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DIFFERENTIATED CULTURES OF PRIMARY HAMSTER TRACHEAL AIRWAY EPITHELIAL CELLS

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SUMMARY

Primary airway epithelial cell cultures can provide a faithful representation of the in vivo airway while allowing for a controlled nutrient source and isolation from other tissues or immune cells. The methods used have significant differences based on tissue source, cell isolation, culture conditions, and assessment of culture purity. We modified and optimized a method for generating tracheal epithelial cultures from Syrian golden hamsters and characterized the cultures for cell composition and function. Soon after initial plating, the epithelial cells reached a high transepithelial resistance and formed tight junctions. The cells differentiated into a heterogeneous, multicellular culture containing ciliated, secretory, and basal cells after culture at an air–liquid interface (ALI). The secretory cell populations initially consisted of MUC5AC-positive goblet cells and MUC5AC/CCSP double-positive cells, but the makeup changed to predominantly Clara cell secretory protein (CCSP)–positive Clara cells after 14 d. The ciliated cell populations differentiated rapidly after ALI, as judged by the appearance of β tubulin IV–positive cells. The cultures produced mucus, CCSP, and trypsin-like proteases and were capable of wound repair as judged by increased expression of matrilysin. Our method provides an efficient, high-yield protocol for producing differentiated hamster tracheal epithelial cells that can be used for a variety of in vitro studies including tracheal cell differentiation, airway disease mechanisms, and pathogen–host interactions.

Key words: goblet; Clara; respiratory virus; trachea; influenza; wound repair; ribonucleic acid virus.

INTRODUCTION

Primary cell culture systems provide a valuable bridge between animal studies and the use of transformed or immortalized cell lines in studies of tissue development and function. It is clear that animal studies provide the best system for studying organ and tissue function; however, the inability to perform real-time observations; precisely control nutrient, toxin, or growth factor concentrations; and difficulty in assessing direct versus indirect effects limit the experimental parameters that can be tested in vivo. In contrast, cell lines are grown easily and have defined characteristics (inducible gene expression, cell type–specific markers, etc.) but are usually derived from a single cell type and cannot perform all the functions attributed to the original organ or tissue.

The airway epithelium consists of a variety of cell types: ciliated cells that propel mucus up through the respiratory tract, secretory cells that are primarily responsible for secreting factors within the mucus layer, and basal cells that are hypothesized to play a structural role and serve as a pool of progenitor cells that can repopulate a damaged epithelial cell layer (Wu, 1997). Studies of function and development of airway epithelial cells are important in understanding the basis of diseases such as cystic fibrosis, cigarette smoke–associated airway injury, and are particularly interesting to us in the study of microbial pathogen–induced injury and disease. Many groups have cultured tracheal epithelial cells (TECs) for evaluating airway growth and development (Wu and Smith, 1982; Lee et al., 1984; Kaartinen et al., 1993; You et al., 2002) as well as pathogen–host interactions (Goldman et al., 1982; Look et al., 2001; Sinn et al., 2002; Walters et al., 2002; Zhang et al., 2002), and protocols have ranged from tracheal ring organ cultures (Collier et al., 1977) to enzymatic dissociation followed by various culture conditions (Goldman and Baseman, 1980; Lee et al., 1984; Wu et al., 1985; Yamaya et al., 1992). The most successful techniques have described culturing at an air–liquid interface (ALI) (Whitcutt et al., 1988; Yamaya et al., 1992; Robison et al., 1993; You et al., 2002). Specific hormone media formulations and ALI culture conditions have allowed researchers to create heterogeneous cultures that differentiate into a variety of cell types, including the major cell types found in the airways (Wu, 1997).

The use of Syrian golden hamsters as a small animal model for a variety of human pathogens has been of particular interest to us. Microbial pathogens ranging from severe acute respiratory syndrome (SARS) coronavirus (Buchholz et al., 2004), West Nile virus (Morrey et al., 2004), Nipah virus (Wong et al., 2003), South American hantaviruses (Hooper et al., 2001; Milazzo et al., 2002), human parainfluenza virus 3 (Tao et al., 1999), *Bordetella pertussis* (Collier et al., 1977), influenza (Ali et al., 1982), Rift Valley Fever virus

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TABLE 1

HAMSTER TEC MEDIA FORMULATIONS[a]

Media	Components	Concentration
TEC Basic[b]	DMEM/F-12	1:1
	$NaHCO_3$	0.03%
	HEPES	15 m*M*
	Penicillin	100 U/ml
	Streptomycin	100 μg/ml
	Glutamine	2 m*M*
	Amphotericin B	250 ng/ml
TEC Plus[c]	TEC Basic	
	Insulin	10 μg/ml
	Transferrin	5 μg/ml
	Cholera toxin	0.1 μg/ml
	Epidermal growth factor	25 ng/ml
	Bovine pituitary extract	15 mg/ml
	Fetal bovine serum	5% v/v
	Retinoic acid	0.05 μ*M*
TEC MM[d]	TEC Basic	
	NuSerum	2% v/v
	Retinoic acid	0.05 μ*M*

[a] TEC, tracheal epithelial cells; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DMEM, Dulbecco modified Eagle medium.

[b] You et al., 2002.

[c] Lee et al., 1984; Wu et al., 1985; Yamaya et al., 1992; Kaartinen et al., 1993; You et al., 2002.

[d] Karp et al., 2002; You et al., 2002.

(Fisher et al., 2003), eastern equine encephalitis virus (Paessler et al., 2004), and leishmania (Melby et al., 2001) have been shown to infect and cause disease in hamsters. Because several of these pathogens interact with the airway epithelium, we sought to optimize and streamline protocols for the isolation and culture of well-differentiated hamster TECs. Our protocol is based on other TEC protocols, using mouse (You et al., 2002), guinea pig (Robison et al., 1993), human (Yamaya et al., 1992, 2002; Karp et al., 2002), and hamster (Lee et al., 1984; Wu et al., 1985; Moller et al., 1987; Whitcutt et al., 1988) tissue. We achieved a high efficiency of cell isolation per hamster (5×10^5 to 6×10^5 cells/trachea), resulting in the seeding of 10–15 wells/animal. The cells are initially devoid of significant numbers of ciliated or secretory cells but rapidly differentiate into a heterogeneous culture containing ciliated, secretory, mucous, and basal cells. Cell types were quantified by immunostaining, characterized by electron microscopy, and possessed secretory and ciliated cell activities. Thus, hamster TECs can provide a faithful in vitro representation of the in vivo tracheal epithelium for studying a variety of systems.

Materials and Methods

Reagents and antibodies. The components in TEC basic media (TEC Basic), proliferation media (TEC Plus), and maintenance media (TEC MM) are described in Table 1. Rabbit anti-hamster Clara cell secretory protein (CCSP) (1:500 immunofluorescence; 1:2000 Western blot) was kindly provided by Gurmukh Singh (VA Medical Center, Pittsburgh, PA). The mouse anti-M_2 antibody, 14c2, (1:500 immunofluorescence) was courtesy of Robert Lamb (Northwestern University, Chicago, IL). Other primary antibodies were purchased as follows: mouse anti-MUC5AC (1:100 immunofluorescence; clone 45M1, NeoMarkers, Freemont, CA) (Bara et al., 1998; Nordman et al., 2002), mouse anti-β Tubulin IV (1:100 immunofluorescence; BioGenex, San Ramon, CA), rabbit anti-zonular occludin 1 (ZO-1) (1:100 immunofluorescence; Zymed, San Francisco, CA), rabbit anti-matrilysin (1:50 immunofluorescence; Ab-4, Oncogene, San Diego, CA), mouse anti-β actin (1:7500 Western blot; Abcam, Cambridge, MA), and goat anti-hemagglutinin (HA-H3 subtype) Aichi/2/68 sera (1:1000 Western blot; NIH/NIAID reference reagent V314-591-157). Secondary antibodies were used as follows: goat anti-mouse fluorescein isothiocyanate (1:250 immunofluorescence), goat anti-rabbit horseradish peroxidase (HRP) (1:7500 Western blot), donkey anti-goat HRP (1:7500 Western blot), goat anti-mouse HRP (1:7500 Western blot), all purchased from Jackson Immunoresearch (Westgrove, PA); goat anti-rabbit Alexa Fluor 594 (1:500 immunofluorescence) and ToPro3 nuclear stain (1:150 immunofluorescence) were purchased from Molecular Probes (Eugene, OR).

Hamster TEC isolation. Six- to eight-week-old female Syrian golden hamsters (Charles River Laboratories, Wilmington, MA) were euthanized by isofluorane inhalation and sodium pentobarbital injection (400 mg/kg injected intraperitoneally). Tracheas were excised from below the larynx to the major bronchi. The explanted tracheas were digested for 12–18 h with 0.3% pronase (Sigma, St. Louis, MO) in Ham's F-12 with 100 U/ml penicillin and 100 μg/ml streptomycin followed by deoxyribonuclease treatment (0.5 mg/ml in Ham's F-12, Calbiochem, EMD Biosciences, San Diego, CA) for 10 min at 4° C (You et al., 2002). Cells were resuspended in TEC Basic (Table 1) with 5% fetal bovine serum (FBS) and plated in a 3.5-cm cell culture plate for 3 h at 37° C, 5% CO_2 to remove contaminating fibroblasts (You et al., 2002). The recovered epithelial cells were plated on thin-layer collagen-coated 0.4-μm pore 0.33 cm^2 Transwell-Clear (Corning Costar, Corning, NY) supported membranes (You et al., 2002). Collagen coating was performed by applying 50 μg/ml rat tail collagen type I (diluted in 0.02 *N* glacial acetic acid) to each membrane, incubating overnight at 4° C, and washing three times with phosphate-buffered saline (PBS) before cell plating. Cells were plated at a density of 1×10^5 cells/cm^2, and typical yields were 5×10^5 to 6×10^5 cells/trachea. Cells were grown in TEC Plus (Table 1), and apical and basolateral media were changed every 2 d (Lee et al., 1984; Wu et al., 1985; Yamaya et al., 1992; Robison et al., 1993; You et al., 2002). Transepithelial resistance (TER) was measured with the Millipore Millicell-ERS (Millipore, Bedford, MA). On reaching a TER of >1000 Ω-cm^2, the apical media was removed to create an ALI (Whitcutt et al., 1988; Yamaya et al., 1992; Robison et al., 1993), and the basolateral media was then replaced with TEC MM (Table 1) (Yamaya et al., 1992; Karp et al., 2002; You et al., 2002). The cells were routinely infected between days 10 and 14 ALI.

Immunofluorescence confocal microscopy. At the indicated d relative to ALI, hamster TECs were washed with PBS (GIBCO Inc., Carlsbad, CA) and fixed in 2% paraformaldehyde for 10 min at room temperature. After fixation, the cells were washed with PBS and permeablized with PBS containing 0.2% Triton X-100, 0.1% sodium citrate for 10 min at room temperature. The cells were incubated with PBS containing 3% normal goat serum and 0.5% bovine serum albumin (blocking buffer) for 30 min at room temperature, with the primary antibody in blocking buffer for 1 h, washed, and incubated with secondary antibodies for 1 h. Nuclei were then stained with ToPro3 for 15 min at room temperature. All washes were performed with PBS containing 0.2% Tween 20. The membranes were mounted with Molecular Probes Prolong antifade (Molecular Probes), and the cells were observed using a Zeiss LSM confocal microscope.

Influenza A virus infection. Air–liquid interface hamster TECs (10 d) and Madin–Darby canine kidney (MDCK, American Type Culture Collection, Manassas, VA) cells were infected at the indicated multiplicity of infection with a recombinant influenza A virus, strain A/Udorn/72 (rUdorn courtesy of Robert Lamb, Northwestern University, Chicago, IL) (Takeda et al., 2002). Mucus was removed by washing twice with warm TEC Basic. Cells were infected through the apical chamber in a total volume of 50 μl for 1 h at 37° C in the presence or absence of 10 μg/ml *N*-acetyl trypsin (Sigma). The inoculum was removed and washed twice with Dulbecco modified Eagle medium (DMEM), 10% FBS, to remove and inactivate trypsin, and once with PBS. The apical inoculum was replaced with 50 μl DMEM, (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO) either with or without 10 μg/ml *N*-acetyl trypsin. The basolateral media was changed at the time of infection to TEC MM. Madin–Darby canine kidney cells were infected in the presence or absence of 10 μg/ml of *N*-acetyl trypsin according to standard procedures (Paterson and Lamb, 1993; McCown et al., 2003). The apical supernatant was collected for plaque assay at the indicated h postinfection (hpi). Plaque assays for hamster TEC infections were performed on MDCK cells as described previously (Paterson and Lamb, 1993; McCown et al., 2003).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting. Briefly, cells were washed once in PBS and lysed in 100 μl of 1% sodium dodecyl sulfate (SDS). Lysates were diluted in 100 μl of 2× SDS

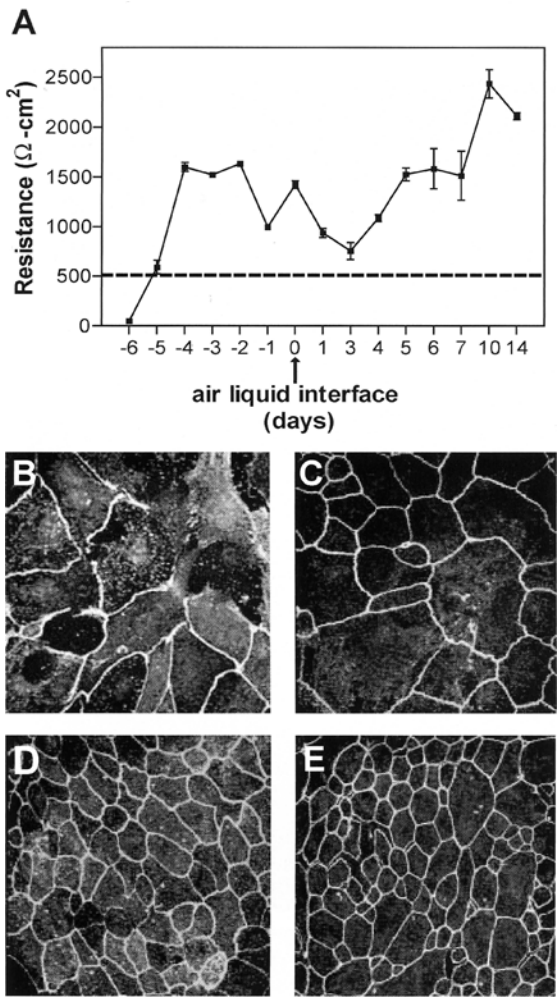

FIG. 1. Hamster tracheal epithelial cell (TEC) tight junction formation. (*A*) Transepithelial resistance was measured across the cell layer at the indicated d relative to the air–liquid interface (ALI). Zonular occludin 1 (ZO-1) staining was monitored by immunofluorescence confocal microscopy at (*B*) −6 d, (*C*) −3 d, (*D*) 0 d, and (*E*) 5 d ALI. All images were acquired at ×63 magnification.

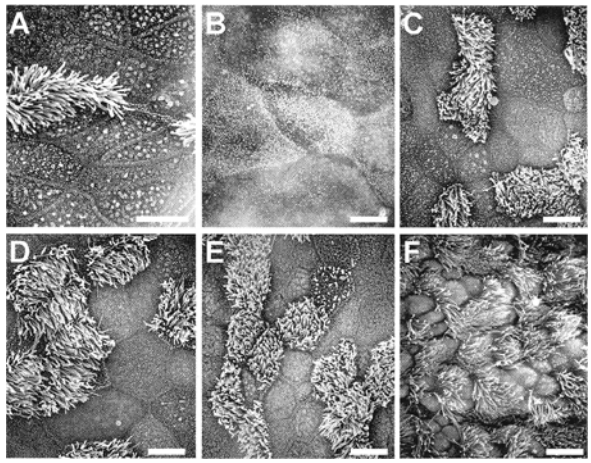

FIG. 2. Scanning electron microscopy (SEM) of hamster tracheal epithelial cell (TEC) differentiation. Scanning electron microscopy was performed on hamster TECs at various d after ALI: 0, 3, 5, 10, and 14 d (*A–E*, respectively) or on tracheal sections from a 6-wk-old female hamster (*F*). *Bar* represents 5 μm.

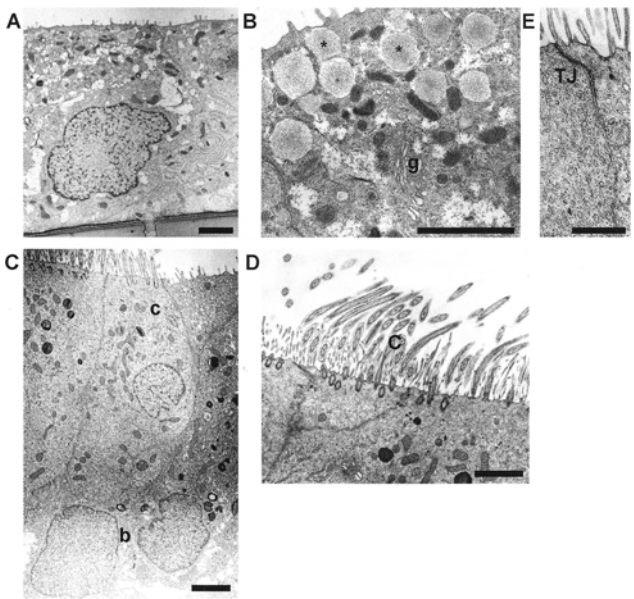

FIG. 3. Ultrastructural analysis of hamster tracheal epithelial cells (TECs) by transmission electron microscopy (TEM). Transmission electron microscopy of 21 d ALI hamster TECs. Nonciliated (secretory) cells at (*A*) low magnification and (*B*) high magnification of secretory vesicles (*) and golgi stacks (g). (*C*) Ciliated (c) and basal cells (b) at low magnification and (*D*) high magnification of cilia (c). (*E*) Tight junction (TJ) between two cells. *Bar* represents (*A–D*) 2 μm and (*E*) 1 μm.

loading buffer (Paterson and Lamb, 1993), run through a 22-gauge needle, sonicated for 15 min at 42 kHz, and boiled for 15 min. For blotting of CCSP, β-mercaptoethanol was added to lysates at a final concentration of 144 m*M* before boiling. Lysates were run on a 15% polyacrylamide mini gel (BioRad, Hercules, CA) for 3 h at 75 V, and for detection of CCSP, proteins were separated on a 17.5% polyacrylamide gel containing 4 *M* urea. Proteins were transferred to a PVDF membrane (Millipore Immobilon-P) at 100 V for 1 h (Mini Transblot, BioRad) and blocked in PBS with 5% dry milk. Blots were incubated in primary and secondary antibodies for 1 h at room temperature, washed with PBS supplemented with 1% Tween 20, and developed using the Amersham (Piscataway, NJ) ECL-Plus substrate.

Wound repair and matrilysin expression. Air–liquid interface hamster TECs (19 d) were injured by scraping the cell layer with a 200-μl pipette tip, taking care not to damage the support filter. Loose cells were removed by washing three times with warm PBS containing Mg^{++} and Ca^{++}, and the cells were left at the ALI. Transepithelial resistance was measured at the indicated times after injury by adding 150 μl of warm TEC Basic to the apical chamber and incubating for 5 min at 37° C in a 5% CO_2. Three measurements were taken for each of three random mock and injured samples.

Clara cell secretory protein secretion assay. Clara cell secretory protein secretion was monitored at the indicated d before and after ALI by applying 50 μl of TEC Basic to the apical chamber and pipetting 10 times to dislodge the mucus from the cell surface. Clara cell secretory protein secretion was measured from the same well for the duration of the experiment. Samples were brought to 1% SDS by addition of 1/10 volume 10% SDS solution. Cell lysates were taken by lysing the cells on the membrane in 100 μl of 1% SDS.

Scanning electron microscopy. Hamster TECs at various d ALI or hamster tracheal tissue was fixed with 2.5% glutaraldehyde in PBS or 0.1 *M* cacodylate buffer followed by postfixation with 1% aqueous osmium tetroxide. Tracheal tissues were further processed using the osmium–thiocarbohydrazide–osmium method (Kelly et al., 1973). The samples were dehydrated in a

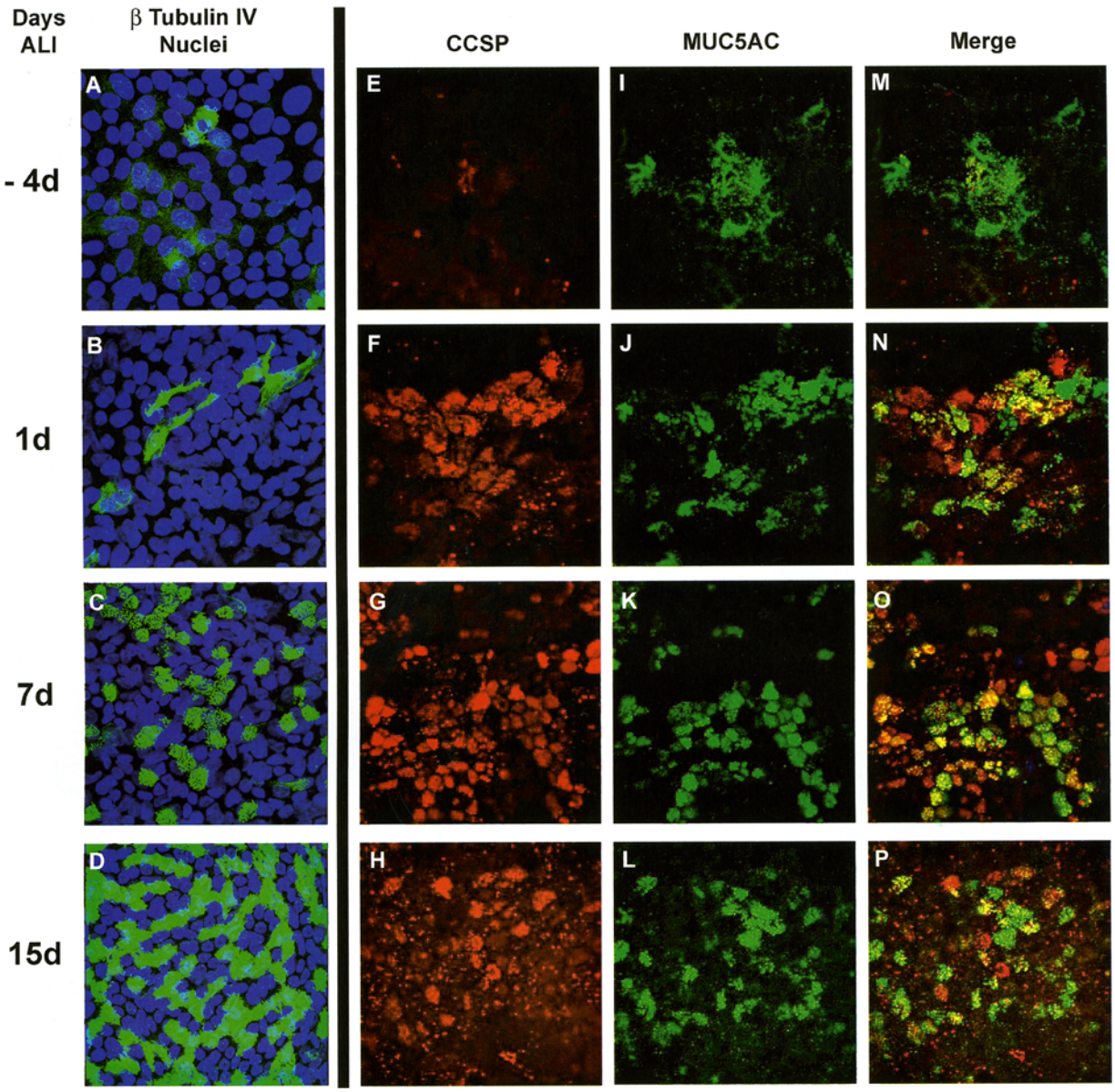

FIG. 4. Hamster tracheal epithelial cells (TECs) differentiate into ciliated and secretory cells after ALI. Hamster TECs were stained for various differentiation markers at the indicated time points relative to ALI. Cells were costained for (*A–D*) β Tubulin IV (green) and nuclei (blue), or (*E–H*) Clara cell secretory protein (CCSP) (red) and (*I–L*) MUC5AC (green), merged images for CCSP and MUC5AC are shown in (*M–P*). All images were acquired at ×63 magnification.

graded series of ethanol and critical point dried with CO_2. The mounted samples were sputter coated with 25 nm of gold and examined in a Hitachi S-450 scanning electron microscope (Tokyo, Japan) operated at 20 kV accelerating voltage.

Transmission electron microscopy. Hamster TECs were fixed at 21 d ALI with 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in a 100 m*M* phosphate buffer, pH 7.2 for 1 h at room temperature. After three washes in phosphate buffer, the cells were postfixed in 1% osmium tetroxide (Polysciences) for 1 h at room temperature. The samples were then rinsed extensively in dH_2O before en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h at room temperature. After several rinses in dH_2O, cells were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella). Sections of 70–80 nm were cut, stained with 2.5% uranyl acetate and 0.66% lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA, Inc., Peabody, MA).

RESULTS

Hamster TEC polarization and tight junction formation. Isolated epithelial cells were plated into Transwell chambers at a density of 1×10^5 cells/cm^2, and the formation of tight junctions was monitored by measuring the TER across the cell layer at the indicated d before and after ALI (Fig. 1*A*). The TER increased postplating and remained >500 Ω-cm^2, decreased slightly after ALI, but quickly regained a TER of >1500 Ω-cm^2 through 14 d ALI (Fig. 1*A*).

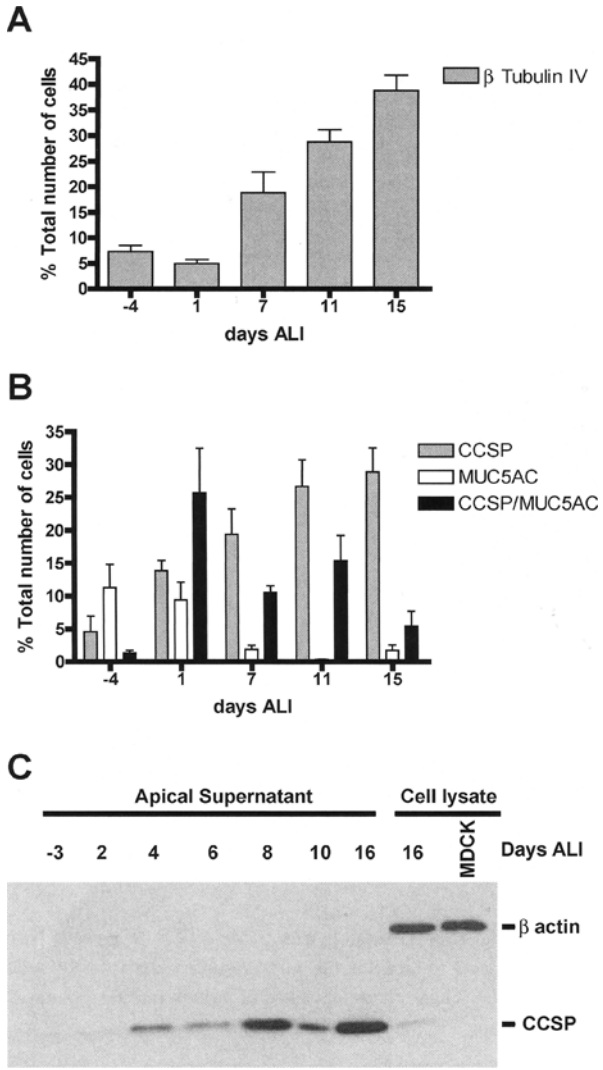

FIG. 5. Ciliated and secretory cell populations and Clara cell secretory protein (CCSP) secretion increase after the air–liquid interface (ALI). The ciliated and secretory cell populations were quantitated as a percentage of the total cell number. Cells were divided into (*A*) ciliated cells (β Tubulin IV–positive), and (*B*) the secretory cell populations CCSP single-positive (*grey bars*), MUC5AC single-positive (*white bars*), and CCSP/MUC5AC double-positive (*black bars*). (*C*) CCSP secretion was measured by Western blot of apical washes taken at the indicated time points ALI. Supernatants and cell lysates were immunoblotted for CCSP and β actin.

Tight junction formation was also evaluated by monitoring the subcellular localization of ZO-1, a protein that specifically localizes to the apical tight junctions of polarized cells (Denker and Nigam, 1998). Zonular occludin 1 staining showed some cell junction localization in addition to a diffuse localization pattern at −6 d ALI (Fig. 1*B*); however, a typical tight junction pattern predominated by −3 d ALI (Fig. 1*C*). This staining pattern remained constant (Fig. 1*D* and *E*) and illustrated the increase in cell numbers that occurred after ALI.

Hamster TEC morphology and ultrastructure. At 0 and 3 d ALI the apical cell surface remained flat, with few ciliated cells (Fig. 2*A* and *B*). However, under high-power magnification, the tight junctions and microvilli could be observed (Fig. 2*A*). Figure 2*A* demonstrates the presence of a ciliated cell, which are found at a low frequency (<5%) postplating. After ALI, total cell numbers increased, and it was evident that ciliated cell differentiation was occurring (Fig. 2*C–E*). By 14 d ALI (Fig. 2*E*), the cellular morphology resembled that of the adult hamster trachea (Fig. 2*F*). Transmission electron microscopy revealed both ciliated and nonciliated cell populations (Fig. 3*A–D*) that had well-formed tight junctions (Fig. 3*E*). Many nonciliated cells contained large apically localized vesicles, characteristic of secretory cells (Fig. 3*B*). Cells that morphologically resembled basal cells were detected below the apical cell layer (Fig. 3*C*). These data indicate that the hamster TEC culture forms polarized cultures consisting of ciliated, nonciliated, and basal cells, with morphology closely resembling the hamster trachea.

Ciliated and secretory cell differentiation. Confocal microscopy with cell type–specific antibodies was used to objectively quantitate the cell populations within the hamster TEC culture during differentiation. The ciliated cell population increased with increasing d ALI (Fig. 4*A–D*), as suggested by scanning electron microscopy (SEM) analysis, and by 15 d ALI constituted 40% of the total cell population (Fig. 5*A*).

Two cell markers were used to evaluate the secretory cell populations: CCSP that is secreted by Clara cells (Singh and Katyal, 1997; Hermans and Bernard, 1999) and the mucin MUC5AC that is secreted by goblet cells (Hermans and Bernard, 1999; Kim et al., 2004). Three distinct secretory cell populations were identified during differentiation (Fig. 4*E–P*): CCSP single-positive, MUC5AC single-positive, and CCSP/MUC5AC double-positive populations. Only 15% of the total cell population expressed either secretory cell marker before ALI. Of the marker-positive cells, MUC5AC single-positive cells predominated before ALI but decreased after ALI (Fig. 5*B*). The CCSP/MUC5AC double-positive cells increased immediately after ALI but decreased by 15 d ALI to less than 5%. The CCSP single-positive population steadily increased to become the predominant secretory cell type at roughly 30% of the total cell population by 15 d ALI (Fig. 5*B*). The basal cells were not identified by immunostaining due to the lack of an appropriate cell differentiation marker. The above data confirms the presence of ciliated, Clara, and goblet cells, as well as the differentiation of these cell types after ALI.

The secretory activity associated with these cultures was evaluated using a CCSP secretion assay. Apical washes at d relative to ALI were collected, and CCSP expression was monitored by Western blot analysis (Fig. 5*C*). The secretion measured in this assay was not cumulative because time points were isolated from a single representative sample well. Clara cell secretory protein secretion into the apical supernatant increased with increasing d ALI. This expression was specific to hamster TECs because CCSP expression was not evident in the polarized canine epithelial cell line MDCK but was present to a small degree in hamster TEC lysates (Fig. 5*C*).

Influenza A virus infection of hamster TECs. The in vitro replication of influenza A virus requires addition of trypsin to the culture media of virtually all cell lines. Trypsin cleaves the influenza A virus HA precursor protein into disulfide-linked subunits and is required to convert the protein into a fusion competent form (Paterson and Lamb, 1993; Steinhauer, 1999). Figure 6*A* demonstrates that exogenous trypsin is not required for influenza A virus replication in hamster TECs, whereas Fig. 6*C* demonstrates that HA cleavage occurs in the absence of exogenous trypsin. Because the

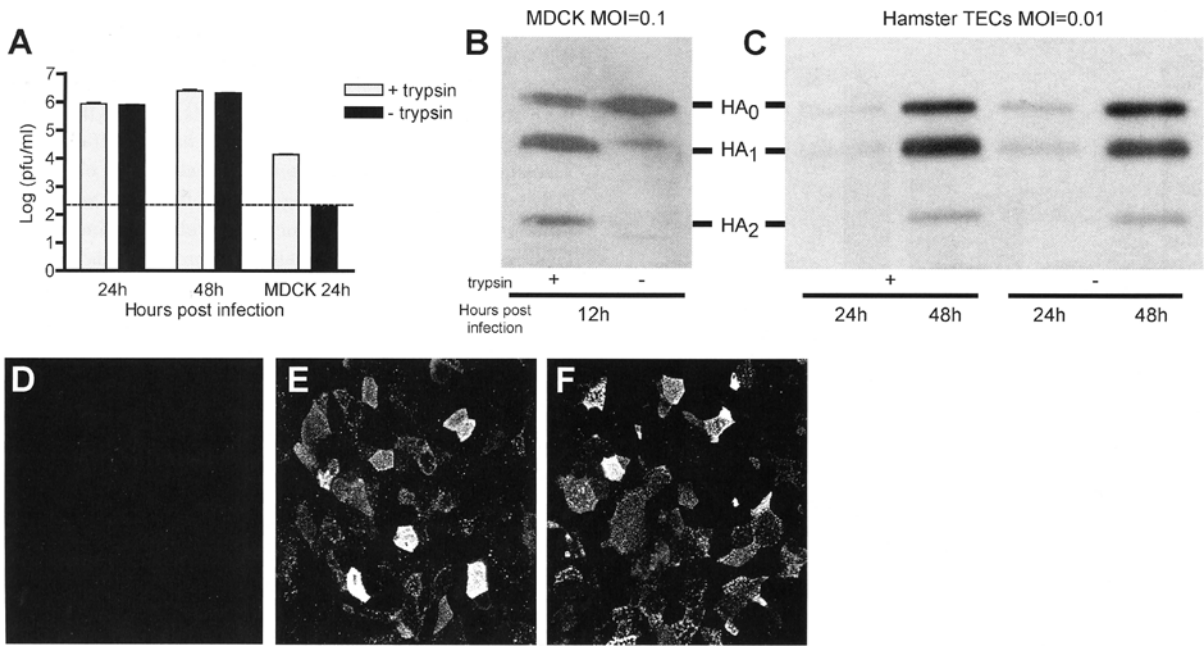

FIG. 6. Influenza A virus infection of hamster tracheal epithelial cells (TECs). (*A*) Madin–Darby canine kidney (MDCK) cells or hamster TECs at 10 d ALI were infected with influenza A virus rUdorn at an multiplicity of infection (MOI) = 0.01 (3000 pfu) in the presence or absence of trypsin, and infectious virus in apical supernatants was measured at 24 and 48 h postinfection (hpi). Hemagglutinin (HA) cleavage was monitored by Western blotting of (*B*) MDCK cells at 12 hpi (MOI = 0.1) or (*C*) hamster TECs at 24 and 48 hpi (MOI = 0.01). HA_0 is the unprocessed form of HA, whereas HA_1 and HA_2 are the subunits produced after cleavage. Viral antigen spread in hamster TEC cultures infected at an MOI = 0.01 was monitored by indirect immunofluorescence confocal microscopy, using a monoclonal antibody (MAb) against the influenza A virus M_2 protein at 24 hpi in cells that were (*D*) mock infected, (*E*) infected and cultured with trypsin, and (*F*) infected and cultured without trypsin.

influenza A virus life cycle takes approximately 12 h to complete, cells expressing viral antigen at 24 and 48 hpi most likely represent cells that were not infected with initial virus inoculum but with infectious virus produced from the initially infected cells. This is in contrast to infection of most transformed cell lines, MDCK cells serving as an example in Fig. 6*A* and *B*. A 12-h time point was taken because HA is not detectable at later time points because the infection of MDCK cells in the absence of trypsin is abortive. Finally, infected hamster TECs produced comparable numbers of viral antigen–positive cells in the presence or absence of exogenous trypsin at 24 hpi (Fig. 6*D–F*). Using various cell markers, we determined that viral antigen was present in ciliated and secretory cells (data not shown). These data indicate that unlike most transformed cell lines, hamster TECs produce a trypsin-like enzyme capable of cleaving the influenza A virus HA protein.

Wound repair. It has been reported that airway epithelial cells express the matrix metalloproteinase, matrilysin, which functions in wound repair (Parks et al., 2001; Li et al., 2002; McGuire et al., 2003; Wielock et al., 2004). To determine if hamster TECs were capable of wound repair, the TER as well as matrilysin expression was monitored after injury. At 1 and 12 h postinjury, the cells had a low TER, which was restored to >500 Ω-cm^2 by 24 h postinjury, after which a high resistance was maintained (Fig. 7*A*). At 12 and 24 h postinjury, the cells were monitored for matrilysin expression using immunofluorescence confocal microscopy (Fig. 7*B* and *D*). At 12 h postinjury, matrilysin expression was detected in cells surrounding the injury (Fig. 7*B*). By 24 h postinjury, matrilysin expression was still present within the scarred region, but the wound had closed, and this correlated with a high TER (Fig. 7*D*). Nuclei staining was used to monitor the migration of cells into the wound (Fig. 7*C* and *E*). These data suggest that hamster TECs are capable of wound repair.

DISCUSSION

In this study, we defined a primary hamster TEC culture system that produces a well-differentiated cellular population that is grown at an ALI, similar to the environment encountered in vivo. Hamster tracheal cultures have been reported (Goldman and Baseman, 1980; Lee et al., 1984; Niles et al., 1988; Whitcutt et al., 1988; Wu, 1997), and in this study we modified, updated, and streamlined the methodology. The use of protease dissociation for cell isolation resulted in a high yield of epithelial cells (roughly 5×10^5 to 6×10^5 cells/trachea), and use of hormone- and serum-supplemented medias led to rapid proliferation and differentiation of the cells at ALI. We chose to limit our cell-type analysis to cells that could be identified with antibodies to well-characterized cell markers in order to objectively characterize the cellular makeup of the cultures and used electron microscopy primarily to assess the ultrastructure of the cultures. We found that our cultures produced a heterogeneous cell population, similar to the trachea in vivo, including ciliated and nonciliated cells. The nonciliated cells were identified as basal (electron microscopy), goblet (MUC5AC-positive), and Clara (CCSP-positive) cells and were capable of producing and secreting mucus and mucus-associated proteins. Interestingly, a high percentage of cells expressed both MUC5AC and CCSP immediately

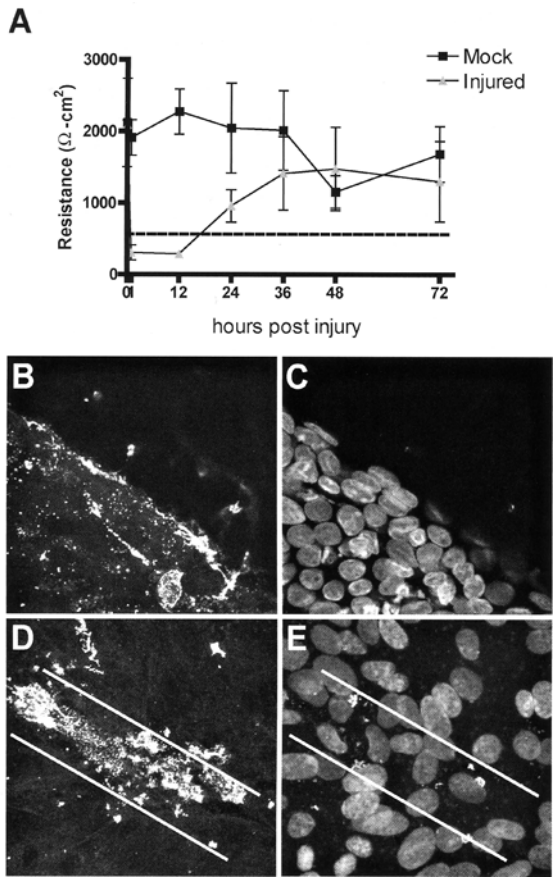

FIG. 7. Wound repair in hamster tracheal epithelial cell (TEC) cultures. Hamster TECs were injured and then monitored for transepithelial resistance (*A*) or matrilysin (MMP-7) expression by immunofluorescence confocal microscopy at (*B*) 12 h and (*D*) 24 h postinjury. (*C*) and (*E*) show nuclei staining from the images in (*B*) and (*D*), respectively. The wound outline at 24 h is shown by the *two lines* in (*D*) and (*E*). All images were acquired at ×63 magnification.

after ALI. Whether Clara cell differentiation is completely independent of MUC5AC expression, proceeds through a MUC5AC/CCSP positive intermediate cell, or goblet cells themselves can differentiate into Clara cells through the double-positive intermediate remains to be investigated. It is interesting to note the mounting evidence indicating that Clara cells can differentiate into MUC5AC-positive goblet cells, perhaps through a MUC5AC/CCSP double-positive population, in response to interleukin-13 or antigen challenge in murine models of airway hyperresponsiveness (Reader et al., 2003; Shahzeidi et al., 2003). The presence of both cell populations in hamster TEC cultures allows for the study of the interdependence of these two cell populations (Boers et al., 1999; Hayashi et al., 2004).

At ALI, there are few differentiated cells, but as d ALI increase, the percentage of differentiated cells increased (Figs. 4 and 5). Although we have not extensively characterized differentiation in the absence of ALI, it is clear from the literature that ALI is an important stimuli for differentiation of mouse (You et al., 2002), human (Yamaya et al., 2002), guinea pig (Robison et al., 1993), rat (Kaartinen et al., 1993), and hamster (Whitcutt et al., 1988) TECs. This differentiation after ALI is consistent with the isolation of a progenitor population as seen in other tracheal isolation protocols (Goldman and Baseman, 1980; You et al., 2002). In vivo, the secretory cells that populate the airways consist of Clara and goblet cells (Wu, 1997). These cell types were present in our cultures and in roughly similar quantities to that observed in hamster tracheas (data not shown). Secretory cells predominated early, as compared with ciliated cells, consistent with reports that suggest that secretory cells may serve as a progenitor population (Wu, 1997). The MUC5AC-positive population was found to be a small percentage, whereas the CCSP-positive population predominated as the major secretory cell in the mature hamster TEC culture (Figs. 4 and 5) as well as in vivo (data not shown). Transmission electron microscopy analysis indicated the cells morphologically consistent with basal cells (Fig. 3*C*), suggesting that this cell type may constitute the remaining 20–30% of the cells in this culture.

Hamster TECs are primary cells and therefore were found to possess many functions that are not found in immortalized cell lines. The hamster TEC cultures produced an enzyme capable of cleaving HA and thereby propagated influenza virus infection. The enzymatic activity may be associated with a trypsin-like protein, tryptase Clara, that was isolated from the airways of rats and localizes to the secretory cells of rat airways (Kido et al., 1992). This emphasizes the uniqueness of primary trachea epithelial cell cultures, which can simulate the in vivo environment when evaluating virus–host cell interactions.

Repair of the respiratory epithelium is vital for maintaining the integrity of the epithelium after damage. Many cellular factors have been implicated in proliferation and migration of cells during wound repair (Kalinichenko et al., 2001, 2003; Parks et al., 2001; McGuire et al., 2003). After injury, hamster TECs quickly regained a high TER, suggestive of establishing cellular tight junction integrity and wound repair (Fig. 7*A*). Under light microscopy, the wound was no longer visible at 24 h postinjury (data not shown). Matrilysin expression is upregulated in response to injury in healthy cells that surround an injury (McGuire et al., 2003), and indeed it appeared to be induced on injury of the hamster TECs. Interestingly, there was a low level of matrilysin expression in mock cells as shown by immunofluorescence microscopy and Western blot analysis (data not shown). This is consistent with reports that in vivo, matrilysin is constitutively expressed in the airways (Dunsmore et al., 1998) and supports the role of matrilysin in other responses, such as immune modulation and airway homeostasis (Parks et al., 2004).

The hamster TEC culture does mimic the in vivo tracheal environment and will be a useful system to evaluate tracheal function. Recently, Syrian golden hamsters have been described as suitable small animal models for many emerging infectious diseases, including SARS coronavirus (Buchholz et al., 2004), Nipah virus (Wong et al., 2003), and the South American hantaviruses, Andes and Maporal viruses (Hooper et al., 2001; Milazzo et al., 2002). These viruses are transmitted through inhalation, result in high morbidity and mortality in human infections, and require high containment facilities (biosafety levels 3 or 4) in which to conduct research (Lopez et al., 1996; Chua et al., 2000; Ksiazek et al., 2003). The respiratory tract is likely to play an important role in the disease progression of these pathogens, whether as a site of virus replication or simply as a barrier preventing virus access to other organs in the animal. Therefore, the use of in vitro systems, such as the hamster TEC culture, could provide a preferred environment to study virus–host interactions, as well as testing of antiviral molecules.

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