Human Bronchial Epithelial Cells Differentiate to 3D Glandular Acini on Basement Membrane Matrix

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To create a model system that investigates mechanisms resulting in hyperplasia and hypertrophy of respiratory tract submucosal glands, we developed an *in vitro* three-dimensional (3D) system wherein normal human bronchial epithelial (HBE) cells differentiated into glandular acini when grown on a basement membrane matrix. The differentiation of primary HBE cells into glandular acini was monitored temporally by light microscopy. Apoptosis-induced lumen formation was observed by immunofluorescence analysis. The acinar cells expressed and secreted MUC5B mucin (marker for glandular mucous cells) and lysozyme, lactoferrin, and zinc- α 2-glycoprotein (markers for glandular serous cells) at Day 22. β -Tubulin IV, a marker for ciliated cells, was not detected. Expression of mucous and serous cell markers in HBE glandular acini demonstrated that HBE cells grown on a basement membrane matrix differentiated into acini that exhibit molecular characteristics of respiratory tract glandular acinar cells. Inhibition studies with neutralizing antibodies resulted in a marked decrease in size of the spheroids at Day 7, demonstrating that laminin (a major component of the basement membrane matrix), the cell surface receptor integrin α 6, and the cell junction marker E-cadherin have functional roles in HBE acinar morphogenesis. No significant variability was detected in the average size of glandular acini formed by HBE cells from two normal individuals. These results demonstrated that this *in vitro* model system is reproducible, stable, and potentially useful for studies of glandular differentiation and hyperplasia.

Keywords: human bronchial epithelial cells; glandular acini; extracellular matrix; three-dimensional culture; submucosal glands

Submucosal glands are a major source of mucus in the respiratory tract. Hyperplasia and/or hypertrophy of submucosal glands contribute to mucus overproduction in chronic airway diseases, such as cystic fibrosis (1–3), asthma (4), chronic obstructive pulmonary diseases (5), and chronic rhinosinusitis (6, 7). Although the morphogenesis of submucosal glands during fetal development is well described (8, 9), glandular hyperplasia in respiratory tract mucosa is markedly understudied, reflecting primarily the lack of an *in vitro* cell model system whereby respiratory tract epithelial cells differentiate into glandular cells.

CLINICAL RELEVANCE

This research demonstrates that primary human bronchial epithelial cells can differentiate into three-dimensional spheroids with a lumen and secretory cells that express and secrete acinar markers when grown on Matrigel, an extracellular basement membrane matrix. This is the first report of an *in vitro* model system of respiratory tract glandular acini and will facilitate investigations into mechanisms that lead to the submucosal glandular hyperplasia manifested in chronic disease of the respiratory tract (chronic obstructive pulmonary disease, cystic fibrosis, chronic rhinosinusitis).

The basement membrane extracellular matrix (ECM) functions as a scaffold for tissue morphogenesis and contains biologically active components that provide cues for cell proliferation and differentiation (10, 11). Various types of primary epithelial cells, including those from salivary and mammary glands as well as cells from the intestine, pancreas and oviduct, have been shown to differentiate *in vitro* into three-dimensional (3D) structures with glandular acini when grown on a basement membrane ECM (12). The most commonly used ECM for *in vitro* 3D cell culture is Matrigel, an extract isolated from Engelbreth-Holm-Swarm murine tumors and composed of laminin (61%), collagen IV (30%) and entactin (7%) (10, 13). It has been extensively used to investigate the differentiation of mammary cells and cell lines into 3D acinar structures (11, 14) as well as branching morphogenesis in murine salivary glands (15). Lung epithelial cells from distal regions of rodent airways have also been grown on Matrigel. Rat lung cells undergo alveolar type II differentiation (16) and those from mice undergo budding (17). However, there are no reports describing whether proximal, e.g., bronchial or tracheal, airway epithelial cells differentiate into glandular acini when grown on a basement membrane matrix.

On the other hand, research over the last 25 years has shown that primary epithelial cells from human bronchi or rodent trachea are capable of differentiating into a conducting airway epithelium. Human bronchial epithelial (HBE) cells grown on collagen-coated Transwell membranes under air-liquid interface (ALI) conditions differentiate to form an epithelium with ciliated, goblet, and basal cells that morphologically mimics human airway epithelium *in vivo* [reviewed in (18, 19)]. Likewise, hamster (20), guinea pig (21) and murine (22) tracheal epithelial cells differentiate *in vitro* to an epithelium with ciliated, secretory, and basal cells that morphologically mimic epithelium observed *in vivo*. More recently, it has been shown that basal cells isolated from murine tracheal and human bronchial epithelium, when immersed in Matrigel plated on Transwell membranes and grown under ALI conditions, differentiate into tracheospheres or bronchospheres that have ciliated cells lining a hollow lumen but lack detectable secretory cells

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(23). Taken together, this information suggests that primary HBE cells, which contain multipotent precursor cells capable of differentiating into a conducting airway epithelium or broncho-spheres, would also differentiate into glandular acini in the proper context.

Submucosal glands are not observed in the ALI *in vitro* model system; however, MUC5B mucin, a gene product whose expression is normally restricted to glandular mucosal cells in human lower respiratory tract tissues *in vivo* (24), is well-expressed and secreted in the ALI system (25). We hypothesized that primary HBE cells grown on Matrigel would differentiate into 3D-structures that would recapitulate features of glandular acini *in vivo*, including the formation of acinar-like spheroids with a hollow lumen, apicobasal polarization of acinar cells, and expression of markers for glandular secretory cells.

MATERIALS AND METHODS

Air-Liquid Interface Culture of HBE Cells

HBE primary cells and culture media were obtained from Clonetics Cell Systems (Lonza, Walkersville, MD) and passage 2 cells were established as differentiated cell cultures, as previously described (26, 27).

3D Culture of HBE Cells

HBE cells (passage 2) from two different individuals, HBE-1 (2-yr-old male) and HBE-2 (27-yr-old female) were used in these studies. HBE-1 cells were used to establish and characterize the model, and HBE-2 cells were used to evaluate stability and reproducibility (Figures 3 and 7). As detailed in the online supplement, ALI medium, (basal medium used for differentiated culture of HBE cells) was supplemented with 2% Matrigel and a higher concentration of epidermal growth factor (EGF) (10 ng/ml) to form a complete medium. The overlay protocol established by Debnath and colleagues (14) and growth factor-reduced phenol red-free Matrigel No. 356231 (BD Biosciences, Bedford, MA) were used, except where indicated. Phenol red-free regular Matrigel No. 356237 (BD Biosciences) was used in experiments to optimize the culture conditions. The Matrigel protein concentration was 8.5–10.5 mg/ml and the endotoxin level was less than 2 U/ml. Matrigel was kept on ice throughout the preparation of the slides; it is a liquid at 4°C and solidifies at warmer temperatures, although it is soluble at a concentration of 2 to 4% at 37°C. Four-well chamber slides with a surface area of 1.7 cm² per well (NalgeNunc International/Thermo Scientific, Rochester, NY) were precooled on ice for 15 to 30 minutes. The bottom of each well was coated with 250 µl of 100% Matrigel. Chamber slides were placed in a 37°C incubator for 30 minutes to allow Matrigel to solidify. HBE cells were diluted in basal medium to achieve a final concentration of 20,000 cells/ml. A separate stock medium (prepared by adding 4% Matrigel and 10 ng/ml EGF to basal medium) was mixed with cells in a 1:1 ratio; 500 µl was plated on top of the solidified Matrigel in each well, corresponding to a final overlay solution of 5×10^3 cells per well in medium containing 2% Matrigel and 5 ng/ml EGF. In experiments where cells were embedded in Matrigel, 5×10^3 cells were mixed with 100% Matrigel or with Matrigel diluted 1:1 (vol/vol) with basal medium and solidified at 37°C for 15 minutes. In all experiments, cells were grown in a 5% CO₂ humidified incubator at 37°C and fed with 400 µl of freshly prepared complete medium (2% Matrigel, 10 ng/ml) every 2 to 4 days. Cells were monitored with a phase contrast inverted microscope (Carl Zeiss Telaval-31, Oberkochen, Germany) starting on Day 1 and then every other day. A 20× objective was used to take brightfield images, which were analyzed using Axiovision Release 4.3 (Carl Zeiss Microimaging GmbH).

Antibodies

See the online supplement for details.

Immunofluorescence

See the online supplement for details.

Periodic Acid Fluorescent Schiff's Staining

See the online supplement for details.

Confocal Microscopy Imaging

See the online supplement for details.

Dot Blot Assays

See the online supplement for details.

Size Quantification of Acini

See the online supplement for details.

Inhibition of Acinar Formation

See the online supplement for details.

RESULTS

HBE Cells Exhibit Different Morphology in Different Cell Culture Conditions

HBE cells were grown for 22 days at two different conditions: on collagen-coated Transwell membranes at ALI (Figure 1A) and on Matrigel (Figure 1B). Marked differences in morphology were observed after differentiation of HBE cells grown under different conditions. As expected, HBE cells grown on a collagen IV-coated Transwell membrane at ALI proliferated into a confluent two-dimensional layer that had a cobblestone appearance (Figure 1A). However, HBE cells grown on growth factor-reduced Matrigel formed 3D spheroid-like structures (Figure 1B). These data demonstrated that HBE cells contain multipotent precursor cells that are capable of undergoing differentiation to form structures with markedly different morphology when grown under different conditions.

Primary HBE Cells Grown on Basement Membrane Matrix Proliferate to 3D Spheroids

HBE cells plated on Matrigel were temporally monitored by brightfield microscopy. HBE cells proliferated to form spheroids that increased in size over 22 days (*see* Figure E1 in the online supplement). Each image in the panel is representative of the overall size of acinar-like structures generated from a single cell or small clumps of cells on the day specified. On Day 3, HBE cells had proliferated to form small spheroids of cells. Individual spheroids continued to proliferate and the spheroids increased in size up to Day 22. Studies were not continued after Day 22 as the spheroids appeared to degenerate.

Figure 1. Human bronchial epithelial (HBE) cells grown under different conditions exhibit different morphology at three weeks. HBE-1 cells were grown according to conditions described in (A) or (B). (A) HBE cells were grown on type IV collagen-coated Transwell membranes for a total of 21 days, the last 14 of which were under ALI conditions. Cells grew into a monolayer of epithelial cells with a cobblestone appearance. Brightfield microscopy, 200× magnification. (B) HBE cells were plated at 5×10^3 cells per well at Day 0 on growth factor-reduced Matrigel, as described in the METHODS. Cells proliferated to form a 3D spheroid ball of cells. Brightfield microscopy, Bar = 200 µm

To optimize culture conditions, we evaluated the effects on spheroid formation of Matrigel type (regular or growth factor-reduced), of mixing HBE cells with Matrigel (overlay vs. embedment), and of cell density. The data (*see* Figure E2) demonstrated that growth factor-reduced Matrigel (*top row*) promoted better formation of larger spheroids at Day 7 than regular Matrigel (*bottom row*) regardless of whether HBE cells were overlaid or embedded in Matrigel. We found that the overlay method was more efficient and reproducible for developing spheroids than embedding HBE cells either in 100% Matrigel or 1:1 diluted Matrigel (Figure E2). For cell density studies, HBE cells were overlaid on growth factor-reduced Matrigel at concentrations over a range of 5 to 80×10^3 cells per well and observed by brightfield microscopy. Our data demonstrated that a plating density of 5×10^3 to 1×10^4 cells per 1.7 cm^2 well provided maximal conditions for HBE cell 3D proliferation (Figure E3).

HBE Cells Differentiate on Matrigel into Acini with Hollow Lumens

Brightfield microscopy demonstrated that HBE cells grown on Matrigel proliferated, aggregated, and formed spheroid-like structures over time (Figure E1). Confocal microscopy was subsequently used to determine whether spheroids from HBE cells, like spheroids from mammary epithelial cells (14), differentiated to form glandular acini with polarized cells and a hollow lumen. At Day 22, spheroids were stained with an antibody for E-cadherin, a component of intercellular junction complexes (28–30), and a nuclear stain. Confocal analysis using z-stack imaging (layered from top to bottom) demonstrated that acini with cell–cell junctions and a hollow lumen had developed (Figure 2A). Two populations of cells within each acinus were evident: an outer layer of cells in contact with the ECM and an inner subset of cells that lacked contact with the matrix. A 3D movie depicting different views of an acinus at Day 22 using only a nuclear stain is provided in the online supplement (Figure E5).

Temporal Analysis of Acinar Development

The temporal progression of acinar development and lumen formation was monitored by confocal microscopy using a nuclear marker (Figure 2B). Acinar development, as evidenced by the formation of a lumen, was evident by Day 9. A mature lumen was distinct by Day 14 and present up to Day 22.

HBE Cell Polarization and Apoptosis Occur before Lumen Formation

The current paradigm is that epithelial cells become polarized during differentiation to glandular acini and that death of the inner cells, that is, those that lack contact with the matrix, is initiated once an outer and inner layer of cells is visible (31). We evaluated this during differentiation of HBE cells to acini. The outer layer of HBE cells expressed the basal polarization marker integrin $\alpha 6$, providing evidence for basal polarization by Day 11 (Figure 3). To determine whether cell death occurred by apoptosis following the appearance of an outer and inner population of cells, we used antibodies that recognize activated (cleaved) forms of caspase-8 and caspase-3, and we monitored their expression by immunofluorescence staining during acinar differentiation on Days 8, 10, 11, 13, and 15. Data showed that caspase-8 was expressed in cells inside the lumen on Day 10 (data not shown) and Day 11 (Figure 3A), whereas caspase-3 was observed in cells located in the acinar lumen only on Day 13 from two different individuals (Figure 3B,C). These data suggested that the lumen formation that occurred during differentiation of primary HBE cells into glandular acini *in vitro* is mediated at least in part by cell apoptosis.

HBE Acinar Differentiation Requires Laminin, Integrin $\alpha 6$, and E-cadherin

To identify Matrigel components and cellular receptors that may be involved in HBE acinar formation, antibody neutralization experiments were performed. No antibodies (Figure 4A) or antibodies against laminin (Figure 4B), E-cadherin (Figure 4C) or integrin $\alpha 6$ (Figure 4D), were added into the complete medium when HBE cells were overlaid on Matrigel. Additional experiments were performed where HBE cells were incubated with antibodies to integrin $\alpha 6$ or to an IgG2a antibody (Figure 4F). Data showed that antibodies to the receptor integrin $\alpha 6$, the cell junction protein E-cadherin, and to laminin, the major component of Matrigel, markedly inhibited spheroid size when compared with the control, thereby preventing normal acinar differentiation. The inhibitory effect was significant whether the controls lacked antibody (Figure 4E) or contained an isotype-specific antibody (Figure 4F).

***In Vitro* HBE Acini Express and Secrete Glandular Acinar Cell Markers**

To characterize the differentiated acinar cells and assess their similarity to glandular acini in respiratory tract glands *in vivo*, we evaluated the expression of submucosal glandular cell markers (32), as well as a ciliated cell marker, on D 22. β -Tubulin IV, a marker of ciliated cells, which are present in the conducting airway epithelium *in vivo* and *in vitro* but not in glandular acini *in vivo* (33), was not detectable in differentiated HBE acini (Figure 5A). Fluorescent Periodic Acid Schiff's staining of differentiated HBE acini on Day 22 (Figure 5B) detected expression of glycoconjugates with vicinal hydroxyl groups typically found in O-glycans in mucins and in some proteoglycans (34). Immunostaining demonstrated expression of MUC5B mucin (Figure 5C), a marker for human glandular mucous cells *in vivo* (35) and of lysozyme (Figure 5D), a marker for human glandular serous cells (32, 36).

To supplement this end point data on Day 22, a time course during differentiation was performed using immunofluorescence to evaluate expression of β -tubulin IV and MUC5B mucin. The results demonstrated that β -tubulin IV, which was strongly expressed in acinar cells on Day 5, was faint by Day 9 and not detectable at later time points (Days 15 and 21) (Figure 6A–D). In contrast, MUC5B mucin was weakly expressed on Day 9, but exhibited strong expression throughout intermediate and late time points (Days 13, 15, and 21) (Figure 6 E–H).

The immunofluorescence data indicated that HBE acinar cells secreted MUC5B and lysozyme into acinar lumens (Figure 5C,D). To further evaluate this, we performed dot blot analyses of acinar lysates and secretions. The data demonstrated that acinar cells both expressed and secreted submucosal gland serous cell markers, e.g., lysozyme, lactoferrin, and zinc- α_2 -glycoprotein (ZAG) (37) and the mucous cell marker, MUC5B mucin (Figure E4). Taken together, these results demonstrated that HBE cells differentiated into glandular acinar cells when grown on Matrigel under the described experimental conditions and that acinar cells expressed and secreted markers of mucous and serous cells.

Reproducibility of the 3D Respiratory Acinar Model from HBE Cells

To evaluate the stability and reproducibility of the HBE glandular *in vitro* model, HBE cells from two different donors of significantly different ages (2-yr-old male and 27-yr-old female) were cultured at the same time under identical conditions. The data demonstrated that the morphological and temporal characteristics of HBE cells from two individuals

Figure 2. Confocal analyses of HBE spheroids on Matrigel. (A) Confocal z-stack images of an HBE-1 acinus grown on Matrigel for 22 days. The spheroid-like structures that differentiated from HBE-1 cells were stained on Day 22 with TO-PRO-3 to visualize nuclear DNA (red) or immunostained with FITC-conjugated E-cadherin antibody (green) to visualize cell-cell junctions. Optical sections traversing from the top to the bottom of the spheroid were acquired and labeled as A-E. A fully formed lumen is shown in C. Bar = 40 μ m. (B) Temporal analyses of acinar development of HBE-1 cells grown on Matrigel during Days 5 to 22. Cells were identified by staining nuclei with TO-PRO-3 (red). Representative sectioned images of the glandular-like structures were acquired by confocal microscopy. Acinar development was evident by Day 9, as evidenced by the formation of a lumen, which was enlarged by Day 14. Bar = 40 μ m

were similar during acinar morphogenesis. The average diameters of acini differentiated from primary HBE cells of two different normal individuals were compared at Day 13, a time when HBE acini manifested a mature lumen. Although there is a continuum of acinar sizes, the median diameter of acini ($n = 31$) formed by HBE-1 cells was 102.45 μ m, whereas that from HBE-2 cells ($n = 41$) was 104.59 μ m. Results showed that the diameters of small and large acini formed by primary HBE cells from two different individuals were not significantly different (t test, small group, $P = 0.23$; large group, $P = 0.13$) (Figure 7), indicating that HBE cells from different individuals differentiated similarly into glandular acini on Matrigel. Additionally, confocal microscopy demonstrated expression of cleaved caspase-3 on the same day, Day 13, but not at earlier time points (data not shown) in acini from HBE-1 (Figure 3B) and HBE-2 (Figure 3C) cells.

DISCUSSION

This study reports for the first time that HBE cells, like salivary and mammary epithelial cells (11, 14, 15), can differentiate into 3D glandular acinar structures when grown on the basement membrane matrix Matrigel under the conditions described. HBE cells also differentiate to form a conducting airway epithelium under ALI conditions (18, 19), thus HBE cells clearly contain multipotent cells that respond to cues from basement membrane components and growth factors to activate various differentiation pathways. This is further supported by a study wherein basal cells isolated from human bronchi were shown to self-renew and differentiate into bronchospheres that contain ciliated cells but lack detectable mature secretory cells (23). The ability to differentiate to various structures appears to be a characteristic of epithelial cells in mammalian respiratory

Figure 3. HBE cell polarization and apoptosis occur during lumen formation. Cell nuclei were stained with DAPI (blue). (A) In HBE-1 acini cultured on Matrigel for 11 days, cleaved caspase-8 (green) was detected in the inner lumen and integrin $\alpha 6$ (red) was polarized to the basal acinar surface. (B) On Day 13, the inner lumen of acini formed by HBE-1 cells immunostained positively for cleaved caspase-3 (green) and integrin $\alpha 6$ (red) remained polarized at the basal acinar surface. (C) The inner lumen of acini formed by HBE-2 cells immunostained positively for cleaved caspase-3 (green) on Day 13. The arrows identify cleaved caspase expression. Bar = 20 μ m.

integrin $\alpha 6$ (red) remained polarized at the basal acinar surface. (C) The inner lumen of acini formed by HBE-2 cells immunostained positively for cleaved caspase-3 (green) on Day 13. The arrows identify cleaved caspase expression. Bar = 20 μ m.

Figure 4. Inhibition of acinar formation by neutralizing antibodies. Brightfield images of HBE-1 acini after culture for 7 days on Matrigel with culture medium that (A) included no antibodies, or (B) included antibodies to laminin, (C) E-cadherin or (D) integrin $\alpha 6$, respectively. Magnification, 200 $\times$. (E) Acinar areas were measured in 10 individual fields with 64 to 166 acini measured in each condition by Image J (NIH). Values are expressed as means of acinar sizes $\pm$ standard error (SE) of three experiments. Significant decreases in acinar size were observed compared with the controls using a *t* test. (**P* < 0.001). (F) Acinar areas were measured by Image J in 15 individual fields from HBE-1 acini grown for 7 days on Matrigel with culture medium that included rat IgG2a isotype negative-control antibodies or antibody to integrin $\alpha 6$. Values are expressed as means of acinar sizes $\pm$ standard error (SE). Significant decreases in acinar size were observed compared with the negative controls using a *t* test (**P* < 0.001).

tracts. Airway epithelial cells from hamsters, guinea pigs, and mice form a conducting airway epithelium when grown under ALI conditions (20–22), as do human nasal epithelial cells (18, 19). Human nasal epithelial cells grown in suspension differentiate to form spheroids of ciliated and secretory cells (38), and basal cells from murine trachea form tracheospheres with ciliated and nonsecretory cells (23).

Matrigel was used both in this study, wherein HBE cells differentiate to acinar cells that express glandular serous and mucous cell markers but do not express a ciliated cell marker, and in the study by Rock and colleagues where basal cells form bronchospheres of ciliated and nonsecretory cells (23). There are distinct differences in the conditions under which cells were cultured in the two studies. Rock and co-workers embedded basal cells isolated from HBE cells in 1:1 Matrigel/ALI medium, plated them on Transwell membranes, and cultured them under ALI conditions, which resulted in the generation of bronchospheres (23). We overlaid HBE cells on Matrigel and covered them with ALI medium containing 2% Matrigel and 10 ng/ml EGF, which drove the differentiation of HBE cells into individual polarized acinar units with a hollow lumen that contained secretory cells and lacked ciliated cells. Furthermore, the EGF concentration in the medium was 20-fold higher in our studies than EGF concentrations used to generate tracheospheres or bronchospheres, suggesting that EGF may be one of the key factors that induce epithelial cells to proliferate and differentiate into glandular cells. Several studies have reported that EGF and EGF-receptor regulate epithelial morphogenesis during development and are important for proper branching in the lung (39) and in mammary (40) and salivary (41) glands. EGF also accelerates differentiation of mucous secretory cells (42). The apparent lack of secretory cells in the tracheosphere

model (23) may reflect the low concentration of EGF (0.5 ng/ml) in the culture medium.

To assess the impact of specific parameters on acinar formation during HBE cell differentiation, various culture conditions were evaluated. Cell densities at the time of plating, overlaying versus embedding, and the use of growth factor-reduced Matrigel were parameters that affected HBE acinar development. The morphological changes that occurred during differentiation of HBE cells to glandular acini were likewise delineated and shown to be similar to those observed with mammary cells (43). Cells proliferated to form small-cell-spheroids during the first 7 days and were followed by apicobasal polarization and lumen formation. Under these conditions, the differentiation of HBE cells to acini on Matrigel appeared to be reproducible and stable, as the spheroids formed by HBE cells from two individuals were similar in average diameter and area on Day 13, when lumens are clearly discernable. Taken together, these data suggest that HBE cells differentiate into acinar 3D structures on Matrigel using mechanisms similar to other epithelial cell types.

Two populations of cells within each acinus were clearly manifested during acinar formation: a polarized outer layer of cells in contact with the matrix and an inner subset of cells that were poorly polarized and lacked contact with the matrix. A similar phenotype where the inner cells undergo apoptosis is observed when mammary gland MCF-10A cells are grown on Matrigel (14). The expression of two activated caspases was monitored during Days 8 to 15, the time period in which the lumen develops. In HBE cells, expression of caspase-8 and caspase-3 cleavage (activation) in the inner cells of the lumen was observed on Day 11 and Day 13, respectively. These data indicated that lumen formation by HBE cells uses apoptosis for

Figure 5. Immunofluorescent staining of HBE glandular-like acini and luminal secretions. HBE-1 cells grown on Matrigel were immunostained or stained with fluorescent markers on Day 21. (A) Immunostaining with antibody to β -tubulin IV, a marker for cilia (green) demonstrated lack of cilia. (B) Fluorescent PAS staining (red) identified glycoconjugates typically found in mucins or proteoglycans. (C) Immunostaining with antibody to MUC5B (green), a marker of mucous cells and secretions, identified MUC5B mucin-expressing cells and secretions in the acinar lumen. (D) Immunostaining with antibody to lysozyme (green), a marker of serous cells and luminal secretions, identified cells and secretions that expressed lysozyme. Images were taken by confocal microscopy. Bar = 40 μ m. In A, C, and D, nuclei were stained with TO-PRO-3 (red).

clearance of the inner cells, a mechanism that has been reported for other *in vitro* models of epithelial gland formation (31, 43, 44). This may reflect anoikis, that is, apoptosis of cells that have lost contact with the ECM when they become internalized in the developing acini. A more detailed time course during the Day 9 to Day 15 window to correlate the activation of caspases and the appearance of the lumen would be important to determine the role of apoptosis in lumen formation by HBE cells.

Differentiation of epithelial cells on Matrigel to 3D structures clearly involves cell–ECM and cell–cell interactions.

Laminin, the major component of ECM *in vivo*, mediates the attachment, migration, and organization of cells into tissues during embryonic development by interacting with other ECM components (45). *In vitro*, blocking laminin decreases the size of the acini formed by a human submandibular gland (HSG) cell line on Matrigel (31). Likewise, inhibiting laminin decreased the size of HBE acini, indicating that laminin is a crucial component for HBE acini formation.

Cell-surface receptors such as integrins and cadherins are also important in cell–cell and cell–matrix interactions, and specific isoforms are implicated in acinar formation by epithelial cells. Integrin $\alpha 2\beta 1$, a cell-surface receptor on Madin–Darby canine kidney (MDCK) cells, binds collagens and laminins (46, 47). The absence of integrin $\alpha 2\beta 1$ in MDCK cells results in reduced cyst formation (48). Additionally, a neutralizing antibody against integrin $\alpha 6$, a laminin receptor, decreases the size of acini from HSG cells cultured on Matrigel (31). Integrins are expressed *in vitro* on the surface of HBE cells where they function as collagen or collagen/laminin receptors (49). In our studies, integrin $\alpha 6$ was expressed in the outer layer of cells during formation of HBE acini. Antibody blocking of integrin $\alpha 6$ markedly decreased the size of spheroids formed by HBE cells, indicating a potential role for interactions between integrin and the ECM in establishing HBE acinar morphogenesis. E-cadherin, the calcium-dependant adhesion molecule expressed in epithelial cells, is also important for the initial cell aggregation and cell–cell interactions in the formation of acini from HSG cells (31). E-cadherin, which was well-expressed on the surfaces of HBE acini and in contact with the ECM, was likewise shown to play a role in the formation of HBE acini. These data demonstrated that laminin, the major component of Matrigel, and the cell surface receptors integrin $\alpha 6$ and E-cadherin, have functional roles during the morphogenesis of HBE acini.

In our model, HBE cells differentiated on Matrigel into polarized acinar cells that expressed and secreted MUC5B mucin, a glandular acinar marker for mucous cells in lung submucosal glands (35), and lysozyme, lactoferrin, and ZAG, markers for lung acinar serous cells (32, 36, 37). Future studies will use electron microscopy to further investigate whether *in vitro* acinar cells possess secretory granules and therefore morphologically mimic *in vivo* serous and mucous acinar cells.

In summary, the kinetics of differentiation, the appearance of apoptosis markers during formation of the lumen, the expression and secretion of acinar markers, as well as the formation of biologically relevant structures, suggest that this model will

Figure 6. Temporal analyses of expression of ciliated and glandular mucous cell markers by HBE-1 cells during differentiation on Matrigel. (A–D) β -tubulin IV, a marker for ciliated respiratory epithelial cells, was strongly expressed by acinar cells on Day 5, less strongly by Day 9, and absent at later time points. (E–H) MUC5B, a mucous glandular marker, was not expressed at Day 5 but was faintly expressed on Day 9 and maintained strong expression at intermediate and later time points (Days 13, 15, and 21).

Figure 7. Comparison of HBE acini diameter from two individuals. Normal primary HBE cells from two different individuals (HBE-1 and HBE-2) were grown on Matrigel for 13 days. Confocal images of 31 HBE-1 acini and 41 HBE-2 acini were taken, and the acini diameters were measured by Zen 2008 Light Edition software (Carl Zeiss Microimaging GmbH). The acini were divided into two groups depending on whether the diameter sizes were smaller or bigger than approximately 100 μm . Fifteen small acini and 16 large acini from HBE-1 and 20 small acini and 21 large acini from HBE-2 were randomly selected and measured. The average acini diameter of each group from the two individuals is shown on the y axis. Values are expressed as means + SE for acini measurements from 3 different wells. There is no significant difference in the acini diameter between HBE-1 and HBE-2.

provide a valuable, reproducible tool for investigating mechanisms important in the pathological pathways that lead to glandular hyperplasia and hypertrophy in the respiratory tract.

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