

Three-Dimensionally Engineered Normal Human Lung Tissue-Like Assemblies: Target Tissues for Human Respiratory Viral Infections

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Abstract

In vitro three-dimensional (3D) human lung epithelio-mesenchymal tissue-like assemblies (3D hLEM TLAs) from this point forward referred to as **TLAs** were engineered in Rotating Wall Vessel (RWV) technology to mimic the characteristics of *in vivo* tissues thus providing a tool to study human respiratory viruses and host cell interactions. The TLAs were bioengineered onto collagen-coated cyclodextran microcarriers using primary human mesenchymal bronchial-tracheal cells (HBTC) as the foundation matrix and an adult human bronchial epithelial immortalized cell line (BEAS-2B) as the overlying component. The resulting TLAs share significant characteristics with *in vivo* human respiratory epithelium including polarization, tight junctions, desmosomes, and microvilli. The presence of tissue-like differentiation markers including villin, keratins, and specific lung epithelium markers, as well as the production of tissue mucin, further confirm these TLAs differentiated into tissues functionally similar to *in vivo* tissues. Increasing virus titers for human respiratory syncytial virus (*wtRSVA2*) and the detection of membrane bound glycoproteins over time confirm productive infection with the virus. Therefore, we assert TLAs mimic aspects of the human respiratory epithelium and provide a unique capability to study the interactions of respiratory viruses and their primary target tissue independent of the host's immune system.

Introduction

The function of respiratory epithelium is critical in protecting humans from disease and acts as a barrier to invading microbes present in the air, defending the host through a complex multi-layered system (1) (Hiemstra and Bals, 2004). This complex system is comprised of pseudo-stratified epithelial cells, a basement membrane, and underlying mesenchymal cells. Ciliated, secretory, and basal epithelial cells are joined by intercellular junctions and anchored to the basement membrane via desmosomal interactions. Through tight junctions and the mucociliary layer, the basement membrane maintains polarity of the epithelium and presents a physical barrier between the mesenchymal layer and the airway (2, 3) (Knight and Holgate, 2003; Gibson and Perrimon, 2003).

Airway epithelial cells defend the host physiology blocking paracellular permeability, modulating airway function through cellular interactions, and transporting inhaled microorganisms away via ciliated epithelial cells (4) (Bals and Hiemstra, 2004) (5) (Cotran et al, 1999). Epithelial cells, regulators of the innate immune response, also induce potent immunomodulatory and inflammatory mediators (cytokines and chemokines) recruiting phagocytic and inflammatory cells thus facilitating microbial destruction. (6, 7, 3, 2) (Garofalo and Haeberle, 2000; Polito and Proud, 1998; Bals and Hiemstra, 2004; Knight and Holgate, 2003).

Optimally, a cell-based model should reproduce the structural organization, multicellular complexity, differentiation state, and function of the human respiratory epithelium. Immortalized human epithelial cell lines (2D), (i.e. BEAS-2B) (8) (Ke et al, 1988), primary normal human bronchial epithelial (NHBE) cells (2D) (9) (Stoner et al,

1980), and air-liquid interface cultures (3D) (10) (Wu et al, 1986) are used to study respiratory virus infections *in vitro*. Traditional monolayer cultures of human immortalized and tumor alveolar and broncho-epithelial cells represent homogenous lineages however, propagated as 2D cultures fail to express the innate tissue fidelity characteristic of normal human respiratory epithelia (11) (Carterson et al.). Thus, their state of differentiation and intracellular signaling pathways differ from epithelial cells *in vivo*. Primary isolates of HBE cells provide a pseudo-differentiated model with structure and function similar to epithelial cells *in vivo*; however, this fidelity is short-lived *in vitro* (9, 12) (Stoner et al, 1980; Gray et al, 1996). Air-liquid interface cultures of primary HBE cells (or submerged cultures of human adenoid epithelial cells (13) Wright et al, 2005) are grown on collagen-coated filters in wells, on top of a permeable filter. These cells receive nutrients basolaterally and their apical side is exposed to humidified air. The result is a culture of well-differentiated heterogeneous (ciliated, secretory, basal) epithelial cells essentially identical to airway epithelium *in situ* (14, 10, 15) (Adler and Li, 2001; Wu et al, 1986; Whitcutt et al, 1988). Although this model mimics fidelity to the human respiratory epithelium in structure and function, maintenance of consistent cultures is difficult, time consuming, and restricted to small-scale production and thus limits industrial pharmaceutical research capability.

Thus cellular differentiation involves complex cellular interactions (16, 17, 18) (Fukamachi et al, 1986; Wiens et al, 1987; Sutherland, 1988), in which cell membrane junctions, extracellular matrices (e.g., basement membrane and ground substances), and soluble signals (endocrine, autocrine, and paracrine) play sustaining roles (19, 20, 21, 22) (Kaye et al, 1971; Buset et al, 1987; Daneker et al, 1987 and Durban, 1990) in tissue

development. This process is also influenced by spatial cellular relationships to one another. Each HBE cell has three membrane surfaces: a free-apical surface, a lateral surface, and a basal surface that interacts with mesenchymal cells (23) (O'Brien et al, 2002). Therefore complex recapitulated 3D models must emulate these characteristics.

In the absence of a reproducible long-term methodology to culture human respiratory epithelium (>3 mm diameter), an established technology developed at NASA's Johnson Space Center is now being used to construct large-scale, 3D, *in vitro* tissue models of human respiratory epithelium (Figure 1) and many other tissues (24, 25, 26) (Goodwin et al, 1988 and 1992) (Table I). This technology, allows the recapitulated TLAs to be used as host targets for viral and bacterial infectivity (27) (Goodwin et al. 2000) in horizontally rotating cylindrical tissue culture vessels, (RWVs) (28) (Schwarz et al, U.S. patent #5,026,650), that provide controlled supplies of oxygen and nutrients, with minimal turbulence and extremely low shear (29)(Schwarz et al, 1992). These vessels rotate the wall and culture media inside at identical angular velocity, thus continuously randomizing the gravity vector and holding particles such as microcarriers and cells relatively motionless in a quiescent fluid (29, 30)(Schwarz et al 1992; Tsao et al, 1992).

The RWV culture system provides ease of manipulation, consistency in culture conditions, and well-differentiated TLAs that share structural and functional characteristics of the human respiratory epithelium. Culturing normal 3D epithelium configurations larger than 3 mm is problematic using traditional *in vitro* culture technology (31) (Chantret et al, 1988). Thus, the factors that control proliferation and differentiation in complex human tissues are largely unknown (32, 33, 34, 35, 36) (Corps

and Brown, 1985; Pyke and Gogerly, 1985; O'Loughlin et al, 1985; Blay and Brown, 1985a and b). Short-term cultures have been accomplished by a variety of methods for animal or human cells (16, 19, 37, 38) (Fukamachi et al, 1986; Kaye et al, 1971; Kleinman et al, 1983; and Reid and Jefferson, 1987) however, long-term growth has required sophisticated, defined culture media (39) (Moyer, 1990) or *in vitro* transformation to increase longevity (40, 41, 42) (Moyer et al, 1990; Moyer 1991; and Shamsuddin, 1990).

When combined with a solid matrix, cocultivation of epithelial and mesenchymal cells in RWVs allow cells to auto assemble into 3D tissue-like masses which we postulate fulfill four of the five basic stages of tissue regeneration and differentiation (Figure 2). Here we report the successful engineering of the first *in vitro* model of the human respiratory epithelium using primary mesenchymal HBTCs as the foundation matrix and an adult HBE immortalized cell line BEAS-2B as the overlying component. Like the air-liquid interface model (23) (O'Brien et al, 2002), the epithelial cell organization of the TLAs improves the expression of airway epithelial characteristics, and also cellular communication. Thus TLAs represent a physiologically relevant model of the human respiratory epithelia that can be used in large-scale production for prolonged periods.

Materials and Methods

Cell Cultures and Media

Mesenchymal cells (HBTC) from human bronchi and tracheae were obtained from the lung mucosa of multiple tissue donors through Cambrex Biosciences (Walkersville, MD). BEAS-2B epithelial cells were obtained from ATCC (Manassas, VA). All were harvested and banked at the NASA Johnson Space Center's Laboratory for

Disease Modeling and shown to be free of viral contamination by survey of a panel of standard adventitious viruses (e.g. HIV, hepatitis, herpes) conducted by the supplier (Cambrex). Cells were initiated as monolayers in human fibronectin coated flasks (BD Biosciences, San Jose, CA) and propagated in GTSF-2 media supplemented with 10% fetal bovine serum (FBS). GTSF-2, a unique media formulated at NASA's Johnson Space Center (43)(Goodwin, TJ U.S. Patent 5,846,807), was found to meet the growth requirements of the coculture system without the need for unique growth factors and most of the other complex components found in previously used culture media. GTSF-2 is a trisugar-based medium, containing glucose, galactose, and fructose supplemented with 10% FBS. All cell cultures were grown in a Forma humidified CO₂ incubator with 95% air and 5% CO₂, and constant atmosphere at a temperature of 37°C. Normal HBTC mesenchymal and BEAS-2B human lung cells were passaged as required by enzymatic dissociation with a solution of 0.1 % trypsin and 0.1 % EDTA for 15 minutes at 37°C. After incubation with the appropriate enzymes, the cells were centrifuged at 800g for 10 minutes in Corning conical 50 ml centrifuge tubes. The cells were then suspended in fresh medium and diluted into T-flasks with 30 ml of fresh growth medium. BEAS-2B epithelial cells were passaged as required by dilution at a 1:4 ratio in GTSF-2 medium in T-flasks.

RWV Cultures

The RWV is a horizontally rotated transparent culture vessel with zero headspace and center oxygenation. Normal mesenchymal cell monolayers were removed from T-75 flasks by enzymatic digestion, washed once with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), and assayed for viability by trypan blue dye

exclusion (Gibco). Cells were held on ice in fresh growth medium until inoculation. The primary inoculum for each coculture experiment was 2×10^5 mesenchymal (HBTC cells) cells/ml in a 55-ml RWV with 5 mg/ml of Cytodex-3 (Type I, collagen-coated cyclodextran) microcarriers 120 μm in diameter (Pharmacia, Piscataway, NJ, USA). Cultures were allowed to grow for a minimum of 24 to 48 hours before the medium was changed. Thereafter, fresh medium was replenished by 65% of the total vessel volume each 20 to 24 hours. BEAS-2B epithelial cells were added at 2×10^5 cells/ml on day 4. As metabolic requirements increased, fresh medium was supplemented with an additional 100 mg/dl of glucose. Coculture experiments in the RWV were grown in GTSF-2 supplemented with 10% fetal bovine serum as per (25, 26) (Goodwin *et al* 1992, 1993). The optimal period of culture was 15-20 days prior to infection with virus. Experiments were cultured for up to 40 days total including post infection. Viable cocultures grown in the RWV were harvested over periods up to 21 days and prepared for various viral infectivity assays. All RWV cell cultures were grown in a Forma humidified CO₂ incubator with 94.5% air and 5.5 % CO₂ providing constant atmosphere, and at a temperature of 35.0°C to mimic that of the nasopharyngeal epithelium (44).

3D Cell Growth Kinetics

The cocultures were sampled over the course of the experiments, generally at 48-hour time points, in order to establish a cellular development profile. The parameters of glucose utilization and pH were surveyed via iStat™ clinical blood gas analyzer to determine the relative progress and health of the cultures and the rate of cellular growth and viability.

Normal Human Lung and 3D hLEM TLA Immunocytochemistry (IHC)

Normal human lung tissue samples and TLA tissue sections designated for histological and immunohistological staining were washed three times with gentle agitation in 1x PBS (Cellox Laboratories Inc, St. Paul, MN, USA) without magnesium and calcium for 5 minutes to remove foreign protein residues contributed by the media. The TLAs were then transferred to 50 ml polystyrene tubes and covered with 10% buffered formalin in PBS (Electron Microscopy Service, Ft. Washington, PA USA) overnight at 4°C and washed three times in PBS. TLAs were centrifuged at low speed (1000x g) to concentrate the bead-cell assembly. Warm noble agar (1 ml) was added for additional stabilization. TLAs were embedded in paraffin-blocks by standard methods, and light sections cut at 3-5 um on a Micron HM315 microtome (Walldorf, Germany). All unstained sections were stored at -20° until stained with haematoxylin and eosin (H & E) or with a panel of differential and developmental membrane receptor antibodies. The sections were deparaffinized by normal procedure (24), antigen retrieved by protease or citrate, and blocked with a normal rabbit or mouse sera – 0.5% Tween 20 blocking solution. The primary antibody (as identified in Table II) diluted in the blocking solution was incubated on sections between 9 and 30 minutes as required, rinsed with distilled water, and incubated with anti- mouse, -goat, or -rabbit-antibodies conjugated with horseradish peroxidase. The second antibody (Dako Envision System) was applied using an automated immunohistochemical stainer (Dako, Carpinteria, CA, USA). Slides were examined under a Zeiss Axioskop (Hamburg, Germany) microscope and images captured with a Kodak DC 290 Zoom (Rochester, NY, USA) digital camera.

Transmission Electron Microscopy (TEM)

TLA TEM samples were washed three times with 0.1 M sodium cacodylate buffer pH 7.4 (# 11652, Electron Microscopy Science, Port Washington, PA, USA) then fixed in a solution of 2.5% glutaraldehyde-formaldehyde in 0.1 M sodium cacodylate buffer (# 15949, Electron Microscopy Science, Fort Washington, PA, USA) – 0.3 M sucrose (Sigma, St. Louis, MO, USA) – 1% DMSO (Sigma, St. Louis, MO, USA) pH 7.4 (Electron Microscopy Science, Fort Washington, PA, USA) overnight at 4°C. The fixed tissue was washed three times in 0.1M sodium cacodylate buffer, pH 7.4 buffer, post-fixed stained in 0.1 M tannic acid (# 21700, Electron Microscopy Science, Port Washington, Pa, USA) in 0.1 M sodium cacodylate pH 7.4 for 3 hours at room temperature. The tissue samples were washed three times in buffer, and then fixed again in 1.0 M osmium tetroxide (# 19152, Electron Microscopy Science, Port Washington, PA, USA) in cacodylate buffer pH 7.4 for 1.5 hours at room temperature. Samples were dehydrated in a series of graded ETOH, and then embedded in EMbed - 812 resin (# 14120, Electron Microscopy Science, Port Washington, PA, USA). Samples were sectioned at yellow-silver (700 Å), mounted on Ni grids and examined under a JEOL-JEM 1010 transmission electron microscope (JEOL, USA) at 80 kV.

Scanning Electron Microscopy (SEM)

Samples from the RWV cultures were taken for SEM at the same times as those taken for growth kinetics and immunocytochemistry. After removal from the reactor vessels, samples were washed once with CMF-PBS. The samples were suspended in a buffer containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate

buffer at pH 7.4 (45) (Luna 1968), then rinsed for 5 minutes with cacodylate buffer three times and post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA, USA) in cacodylate buffer for 1 hour. Samples were then rinsed three times for 5 minutes each with distilled water and then treated for 10 minutes with a Millipore (Millipore Corp., Bedford, MA, USA) (0.2- μ m)-filtered, saturated solution of thiocarbohydrazide (Electron Microscopy Sciences), then washed five times for 5 minutes each with distilled water and fixed with 1% buffered osmium tetroxide for 10 minutes. This last step was necessary to prevent the microcarriers from collapsing. Samples were then rinsed with distilled water three times and dehydrated with increasing concentrations of EtOH, followed by three changes in absolute methanol. After transfer to 1,1,1,3,3,3-hexamethyldisilazane (Electron Microscopy Sciences), samples were allowed to soak for 10 minutes, drained, and air-dried overnight. Dried samples were sprinkled with a thin layer of silver paint on a specimen stub, dried, coated by vacuum evaporation with platinum-palladium alloy, and then examined in the JEOL T330 scanning electron microscopy at an accelerating voltage of 5 to 10 kV.

Viral infection of 3D hLEM TLAs

TLAs were infected as described previously. Briefly, TLAs were inoculated with *w*tRSV A2 (46) (Lewis et al, 1961) and (47) (Belshe et al, 1982) at a MOI of 0.01. After virus absorption at room temperature for one hour, monolayers and TLA cultures were washed 3 times with DPBS (Invitrogen, Carlsbad, CA) and fed with media specified above. All air bubbles were removed from the RWV before rotation to eliminate shearing of the cells (24) (Goodwin et al, 1988) and before placing in a humidified incubator with

5% CO₂ at 35.0°C. Approximately 65% of the culture media was replaced every 48 hours for both monolayer and TLA cultures. Samples were collected at days 0, 2, 4, 6, 8, and 10 for virus titration. For RSV titration, 1 mL samples of the TLA cultures were flash-frozen with 1X SPG. The titer was determined by immunostaining in HEp-2 cells at 32°C as previously described (48)(49) (Randolph et al, 1994 and Karron et al. 1995).

Immunostaining fixed RSV-infected 3D hLEM TLAs

Uninfected and TLAs (10⁶ cells) infected with wtRSV A2 were fixed at different times post infection (pi) as described (50) (Cheutin et al, 2003). Briefly, paraformaldehyde (EM Grade from Electron Microscopy Sciences, cat #1570) was added to a final concentration of 4% after the TLAs were washed three times in DPBS (Cellgro cat #21-030-CV). After one hour, the TLAs were washed 3 more times with DPBS. 0.1% The TLAs were permeablized in Triton X-100 (Sigma #T9284) for 5 minutes on ice. To avoid nonspecific binding the samples were incubated with 1% BSA for 5 minutes followed by cold water fish gelatin (Fluka #48717) in PBS at room temperature for 10 minutes. The TLAs were incubated with 0.02 M glycine (Fluka Biochemical #1050586) for 3 minutes to reduce autofluorescence. A 1:1000 dilution of RSV F (133-1H and 143-6C) and G (131-1G) monoclonal antibodies (51) (Anderson et al, 1988) were incubated for one hour; then the TLAs were washed 5 times with 1% BSA. Texas Red dye conjugated AffiniPure Goat anti-mouse IgG H + L (Jackson ImmunoResearch Laboratories #115-075-146) was diluted 1:100 and 500 µL was added to each sample for 1 hour, then washed 4 times with DPBS. TLAs were observed with an Olympus IX70 fluorescent microscope.

Results

RWV Cultures

Growth Kinetics of 3D hLEM TLAs

3D hLEM TLAs were produced as illustrated in Figure 1 using GTSF-2 media, and then monitored at 24-hour time points for glucose utilization and pH. Figure 3 reflects a typical metabolic profile for these cultures. These data clearly demonstrate rapid uptake of glucose by TLAs with a slight decrease in pH over the initial growth period. Together these factors indicate an increase in cellular metabolism commensurate with an increase in the size of the aggregates.

3D hLEM TLAs Express Specific Markers of *in vivo* Respiratory Epithelium (IHC)

To compare the cellular composition and differentiation state of TLAs to normal human respiratory epithelium, fixed TLAs and normal human lung sections were immunostained for epithelial specific cell markers (Figure 4, Table II). The cytokeratins (8 and 18; Fig 4G,H,O,P) and Factor VIII (Fig. 4I, J) antibodies detect epithelial, mesenchymal, and endothelial cells, respectively (30, 40, 52, 53, 54) (Tsao, et al, 1992, Moyer, 1990, Woodcock-Mitchell, et al, 1982, Vogel, et al, 1984, Shima, et al 1988). Tubulin (Fig. 4E, F), is a cytoskeletal protein found in epithelial cells (12, 25). Endothelial markers, PECAM-1 (Fig. 4A, B) and Factor VIII (Fig. 4I, J), are present in subsets of precursor endothelial cells, particularly dividing cells. Basement membrane and extracellular matrix components (e.g., collagen IV; Fig. 4Y, Z) were also assayed to determine their expression in the TLAs. Expression of endothelial specific and basement membrane components (Fig. 4 J, Z) were frequently seen at cell-bead-aggregate interfaces. Other markers were also selected to highlight epithelial characteristics

including microvilli (Villin; Fig. 4M, N) tight junctions (ZO-1; Fig. 4Q, R), and polarization (Epithelial Membrane Antigen; Fig. 4C, D). Expression of ICAM-1 (Fig. 4S, T) and cytokeratin 18 (Fig. 4O, P) reflect a differentiated state. Positive staining for mucin (Fig. 4 K, L) indicates production of mucus in the tissue. Of particular interest, Figure 4 T, N, and F illustrate homogenous staining for cytoskeletal markers, ICAM-1, villin, and tubulin at the surfaces of most areas of the cell/microcarrier TLAs. Each of the cell specific cellular stains applied to TLAs compared favorably with the 3D human tissue controls shown in Table III.

3D hLEM TLAs Display Structural Characteristics of the Human Respiratory Epithelia

TEMs of uninfected TLAs (Fig. 5 A-F) illustrate many features of normal tissue and demonstrate recapitulated respiratory epithelium polarized with apical and basolateral sides reinforced the IHC data. TEMs of thin sections of TLAs illustrate human respiratory epithelial characteristics including a multi-layered structure punctuated by extracellular matrix and pseudo-stratified mesenchymal and epithelial layers (Fig. 5 A, B). Multiple cell types are shown in (Fig. 5 C, D); the nuclei of mesenchymal cells (on bead) are elongated and the nuclei of epithelial cells are rounded. Figure 5 (E and F), the center of both micrographs demonstrates conformational data showing tight junctions (TJ) also represented by ZO-1 IHC staining. Microvilli, stained by villin and tubulin on IHC can be seen in Figure 5 F. Further successful villin and tubulin reflects the presence of microvilli as demonstrated in Fig. 5 F (MV).

3D hLEM TLAs are Susceptible to Infection by Respiratory Viruses

Scanning Electron Microscopy (SEM)

TLAs were infected with *w*RSV A2 at a multiplicity of infection (MOI) of 0.01 at 35.0°C, the upper temperature of the human respiratory epithelium. TLA samples were collected at intervals across the initial growth experiment (Fig. 6A, B uninfected) and post infection (pi) (Fig.6 C-F) and were prepared for scanning electron microscopy as stated previously. Photomicrographs taken of day 2-12 cocultures pi showed viral presence and cellular damage (Figure 6C, D). Figure 6E demonstrates cell surface damage analogous to pockmarks at 8 days pi. In Figure 6F, 12 days pi, an insert of budding virus is visible. Samples harvested at approximately 12 days of culture contained small microcarrier bead packs that were totally engulfed in proliferating TLA epithelium despite viral infection (Fig. 6 E, F). Additionally, at 20 days large proliferating masses of TLAs (>3.5mm) were evident, growing on the microcarrier bead packs pi.

Transmission Electron Microscopy (TEM)

TLAs were infected as previously stated. (Fig. 7A-F) illustrates the time course of infection into the 3D hLEM TLAs from 0-12 days respectively. TEMs of all TLAs subjected to virus demonstrated infection beginning as early as 1 hour pi, Figure 7B, and continuing through day 12 pi Figure 7F. Viral nucleocapsids (VNC) were found to locate through out the cells and in the perinuclear regions (Fig. 7 B, E and F) and were overtly apparent in all RSV infected TLAs. Mature virus particles are formed when VNCs bud from the cell membrane containing the viral glycoproteins thus budding virus was present

beginning at day 2 (Fig. 7C) and day 4 (Fig. 7D) and continuing throughout the course of the infection.

Viral Protein and Titer Data

Photographs of fluorescently stained TLAs, specific for RSV glycoprotein that increased in concentration (Days 2-10), are shown in (Fig. 8 A-D). Figure 9 illustrates viral growth kinetics up to day 20 pi with *wtRSV* A2. As illustrated, *wtRSVA2* replicated well in TLAs with peak replication occurring on day 10 (approximately $7 \log_{10}$ particle forming units (pfu) per mL).

Discussion

The data presented constitute a major advance in the construction of a functionally accurate, large-scale $> 3\text{mm}$, three-dimensional *in vitro* tissue model of the human airway. The recapitulation of large TLAs that express differentiated epithelial and mesenchymal cell markers offers a multitude of possibilities for cell biological investigations. Functional epithelial cell brush borders with extracellular matrix and basal lamina components represent ordering of tissue and cellular polarity nurtured by the molecular conditions and physical orientations of the culture system. These data are confirmed in Figures 4 (IHC) and 5 (TEM) and represent concomitant cellular differentiation marker expression and architectural ordering when compared to normal human tissue. Additionally, this three-dimensional model demonstrates a significantly diminished requirement for complex culture media in the RWV culture system. The growth of mesenchymal and epithelial cells in the absence of complex media infers specific cell-cell interactions and the production of the paracrine and autocrine factors essential to the growth, development and differentiation of these fragile tissues. The

nature of these factors, cytokines, cellular interactions and their roles at the molecular and genetic levels are a subject for further investigations..

The role of basement membranes and extracellular matrix and their relationship to epithelio-mesenchymal development and differentiation and infectivity are the subject of considerable research. Studies indicate for example the stromal component exerts a driving influence over developing intestinal mucosa (55, 56, 57) (Haffen et al 1987, Kedinger et al, 1986, Kedinger et al, 1987). Stallmach et al (58) have shown that only organ-specific mesenchyme will produce differentiation in epithelium from a given organ site and that embryonic mesenchyme of the same age but from different organs is ineffective (59, 60) (Quaroni, 1985a, Quaroni, 1985b). Finally, a recent publication demonstrated three-dimensional aggregates derived from an alveolar epithelial tumor cell line (A549) were used as targets for bacterial infection (11) (Carterson et al. 2005). These aggregates, while far superior to two dimensional cultures (as demonstrated in the text) lacked the some of the functional and structural characteristics we report with TLA cocultures. Additionally, the air liquid interface (ALI) models reported by (Zhang et al., 2005) (61) show cellular differentiation, basolateral orientation, and cilia, but lack the fidelity of *in vivo* tissues as the ALI tissue density is approximately 3-5 cell layers versus dozens achieved in TLAs.

The TLA model of human lung embodies most aspects of differentiation and cellular organization observed in other *in vitro* and *in vivo* cell and organ models including the presence of microvilli. Primary distinctions for this model are: (i) the overall scale of the model > 3.5mm diameter inclusive of cellular density translating to in excess of 20 cell layers, a distinct benefit for industrial utility (ii) the ability to culture

epithelium for periods in excess of 35 days without loss of functional cell markers, (iii) the ability to maintain viral production for 20 days pi and cellular repair while maintaining the model, and (iv) the ability of the system to respond to extensive analyses and manipulations without the termination of a given experiment. Future experiments will use genomic and proteomics technologies to clarify and characterize the potential of this new model system. Of particular interest will be regulation of unique cytoskeletal proteins such as villin, functional markers such as tubulin, ZO-1, EMA, ICAM-1, a myriad of inflammatory response modifiers, and other markers that may be represented more accurately by large-scale 3D modeling.

The molecular basis of inflammatory responses and pathogenesis of the human lung to many airborne and blood borne infections may be investigated with the advent of this new technology. Further, clinical response and treatment of diseases may be accomplished more efficiently as a result of rapid vaccine development (Deatly et al, submitted). Analogous to the data presented for RSV, the human immunodeficiency virus (HIV) is shown to replicate in human 3D lymphoid tissues and complex epithelium maintained in the RWV, thus immunodeficiency virus-host interactions in the RWV culture system are possible (41, 62, 63) (Moyer et al, 1990, 1990b, Margolis 1997). This hypothesis is being investigated at the NIH. On this basis, we propose the potential broad application of this culture model may lead to advances in understanding the developing human lung, the potential treatment of a myriad of clinical conditions, and advances in regenerative medicine.

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Figure Legends

1. **Figure 1** Tissue assembly process in a Rotating Cell Culture System
2. **Figure 2** Five stages of tissue development and assembly.
3. **Figure 3** Glucose utilization and pH curves for a healthy three-dimensional culture. Standard error of the Mean for the pH data is < 0.08
4. **Figure 4** Comparative IHC staining of normal human lung tissue samples (A, C, E, G, I, K, M, O, Q, S, U, W, and Y) and recapitulated TLAs (B, D, F, H, J, L, N, P, R, T, V, X, and Z) formed in the Rotating Wall Vessel. Photos are arrayed in matched pairs showing the normal human tissue and the TLAs were stained for PECAM-1 (A and B), EMA (C and D), tubulin (E and F), cytokeratin 8 (G and H), Factor VIII (I and J), mucin (K and L), villin (M and N), cytokeratin 18 (O and P), ZO-1 (Q and R), ICAM-1 (S and T), and collagen IV (Y and Z) . Sample pairs U and V and W and X are H&E histologies demonstrating human tissue organization and TLA cell density. All samples are shown at 400X magnification.
5. **Figure 5 TEMs of uninfected TLAs**, A and B (mag. X 7,500) show TLAs which are multilayered (6 or 7 layers of long thin cells with dark nuclei) and demonstrate extracellular matrix material between the cells; C and D (mag. X 7,500) demonstrate both mesenchymal and epithelial cells (oval and elongated nuclei) lying close to the bead surface; E and F (mag X 50,000) demonstrate cellular tight junctions (TJ) and microvilli (MV) are visible in F.
6. **Figure 6 SEMs of TLAs infected with wtRSVA2**, A and B demonstrate healthy non-infected (smooth) epithelium; C and D demonstrate clusters of

budding virus (BV) atop the epithelium on day 2 and 4 pi; E illustrates the result of viral infection of the epithelial layer on day 8 pi. Notice the pock-marked appearance of the once smooth epithelium. F demonstrates an inset of budding virus masses from an infected epithelium on day 12 pi.

7. **Figure 7 TEMs of *wtRSVA2* infected TLA epithelium.** A is an uninfected micrograph showing a tight junction (TJ) between cells at time zero. B demonstrates viral nucleocapsids (VNC) present in the perinuclear area of the cell at 1 hr. pi. Both A and B shown at mag. X 50,000. C (mag. X 50,000) and D (mag. X 12,000) illustrate the presence of budding virus (BV) at 2 and 4 days pi, respectively, and vacuoles (Vs) in D at day 4 pi. E (mag. X 50,000) and F (mag. X 25,000) show VNC present in the cells at days 8 and 12 pi, respectively.
8. **Figure 8** illustrates the increase in expression of RSV F and G glycoproteins from day 2 to 10 pi.
9. **Figure 9** illustrates the growth kinetics of *wtRSVA2* in recapitulated TLAs up to day 20.

Table Legends

1. **Table I** is a listing of the 3D tissues successfully engineered in the Rotating Wall Vessel
2. **Table II** is a listing of all IHC antibodies used for identification of developmental and differential cell and tissue characteristics
3. **Table III** demonstrates the IHC staining of normal human lung tissues compared to recapitulated human lung TLAs

Table I. Human and Animal TLAs Successfully Engineered In the RWV System

NORMAL	CANCER
Bovine Cartilage (chondrocytes) (64)	Human Colon (24, 25)
	Human Lung (76)
Rat Cardiomyocytes (65)	Human Ovarian (69)
	Human Prostate (67)
Human Bone (Osteoblast) (66, 67)	
Human Cornea (68)	
Human Kidney (26, 70)	
Human Liver (71)	
Human Lymphoid (63,72)	
Human Neural Progenitor (73, 74)	
Human Renal Proximal Tubule (70)	
Human Small Intestinal Epithelial (75)	

Table II. Developmental and Differential Human Immunohistochemistry Antibodies

Antibody	Manufacture	Dilution
Rabbit anti- ZO-1	Zymed, # 61-7300	1:3000
Mouse anti- Human Villin	Neomarkers, Ezrin p81/80K Cytovillin Ab-1, Clone 3C12	1:40
Mouse anti-Human Epithelial Membrane Antigen	Dako, #N1504, Clone E29	1:1500
Mouse anti-Human Endothelial Cell Membrane PECAM-1 (CD 31)	Dako, #N1596, Clone JC70A	1:500
Mucin Stain Kit	Ventana Medical Systems	NA
Mouse anti-Human Cytokeratin 8	Dako, #M0888, Clone RCK 108	predilute
Mouse anti-Human Laminin	Dako, #M0638, Clone 4C7	1:1000
Mouse anti-Swine Vimentin	Dako, #M0725, Clone V9	1:2000
Mouse anti-Human Cytokeratin 18	Dako, #N1589, Clone LP34, 34 beta E12	predilute
Rabbit anti-Human Von Willebrand Factor	Dako, # N1505	1:75
Fibronectin	Dako	1:500
Tubulin	ProMega Cat. No. #946, clone 5G8	1:1000
Collagen IV	Dako #N1536 clone CIV 22	predilute

Table III Native Cellular Differentiation

Tissue Characterization Stains	3D/Nor Hu Lung Tissue	3D/TLA/ BEAS- 2B/
ICAM-1	4+	3+
Villin	2+	3+
Tubulin	3+	4+
Cytokeratin 8	4+	3+
Cytokeratin 18	3+	4+
PECAM-1	3+	4+
ZO-1	2+	3+
EMA	4+	2+
Hu Mucin	4+	4+
VWR/ Factor VIII	4+	3+
Collagen IV	4+	4+

Slides were scored on a relative scale: 0 (no staining), 1+ (weak staining), 2+ weak staining for 25-50% of the cells, 3+ indicates moderate staining for 50-75% of the cells, and 4+ indicates staining of 99% of the cells.