in specific locations in the mouse trachea and that cells lose K5 expression during outward migration from these foci. The half-life of EGFP protein is estimated to be 24–36 h (8), much shorter than is suspected for native K5, and EGFP protein may reflect the RNA expression pattern more closely than the long-lived K5 protein. Nevertheless, it is interesting that EGFP was present in a subset of basal cells that apparently had the highest levels of K5 promoter activity and that the location of these cells appeared to correspond with the positions of previously identified BrdU LRCs thought to represent the stem cell compartment (3). Further studies are needed to systematically address the three-dimensional distribution of EGFP-positive cells in the K5EGFP mouse and cell migration during cell replacement in the airway epithelium and to definitively establish whether cells with high K5 promoter activity are identical to BrdU LRCs or whether these cells are simply located close to one another.

Flow cytometry of K5EGFP mouse tracheal epithelial cells. We next examined whether flow cytometry would be useful for sorting dissociated tracheal epithelial cells from the K5EGFP mice. As shown in Fig. 7, a distinct population of green fluorescent cells was visible by flow cytometry in cells from K5EGFP transgene hemizygous mice but not in littermate wild-type controls. The fluorescent cells tended to be smaller and less granular than other epidermal cells as judged by relatively low forward and side scatter values, respectively. This pattern is similar to that previously observed for rat tracheal basal cells (27). We were able to consistently sort very pure populations of EGFP-positive or -negative cells as evidenced by a very low cross-contamination rate, 0.5 ± 0.1 and 0.1 ± 0.2% for EGFP-positive and -negative cells, respectively (mean ± SE, n = 4 sorts). Twelve to fourteen mice were used for each of the four sorts, resulting in total presort cell yields ranging from 5.2 to 8.4 × 10⁶ tracheal epithelial cells. The number of single, viable EGFP-positive and -negative cells per sort was nearly equal and ranged from 0.27 to 0.73 × 10⁶ cells per sort, per fraction. It is likely that some of the apparent cross-contamination from EGFP-positive sorted cells in the negative gate was due to cell death or fragmentation and EGFP leak during rerunning of the cells in the flow cytometer, causing the cells to "fall into" the zone of lower green fluorescence. The occasional cross-contaminating cell may have also been part of live cell doublets or triplets or dead cell-live cell clumps that were not screened out by gating and then released from each other upon being rerun in the flow cytometer.

Table 1. Composition of cell fractions

<table>
<thead>
<tr>
<th>Marker</th>
<th>Presort</th>
<th>EGFP+</th>
<th>EGFP−</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSIB4 lectin</td>
<td>54.8±3.5</td>
<td>97.8±2.8</td>
<td>73.7±8.5</td>
</tr>
<tr>
<td>β-Tubulin 4</td>
<td>48.2±9.3</td>
<td>0.3±0.1</td>
<td>5.5±4.4</td>
</tr>
<tr>
<td>CCSP</td>
<td>2.2±1.7</td>
<td>0</td>
<td>14.9±5.0</td>
</tr>
</tbody>
</table>

Values are means ± SD. 500 cells were scored per sample, n = 3 sorts for all values. EGFP, enhanced green fluorescent protein; GSIB4, Griffinia simplicifolia isolecitin; CCSP, Clara cell secretory protein.

Fig. 8. Qualitative electron microscopic characterization of EGFP-positive and -negative cells. Representative electron micrographs illustrate diversity among the EGFP-negative population including cells with abundant organelles suggestive of secretory cells and ciliated cells as well as small basal-like cells. The EGFP-positive fraction was almost entirely small cells with a high nuclear to cytoplasm ratio. Original magnification ×4,400.
Characterization of the sorted cell populations. To characterize the sorted cell populations, we examined cell smears using routine histological stains and performed immunostaining for a panel of conditional “cell type-specific” markers also studied in intact tracheas. The EGFP-positive cells were generally smaller and more consistent in size than EGFP-negative cells. The EGFP-positive cells were nearly 100% positive for the sensitive basal cell marker GSIB4 lectin, and this population contained very few Clara or ciliated cells as indicated by CCSP and β-tubulin staining, respectively (Table 1). Close to three-fourths of the EGFP-negative cells were also basal cells, and this population was enriched with Clara and ciliated cells. On the basis of our prior experience (27), EM is a less informative tool for quantitative morphometry of sorted cell populations due to the high probability of nondiagnostic profiles in random ultrathin sections. Nevertheless, we qualitatively examined the cells ultrastructurally as shown in Fig. 8. These results confirmed a rather homogeneous population of basal-like cells with high nucleus-to-cytoplasm ratio in the EGFP-positive fraction. The EGFP-negative fraction was more diverse, containing cells with abundant cytoplasm, including readily identifiable ciliated and secretory cells. In combination, the in vivo EGFP expression pattern in K5EGFP mice, the flow cytometry dot plots, lectin and immunostaining results on cell smears, and ultrastructural observations strongly suggest that the EGFP-positive fraction consists of subset of basal cells and that the EGFP-negative fraction represents all remaining cells. The low proportion of ciliated cells is not unexpected since it is widely appreciated that this cell type is more fragile and lost during cell dissociation and especially during sorting. The overall number of Clara cells viewed in CCSP-stained tracheal sections was consistent with the proportion of Clara cells noted in the dissociated primary cell population (Table 1). We also observed relatively few CCSP-positive Clara cells in the upper mouse trachea and a greater proportion in the lower trachea (not shown).

CFE of sorted populations. To directly test whether EGFP-positive or -negative cells had different progenitorial capacities, we performed four consecutive CFE experiments with sorted cells. As seen in Fig. 9, the CFE of the EGFP-positive cells was consistently greater than that of the EGFP-negative cells with the average CFE being 4.5-fold greater. The differences were more profound when colony size was considered. The ability to generate small colonies was, on average, only two- to threefold different. However, in two of four experiments, the EGFP-negative cells generated no large colonies, and overall, the large colony CFE was 12-fold less. These results are consistent with a diverse progenitorial capacity among different mouse tracheal epithelial cell types and even within the basal cell compartment. Apparently, cells with high levels of K5 promoter activity, namely a subset of basal cells, could generate large colonies.

Are adult stem cells of the mouse tracheal surface epithelium a subset of basal cells? Many factors contribute to the perennial debate about which cell types constitute the stem cell compartment of the airways. Different lineage pathways during development versus steady-state renewal in the adult, low basal rates of proliferation, regional cellular heterogeneity, great plasticity in growth capacity among resident airway cells in response to injury, and potential involvement of blood-borne progenitors all contribute toward making this a difficult system to understand fully. We show, for the first time, greater CFE of a subpopulation of airway epithelial cells in a model system supporting ciliated cell differentiation. Obviously, many technical factors must be considered when interpreting these results, especially the impact of removal from the in vivo environment and suitability of the in vitro assay to reveal stem cells. It is clear that growth in the model used is affected not only by the cells’ intrinsic properties but also by growth conditions. However, the most direct interpretation of our results is that stem cells or early transient amplifying cells are enriched in a subset of basal cells defined by high-level K5 expression.
promoter activity. This notion is supported by the observation that EGFP expression in the mouse tracheas in vivo appeared to correspond with the position of BrdU LRCs as seen in a prior study (3). However, additional studies are necessary, involving injury and chronic BrdU labeling of K5EGFP mice, to definitively prove this point.

In vitro growth of pseudostratified versus Clara cell-based cell lineage systems. The mouse trachea is a convenient unit to study, and the ease of mouse genetic manipulation enables the present approach. However, as noted in our prior study (3) and confirmed by CCSP immunostaining in the present study, the mouse trachea likely represents at least two related but distinct cell lineage “zones.” Cell renewal in the upper trachea may rely more on gland duct and surface basal cell progenitors, whereas the lower trachea may also involve a Clara cell-based lineage system, with a degree of overlap. Two prior studies show that the upper trachea was a more potent generator of in vitro cultures (9, 37). As also suggested by the numerous upper airway in vitro cell culture models versus few reports of proliferative lower airway, Clara cell-based cultures, pseudostratified airway cells are easier to grow in vitro than cells from more distal airways. The culture model we used may not have supported the growth of Clara cell-based progenitors and thus may have underestimated the growth capacity of Clara cells in the EGFP-negative population. A robust cell growth and differentiation model for Clara cells will be necessary to answer this question.

Implications for gene therapy. Although there are many obstacles to successful gene therapy, there remains hope that it may ultimately be useful for the treatment of airway diseases. Assuming that the pseudostratified airway epithelium is an important gene therapy target, the present study has both positive and negative implications. Basal progenitor cells may be inaccessible to luminal applied vectors, and a lasting change may be difficult to achieve without disrupting epithelial integrity to access progenitor cells. Alternatively, if integrating vectors put cells at risk for insertional mutagenesis (7, 15), and integrity to access progenitor cells. Alternatively, if integrating change may be difficult. Basal progenitor cells may ultimately be useful for the treatment of airway diseases. In vitro growth of pseudostratified versus Clara cell-based lineage systems. The mouse trachea is a convenient unit to study, and the ease of mouse genetic manipulation enables the present approach. However, as noted in our prior study (3) and confirmed by CCSP immunostaining in the present study, the mouse trachea likely represents at least two related but distinct cell lineage “zones.” Cell renewal in the upper trachea may rely more on gland duct and surface basal cell progenitors, whereas the lower trachea may also involve a Clara cell-based lineage system, with a degree of overlap. Two prior studies show that the upper trachea was a more potent generator of in vitro cultures (9, 37). As also suggested by the numerous upper airway in vitro cell culture models versus few reports of proliferative lower airway, Clara cell-based cultures, pseudostratified airway cells are easier to grow in vitro than cells from more distal airways. The culture model we used may not have supported the growth of Clara cell-based progenitors and thus may have underestimated the growth capacity of Clara cells in the EGFP-negative population. A robust cell growth and differentiation model for Clara cells will be necessary to answer this question.

Conclusions. We describe a reproducible CFE assay useful for quantitating the growth capacity of mouse tracheal epithelial cells. We found diverse progenitorial capacity among different airway epithelial cells. Only a small subset of cells was capable of generating large colonies, possibly representing stem cells or early transient amplifying cells. These cells were enriched in the subcompartment of airway basal cells with the greatest K5 promoter activity. This report represents an initial step toward the ultimate purification and characterization of airway epithelial stem cells.

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REFERENCES