Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population

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You, Yingjian, Edward J. Richer, Tao Huang, and Steven L. Brody. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. Am J Physiol Lung Cell Mol Physiol 283: L1315–L1321, 2002. First published August 9, 2002; 10.1152/ajplung.00169.2002.—Highly regulated programs for airway epithelial cell proliferation and differentiation during development and repair are often disrupted in disease. These processes have been studied in mouse models; however, it is difficult to isolate and identify epithelial cell-specific responses in vivo. To investigate these processes in vitro, we characterized a model for primary culture of mouse tracheal epithelial cells. Small numbers of cells seeded at low density (7.5 × 10⁴ cells/cm²) rapidly proliferated and became polarized. Subsequently, supplemented media and air-liquid interface conditions resulted in development of highly differentiated epithelia composed of ciliated and nonciliated cells with gene expression characteristic of native airways. Genetically altered or injured mouse tracheal epithelial cells also reflected in vivo patterns of airway epithelial cell gene expression. Passage of cells resulted in continued proliferation but limited differentiation after the first passage, suggesting that transit-amplifying cell populations were present but with independent programs for proliferation and differentiation. This approach provides a high-fidelity in vitro model for evaluation of gene regulation and expression in mouse airway epithelial cells.

airway; cilia; Clara cell; progenitor cell

AIRWAY EPITHELIAL CELL proliferation and differentiation during lung development and repair are highly regulated, complex processes (6, 24). In lung disease, these processes are influenced by growth factors, cytokines, or proteases released by mesenchymal and inflammatory cells (11, 24). To determine epithelial cell-specific function in diseases such as asthma and cystic fibrosis, primary human airway epithelial cell culture has been valuable (11, 21, 28), although defined mutations and phenotypes are limited and analysis is complicated by genetic variation between individuals.

To characterize the role of a single gene in airway epithelial cell function during lung development or disease, isogenic animals have been generated with deficient or augmented gene expression. Like human disease, these mice often have complex responses involving airway epithelial cells (11, 30). To identify a cell-specific response in airway epithelial cells from genetically defined mice, it would be desirable to culture and manipulate these cells in vitro. However, in contrast to human and other species, there are few approaches for primary culture of differentiated mouse airway epithelial cells (5, 7, 15). Available primary culture protocols for mouse tracheal epithelial cells (MTEC) require large numbers of cells and result in minimal cell differentiation (5, 7, 15). Problems in primary culture include the inherent low cell yield from each mouse trachea and possible differences in growth factor requirements for amplifying progenitor cell populations and induction of specific cell phenotypes.

We have defined conditions for primary culture of MTEC that result in rapid proliferation and generation of a highly differentiated epithelium reflecting wild-type and mutant phenotypes. In vitro passage and subsequent regeneration of differentiated epithelial cell populations suggest the presence of progenitor-like cells located in the proximal trachea.

MATERIALS AND METHODS

Culture media and supplements. Media formulations were modified from previously described methods (14, 17, 19, 26–28). Supplements were from Sigma-Aldrich (St. Louis, MO) unless indicated. “Hams F-12 pen-strep” is Ham’s F-12 media with 100 U/ml penicillin and 100 μg/ml streptomycin. “MTEC Basic” media is DMEM-Ham’s F-12 (1:1 vol/vol), 15 mM HEPES, 3.6 mM sodium bicarbonate, 4 mM L-glutamine, 100

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U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone. “MTEC/Plus” is MTEC basic media supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 0.1 μg/ml chola-
toxin, 25 ng/ml epidermal growth factor (Becton-Dickinson, Bed-
ford, MA), 30 μg/ml bovine pituitary extract (17), 5% PBS, and freshly added 0.01 μM retinoic acid. “MTEC/NS” is
MTEC basic media supplemented with 2% NuSerum (Bec-
ton-Dickinson) and freshly added 0.01 μM retinoic acid.

Mouse tracheal cell isolation. Tracheal cells from wild-type C57Bl/6, SV129/J, C57Bl/6-SV129/J hybrid, Friend Virus B-
Type (FVB), Clara cell secretory protein (CSP)-deficient
(kindly provided by B. Stripp, University of Pittsburgh), and
FoxJ1-deficient mice were evaluated (3, 22). For most studies, wild-type C57Bl/6 or hybrid mice, 3–6 wk of age were used.
MTEC harvest was modified from previously described meth-
ods (7, 19, 28). Mice were killed and then briefly immersed in
70% ethanol (avoiding airway submersion). With the use of a
scissors, tracheas were dissected from the larynx to the bronchal
main branches and branches in ice-cold Ham’s F-12 pen-strep.
In a tissue culture hood, muscle and vascular tissues were
dissected from tracheas in cold media. Tracheas were
washed with media, opened longitudinally, and then
incubated in Ham’s F-12 pen-strep containing 1.5 mg/ml
pronase (Roche Molecular Biochemicals, Indianapolis, IN) for
18 h at 4°C. The tube was then put on ice, and FBS was added
to a final concentration of 10%. The tracheas were inver-
ted 12 times, transferred to another tube of Ham’s F-12 pen-
strep with 10% FBS, inverted again, placed in one-third tube
of media, inverted to further release cells, and then dis-
carded. Contents of the three tubes were pooled and collected
by centrifugation at 400 g for 10 min at 4°C. Cells were
resuspended in 200 μl/trachea of Ham’s F-12 pen-strep con-
taining 0.5 mg/ml crude pancreatic DNase I (Sigma-Aldrich)
and 10 mg/ml BSA. The cells were then incubated on ice for
5 min, centrifuged at 400 g for 5 min at 4°C, and resuspended
in MTEC basic media with 10% FBS. After incubation in
tissue culture plates (Primera; Becton-Dickinson Labware,
Franklin Lakes, NJ) for 3–4 h in 5% CO2 at 37°C to adhe-
er fibroblasts, nonadherent cells were collected by centrifu-
gation, resuspended in 100–200 μl MTEC/Plus per trachea, and
counted. No attempt was made to achieve a single cell sus-
pension from cell clumps. The average yield of tracheal cells
was 1.81 × 106 cells/trachea (±0.58 × 106 (SD)) from 34
preparations obtained from 162 mice weighing 10–20 g. Cell
viability determined by trypan blue exclusion was >90%.

In vitro culture of mouse tracheal cells. Supported polycar-
bionate and polyester porous (0.4 μM pores) membranes
(Transwell and Transwell Clear; Corning-Costar, Corning,
NY) were coated with filter-sterilized 50 μg/ml type I rat tail
collagen (Becton-Dickinson) in 0.02 N acetic acid using 1.0
ml/cm2 membrane for 18 h at 25°C. Membranes were seeded
with 7.5 × 104 cells/cm2 and incubated with MTEC/Plus
filling upper and lower chambers in 5% CO2 at 37°C. Media
were changed every 2 days until the transmembrane resist-
tance (Rt) was >1,000 Ω-cm2, as measured by an epiphy-
thal Ohm-voltmeter (World Precision Instruments, Sarasota, FL).
Media were then removed from the upper chamber to estab-
lish an air-liquid interface (ALI), and lower chambers only
were provided fresh MTEC/NS or MTEC/SP media every 2
days. Cells on Transwell Clear membranes were monitored by
inverted-phase microscopy. To remove epithelial cells
from membranes for enumeration or passage, cells were
incubated in Cell Dissociation Solution (Sigma-Aldrich) sup-
plemented with 0.25% trypsin and 2.7 mM EDTA at 37°C for
15 min and resuspended in Ham’s F-12 pen-strep with 10% PBS.
Cells were collected by centrifugation and resuspended
in MTEC/Plus for reseeding.

Immunofluorescent labeling and analysis. Membranes were
fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10
min at 25°C, washed in PBS, cut from supports into two to six
pieces, and processed for immunodetection in 24- or 96-well
dishes. Cell sections (6 μm) were obtained after fixed mem-
branes were submerged in warm 2% agarose and then em-
bedded in paraffin. Nonspecific antibody binding was blocked
using 5% donkey serum and 3% BSA in PBS for 30 min at
25°C. Samples were incubated for 18 h at 4°C with isotype-
matched control antibody or primary antibody in blocking
solution. Control or primary antibody binding was detected
using FITC- or indocarbocyanine-labeled secondary antibody
(Jackson ImmunoResearch Laboratories, West Grove, PA). 25% goat serum
in PBS was used to block endogenous peroxidase activity, and
control membranes. Membranes were mounted on slides with
Vectorshield (Vector, Burlingame, CA) containing 4’,6 di-
aminidino-2-phenylindole to stain intracellular DNA. Photomi-
icroscopy was performed using an Olympus BX51 camera
(Melville, NY) to acquire images with a CCD (MagniFire;
Olympus) interfaced with MagniFire software. At least three
fields of cells were counted directly or analyzed using Na-
tional Institutes of Health Image software (version 1.25;
National Institutes of Health; http://rsb.info.nih.gov/nih-
image). Primary antibodies and dilutions or concentrations
were as follows: rabbit pan-cytokeratin (1:500; Biomedical
Technology, Stoughton, MA), rabbit anti-20–1 (0.5 μg/ml;
Zymed, San Francisco, CA), rabbit anti-TTF-1 (2.0 μg/ml;
Biopat, Caserta, Italy), rabbit anti-aquaporin 4 (2.5 μg/ml;
Chemicon, Temecula, CA), mouse anti-Muc5AC (0.4 μg/ml;
Lab Vision, Fremont, CA), mouse anti-β-tubulin-IV (30 μg/
ml; BioGenex, San Ramon, CA), and rabbit anti-mouse CCSP
(1:500; kindly provided by F. DeMayo, Baylor College of
Medicine, Houston, TX). Staining with bionylated lectin
Bandeiraea (Griffonia) simplicifolia BS-1 isoelectin S4 (50
μg/ml; Sigma-Aldrich) detected by Texas red-labeled avidin
(Vector) was used to identify basal cells, as described pre-
vously (20).

Cell proliferation assay. Cells were incubated in media
containing 10 μM 5-bromo-2’-deoxyuridine (BrDU; Sigma-
Aldrich) for 2 h, fixed as above, treated with 4 N hydrochloric
acid for 15 min, and then neutralized in 0.1 M sodium borate,
PH 8.5 for 20 min. Cells were incubated with rat anti-BrDU
antibody (0.5 μg/ml; Accurate Chemical, Westbury, NY) in
PBS containing 0.2% Triton X-100 for 30 min at 25°C, and
primary antibody was detected using rabbit anti-rat FITC-
labeled antibody (Jackson ImmunoResearch Laboratories).
Cells expressing BrDU were counted as described above. To
determine responses after wounding, cells on membranes were
embossed with the proximal end of an 8-mm glass Pasteur
pipette to denude a 1-mm ring of cells before BrDU labeling.

Scanning electron microscopy. Tracheas and membranes
were prepared for scanning electron microscopy (SEM), as
previously described (18). Briefly, samples were fixed with
2.5% glutaraldehyde stained with 1.25% osmium tetroxide,
critical point dried under liquid carbon dioxide, gold sputter
coated, and visualized on a Hitachi S-450 microscope (Tokyo,
Japan).

Statistical analysis. R and cell numbers were analyzed for
statistical significance using the Student’s t-test and a one-
way ANOVA for a factorial experimental design. The two-sample and multicomparison significance level for one-way ANOVA was 0.05. If significance was achieved by one-way analysis, post-ANOVA comparison of means was performed using Scheffe's F-test (29).

RESULTS

Formation of tight junctions and polarization of cultured mouse tracheal cells. MTEC seeded at low density on membranes developed a polarized cell layer demonstrated by development of $R_t$, exclusion of media from the upper chamber during ALI conditions, and expression of tight-junction protein ZO-1 (Fig. 1). In the presence of supplemented, FBS-containing media (MTEC/Plus), $R_t$ increased over the first 5–6 days as cells became confluent (Fig. 1A). ALI was created for each preparation when $R_t$ of all membranes was $>1,000\ \Omega\ \text{cm}^2$. At this time (ALI day 0), media were removed from the upper chamber, and cells were cultured in MTEC/NS or MTEC/SF media. $R_t$ gradually decreased after creation of ALI, as noted by others (7). Fewer than 4% of Transwell inserts never formed electrically tight junctions. To correlate the increase in $R_t$ with gene-specific expression, ZO-1, a component of tight-junction assembly, was evaluated (13). ZO-1 was expressed in portions of the peripheral cell membranes (Fig. 1B) before development of high $R_t$; however, after ALI was created, peripheral membranes uniformly expressed ZO-1, consistent with the establishment of a layer of polarized epithelial cells.

MTEC growth kinetics. ZO-1 expression also suggested changes in cell size and number. We found that cell number increased markedly within the first week of culture, reflecting proliferation as detected by BrDU incorporation and resulting in a multilayered epithelium (Fig. 2). Although $7.5 \times 10^5\ \text{cells/cm}^2$ cells were seeded on the membrane, after 3 days, total cell number was approximately one-half (40.6%), indicating that only a fraction of seeded cells adhered to the membrane, as described in tracheal cell culture from other species (25). During a discrete proliferation phase in the first 7–10 days, cells grown in MTEC/SF resulted in a greater number of cells than in MTEC/NS. After this phase, BrDU incorporation in MTEC was 0.1–0.2% in MTEC/NS and 1–2% in MTEC/SF. In either media, multiple cell layers developed with tall ciliated and Clara cells apparent at ALI day 5 (Fig. 2B). MTEC grown in MTEC/NS typically had one to two layers, whereas MTEC in MTEC/SF had two to three layers.

Fig. 1. Polarization of cultured mouse tracheal cells. A: transmembrane resistance ($R_t$) during mouse tracheal epithelial cell (MTEC) culture. Cells were seeded ($7.5 \times 10^5\ \text{cells/cm}^2$) on 0.33-cm$^2$ polycarbonate membranes and submerged in serum- and supplement-enriched MTEC/Plus (Plus; $\bullet$) media until $R_t$ was $>1,000\ \Omega\ \text{cm}^2$ when the air-liquid interface (ALI) was established. After ALI day 0, $R_t$ was measured in cells cultured in NuSerum-containing MTEC/NS (NS; $\bullet$) or serum-free MTEC/SF (SF; $\circ$) media at indicated days. Values are means $\pm$ SD ($n = 16–50$ samples/day) from 3 preparations. $^{*}P < 0.05$, significant difference compared with ALI day 0, 3, 5, and 7. B: expression of ZO-1 in cell membrane junctions detected by immunofluorescence at indicated days. Representative samples are shown. Bar = 10 $\mu$m.

Fig. 2. MTEC growth kinetics. MTEC were cultured as in Fig. 1A. A: cell number (solid lines) and proliferation (broken lines) of MTEC cultured in MTEC/Plus media (Plus; $\bullet$) until ALI and then in NuSerum-containing (●) or serum-free (○) media. Proliferation measured by 5-bromo-2'-deoxyuridine (BrDU) incorporation was expressed as a percentage of total cells, as described in MATERIALS AND METHODS. Values are means $\pm$ SD ($n = 3–9$ samples) from 5 preparations. $^{*}P < 0.05$, significant difference between cells grown in different media. B: histological appearance of MTEC cultured in different media compared with normal mouse trachea. Arrows, ciliated cells; arrowheads, Clara cells. Cells fixed on membranes at indicated days, embedded in paraffin, and sectioned (6 $\mu$m) were stained with hematoxylin and eosin. Bar = 20 $\mu$m.
The multicell layers at ALI day 14 resembled native trachea (Fig. 2B) and tracheal cell cultures from other species (8, 25, 28).

MTEC apical surface differentiation. To further characterize changes in cell histology, epithelial cell apical surface morphology was observed by scanning EM (Fig. 3). This showed large, flat, uniform cells with microvilli at ALI day 0 (Fig. 3A). From ALI day 0 to day 10, cells became smaller, more dome-like, and ciliated. Thus, after proliferation, there was a distinct phase of epithelial cell differentiation. By ALI day 10, the surface of the cultured MTEC morphologically resembled mouse trachea (Fig. 3B).

Expression of differentiation markers in MTEC. Changes in histology and morphology correlated with known markers of airway epithelial cell differentiation (Fig. 4). As observed in vivo, airway epithelial cell transcription factor TTF-1 was expressed in all MTEC evaluated at ALI days 0, 7, and 14 (6, 24). Lectin B. simplificofolia isolecitin B4 (BS-B4) staining was used as a marker of basal cells and had an affinity for cells in the layer adjacent to the membrane but not upper layers, also as observed in vivo (20). Similarly, aquaporin 4 expression was detected in the basolateral domain of cells after ALI day 0 (1). Unlike normal lung (10, 25), Muc5AC was expressed in large numbers of cells at ALI day 5 and in fewer cells by ALI day 14, but typical mucus cells were not found in samples processed for histological evaluation (Fig. 2B).

The expression of β-tubulin-IV in ciliary axonemes was used to detect ciliated cells, and CCSP was used as...
a marker of secretory cells (Fig. 4, B and C). The abundance of ciliated cells mirrored the mouse trachea, but the number of CCSP-expressing cells was less than in vivo (18). After ALI day 14, ciliated cells covered about one-third of the apical cell surface [MTEC/NS, mean 30.9 ± 8.6% (SD); MTEC/SF, mean 39.1 ± 10.5% (SD)]. In contrast, CCSP expression varied with time and media, but in both media were significantly less abundant after ALI day 14, suggesting that the cell microenvironment changed over time. In vitro gene expression analyzed in MTEC cultured from CCSP or Foxj1 knockout mice (−/−) (Fig. 4B) mirrored their in vivo patterns of expression. CCSP(−/−) MTEC had no detectable CCSP expression; however, ciliated cell numbers were similar to wild-type preparations. MTEC from Foxj1(−/−) mice (with absent cilia) lacked apical staining of β-tubulin-IV, but had CCSP-expressing populations similar to the wild type.

**MTEC proliferation and differentiation during injury and passage.** To determine if MTEC were capable of proliferation and redifferentiation in vitro as required for wound healing, mature cells (ALI day 18) grown in MTEC/NS were wounded and observed for proliferation and differentiation. Uninjured MTEC exhibited a low percentage of BrDU incorporation (as shown in Fig. 2), but 1 day after injury BrDU incorporation was increased markedly at the wound edge (Fig. 5A). Later (3 days), nearly the entire denuded region was replaced by large numbers of ciliated cells, suggesting the presence of an MTEC population capable of epithelial cell proliferation and differentiation, similar to in vivo repair (16).

Serial passage of primary cells was used to identify the presence of transit-amplifying cells capable of proliferation and differentiation after a quiescent period. Cells grown in MTEC/SF could be passaged, whereas cells in MTEC/NS media did not form new confluent layers. To determine if cells could continue to proliferate and differentiate after passage, cells cultured in MTEC/SF were harvested at ALI day 10 and reseeded (7.5 × 10^4 cells/cm^2) and cultured as in passage 0. During the first passage, cells lost CCSP and β-tubulin-IV differentiation markers before the ALI and then developed ciliated cells numbers similar to passage 0 but fewer CCSP-expressing cells (Fig. 5B). In passages 2 and 3, rare cells expressed either marker, demonstrating that MTEC retained its proliferative capacity, independent of programs for differentiation.

Cell populations residing in or near the tracheal glands have been implicated as a source for tracheal epithelial progenitor cells (2, 7). Serial sections of trachea showed glands only in the most proximal segments of the trachea, as previously noted (2). Therefore, we divided tracheas in half and cultured MTEC harvested from proximal and distal tracheal segments. Compared with MTEC from total trachea or the proximal segment, distal trachea MTEC cultures had a 1- to 2-day delay in achieving tight junctions and fewer cells at ALI day 12 (Fig. 5C). At ALI day 12, the percentage of ciliated and CCSP-expressing cells was similar in MTEC from proximal and total segments, but the degree of differentiation was significantly less in distal trachea-derived cells. These observations suggest that, although small numbers of cells with progenitor capacity may reside in the distal trachea, cells with the potential to proliferate and differentiate are primarily within the proximal trachea.

**DISCUSSION**

We found that MTEC can be differentiated rapidly using defined conditions to model native mouse trachea.
with several advantages. First, in the presence of a supplemented media, cells rapidly proliferate, permitting successful initiation of cultures from a small number of cells harvested from trachea (7.5 × 10⁴ cells/cm²; less than one-half a trachea). This allows evaluation of MTEC when numbers of mice are limited and facilitated analysis of MTEC from surviving Foxj1(−/−) mice that are runted and die at <3 wk of age (3, 22). In contrast, prior MTEC protocols used a seeding density of 1.2 × 10⁶ cells/cm² (6 adult mouse tracheas; see Ref. 7). Second, selective media supplementation and ALI conditions also rapidly led to a high degree of differentiation with large numbers of ciliated, CCSP-, mucin-, and aquaporin-expressing cells that have not been described previously in MTEC cultures. This makes possible in vitro study of gene regulation using approaches not feasible in vivo. Third, culture conditions allow proliferation and redifferentiation in vitro for the study of airway repair in the absence of immune cell mediators. Finally, primary culture of cells from genetically deficient mice mirrored in vivo phenotypes in Foxj1 and CCSP null MTEC. This provides a potent alternative approach for in vitro analysis of airway epithelial cells from isogenic mice with deficient or augmented gene expression to characterize and manipulate cell-specific responses.

Conditions used for MTEC proliferation and differentiation were similar to those used to culture tracheal cells from human, rat, and other species. Those studies showed that ALI, insulin, epidermal growth factor, and retinoic acid were important for cell proliferation, ciliogenesis, and mucus cell differentiation (4, 8, 17, 23, 27, 28). We also found that a simple media with a modified serum supplement (NuSerum; see Ref. 9) compared with Ultroser G (see Refs. 7 and 28) resulted in many more ciliated cells, increased cell layer number, and increased cell height (unpublished observations), likely because of differences in proprietary additives. Conditions we used favor ciliogenesis but were associated with a rise then fall in numbers of CCSP-expressing cells. This change in secretory cell populations was seen by others during human airway cell culture (8) and could be a result of undefined secreted factors or changes in matrix composition that support different cell types. Although we noted Muc5AC expression in many cells, we did not specifically create conditions found by others to favor mucus cell differentiation such as lowering epidermal growth factor concentrations or using a collagen gel substratum (10, 25). In mature cells, mucus was present on the apical aspect of cells, and electron-dense granules were found by transmission EM in nonciliated cells that may contain mucin or CCSP (data not shown), suggesting that functional secretory cells are present. Characterization of secretory cells and the effect of varying concentrations of retinoic acid, epidermal cell growth factor, or cytokines on changes in cell populations or gene expression may be addressed in future studies.

Passage, proliferation, and subsequent differentiation of the primary culture MTEC suggest that populations of transit-amplifying cell populations are present, similar to progenitor-like cells found in vivo (2, 12) and in vitro rat and human airway cell cultures (27). We found that, after 3 days, only a small percentage of cells initially seeded on membranes became adherent and proliferated, indicating a subpopulation of progenitor-like cells survived in the culture conditions (27). Molecular markers of airway progenitor cells are not defined; however, at ALI days −3, 0, and 3, these cells expressed fundamental airway epithelial cell transcription factors TTF-1 and hepatocyte nuclear factor-3β (data not shown) but not CCSP or β-tubulin-IV. B. simplicifolia BS-1 isoelectric S4 (BS-B4), a putative basal cell marker associated with repairing epithelial cells (20), was detected in over one-half of the cells at this time. However, BS-B4 also stained many cells at ALI days 7 and 14 when proliferation was low, suggesting that BS-B4 may not be a specific marker of transit-amplifying cells. Consistent with the observations of others, we also found that, under the growth conditions provided by our in vitro culture system, cells most capable of proliferation and differentiation appeared to reside in the proximal trachea, possibly in or around tracheal glands (2, 7); however, we did not evaluate gland cell marker expression. Finally, these findings show that programs for proliferation are not directly linked to differentiation, since, despite the presence of a transit-amplifying population that permitted proliferation, differentiation was limited after the first passage. It is possible that an additional cell population capable of proliferation and differentiation has limited survival within the conditions established.

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