Published in final edited form as:

Exp Lung Res. 2009 March ; 35(2): 155–174. doi:10.1080/01902140802495870.

CHARACTERIZATION OF ALVEOLAR EPITHELIAL CELLS CULTURED IN SEMIPERMEABLE HOLLOW FIBERS

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Abstract

Cell culture methods commonly used to represent alveolar epithelial cells in vivo have lacked airflow, a 3-dimensional air-liquid interface, and dynamic stretching characteristics of native lung tissue—physiological parameters critical for normal phenotypic gene expression and cellular function. Here the authors report the development of a selectively semipermeable hollow fiber culture system that more accurately mimics the in vivo microenvironment experienced by mammalian distal airway cells than in conventional or standard air-liquid interface culture. Murine lung epithelial cells (MLE-15) were cultured within semipermeable polyurethane hollow fibers and introduced to controlled airflow through the microfiber interior. Under these conditions, MLE-15 cells formed confluent monolayers, demonstrated a cuboidal morphology, formed tight junctions, and produced and secreted surfactant proteins. Numerous lamellar bodies and microvilli were present in MLE-15 cells grown in hollow fiber culture. Conversely, these alveolar type II cell characteristics were reduced in MLE-15 cells cultured in conventional 2D static culture systems. These data support the hypothesis that MLE-15 cells grown within our microfiber culture system in the presence of airflow maintain the phenotypic characteristics of type II cells to a higher degree than those grown in standard in vitro cell culture models. Application of our novel model system may prove advantageous for future studies of specific gene and protein expression involving alveolar epithelial or bronchiolar epithelial cells.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Keywords

airway model; lung cell culture; lung epithelial cells; surfactant protein; type II pneumocytes

The mammalian respiratory system represents a heterogeneous tissue that is uniquely adapted to a number of dynamic stresses. The distal alveolar surface is defined by two types of epithelial pneumocytes: alveolar type I (ATI) and alveolar type II (ATII) cells. ATII cells are distinguished by a cuboidal morphology, are confined to the corners of the alveolus, and have special clinical significance in that their function involves the production, secretion, and recycling of pulmonary surfactant [1, 2]. Due to the extremely complex nature of lung architecture that renders the distal alveolar epithelium relatively inaccessible, reproducible and quantitative molecular and mechanistic *in vivo* studies involving the analyses of individual epithelial cell types and their interactions with the environment and other cell types is challenging. In order to examine the cellular and molecular mechanisms involved in surfactant regulation, the application of *in vitro* culture systems is required. Ideally, model systems should incorporate the features of native lung tissue including a 3-dimensional nature, airflow, an air-liquid interface, and dynamic stretching characteristics. Currently used experimental methodologies may not adequately represent all of these conditions likely to be crucial for normal gene expression and cellular function that exist in the *in vivo* alveolar microenvironment.

Several attempts have been made to develop *in vitro* experimental model systems using cultures of alveolar epithelial cells with variable success. These methodologies include the use of primary cultures of isolated ATII cells grown on floating collagen gel or semipermeable polycarbonate membranes and the use of alveolar epithelial cell lines derived from tumors or immortalized cells [3–10]. Primary isolates of ATII cells obtained from excised lung tissue have been used most frequently in studies that attempt to represent alveolar epithelial cells *in vivo*. Typically, methods involve purifying cells directly from the lung and culturing them for 5 to 8 days on semipermeable polycarbonate membranes using Transwell inserts associated with extracellular matrix components or floating collagen gel [4, 5, 11, 12]. This approach has been shown to be effective in allowing cells to generate monolayers that mimic those seen in the *in vivo* lung epithelium; however, primary ATII cells terminally transdifferentiate in culture, resulting in the loss of ATII markers and rapid gain of ATI phenotypes. Variations of experimental conditions (e.g., matrix composition, growth factors, steroids, or mechanical stretch) have been shown to effect the morphological, biological, and molecular expression of markers of ATII cells [10, 13–19]. Additionally, it has proven to be extremely difficult to isolate cultures of ATII cells with greater than 80% to 89% purity, yielding preparations that may be less than ideal for studies of cell-specific gene expression [11].

Immortalized or tumor cell lines are often used for study of ATII cells due to availability and experimental reproducibility, but often lack the fully differentiated phenotype of the *in vivo* ATII cell. Cultures of ATII-like cell lines rarely grow in restrictive monolayers characterized by tight junctions and limited permeability [20, 21]. The development of such a monolayer is crucial in studies that aim to mimic the *in vivo* alveolar epithelium. Additionally, immortalized cell lines often lack distal respiratory epithelial cell characteristics such as apical microvilli and lamellar body inclusions, and rarely secrete physiologically normal levels of surfactant proteins or phospholipids [9].

As described in previous reports, hollow fiber cell culture systems have been applied in studies that aim to grow cells at high densities (10^8 cell/mL or higher) in order to engineer tissues for organ replacement or in applications that involve the production and collection of

secreted proteins, monoclonal antibodies, or viruses [22–25]. Additional studies have used such culture systems, or similarly designed porous microdevices, to culture endothelial cell lines alone and in coculture with other cell types with the goal of improving upon standard cell culture systems where monolayer and tight junction formation remain limited [26–28]. A number of these systems incorporate liquid medium flowing over the cells but are restricted in that cells grown in this manner do not form the air-liquid interface that exists in the alveoli in vivo. In this report, we present a novel approach to culture and maintain a mouse alveolar epithelial cell line in vitro exposed to constant airflow over the cells, while having access to culture medium via the permeable membrane. Using a mouse ATII-like cell line, we demonstrate that the hollow fibers provide a microenvironment that mimics that of the mammalian alveolar epithelium in vivo and enables the maintenance of an ATII cell phenotype, including secretion of mature surfactant protein.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Murine lung epithelial cells (MLE-15) were a gift from Dr. Jeffrey A. Whitsett (Children's Hospital Medical Center, Cincinnati, OH) [9]. Cells were maintained in HITES medium (RPMI 1640 with 5 $\mu\text{g/mL}$ insulin, 10 $\mu\text{g/mL}$ transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β -estradiol, and 10 mM HEPES) supplemented with 4 mM L-glutamine, 2% fetal bovine serum (FBS; Hyclone, Provo, UT, USA), 100 U/mL penicillin G, and 100 $\mu\text{g/mL}$ streptomycin. All media and supplements were obtained from Invitrogen (Carlsbad, CA, USA) unless otherwise indicated. Cells were maintained at 37°C in a humidified atmosphere of air with 5% CO₂.

Hollow Fiber Model System

Production of hollow semipermeable microfibers was performed from polyurethane extrusion as previously described. Briefly, a medical-grade aromatic based polyurethane manufactured by Noveon (40,000 to 70,000 Da; Thermedics Polymer, Wilmington, MA) under the registered trademark name Tecothane was used as a model nondegradable polymer for textured hollow fiber membrane fabrication. Polyurethane solution was prepared by mixing 13 wt % polymer and 87 wt % dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) with constant stirring and hollow fiber membranes were formed with the use of an annular spinneret with a bore liquid precipitant of deionized water. Resulting polyurethane fibers are approximately 13 cm long, having a diameter of 0.9 mm and an internal surface area of about 3.7 cm². The fibers are sealed within a Plexiglas tube associated with a number of connections that provide the ability to modulate the environment both within and surrounding the microfiber (Figure 1A; see Results for additional detail). MLE-15 cells at densities of approximately 8.6×10^4 cells per cm² were seeded via direct injection into sterilized polyurethane microfibers that had been coated with 250 $\mu\text{g/mL}$ rat-tail type I collagen (Sigma) in .1% (v/v) acetic acid. Coated fibers were allowed to dry overnight and then flushed several times with phosphate-buffered saline (PBS) and HITES medium. After seeding, the entire fibers were incubated at 37°C for 48 hours, submerged in HITES medium both inside and outside of the hollow fiber in order to allow for cell attachment and division. After the 48-hour seeding period, airflow consisting of 5% CO₂, 21% O₂ was introduced at a rate of 10 $\mu\text{L/min}$, via a peristaltic pump (Gilson Minipuls 2, Middleton, WI, USA), through the interior of the microfiber for an additional 24 or 48 hours at 37°C. HITES medium surrounding the outer surface of the fiber was circulated continuously using a peristaltic pump. Upon completion of the culturing period, fibers were removed and cells were fixed in either 3% paraformaldehyde (PFA) or 2% glutaraldehyde and cut on an oblique angle for microscopy. For comparison to standard culture conditions, additional sets of control cells were plated at 8.6×10^4 cells per cm² on

polycarbonate 0.4- μ m porous Transwell inserts with a surface area of 0.33 cm² (Corning Costar, Cambridge, MA, USA) or 22-mm round coverslips similarly coated with rat-tail type I collagen. Cultures were grown to confluence in HITES medium during a time course identical to that of the fibers and similarly fixed and stained. Specifically, this involved incubating the Transwell cultures at an air-liquid interface in a minimal amount of culture medium for the final 24 or 48 hours after the initial 48-hour seeding period. Cultures maintained on coverslips were submerged in HITES medium for the entire 72-hour incubation time course. HITES medium was changed every 24 hours in both static cultures. Sets of experiments were replicated a minimum of 5 times.

Immunocytochemistry and Confocal Microscopy

Immunofluorescence analysis was performed according to standard protocols. Briefly, after washing with cold phosphate-buffered saline (PBS), cells were directly fixed within the microfibers, coverslips, or Transwells using 3% PFA. Fixed cells were then further washed with PBS and permeabilized using 0.2% Triton X-100 for 5 minutes. Samples were then blocked in 2% BSA in PBS for 2 hours at room temperature and stained with nuclear stain (TO-PRO-3; Invitrogen) and either phalloidin–Alexa Fluor 488 (Invitrogen) for staining F-actin or with polyclonal rabbit antibody ZO-1 (zonus occludens-1; Zymed Laboratories, South San Francisco, CA, USA) for the detection of intracellular tight junctions. For ZO-1 staining, Alexa Fluor 546 goat anti-rabbit secondary antibody (Invitrogen) was used in an additional 2-hour incubation at ambient temperature. After washing, fibers were sliced at an oblique angle and submerged in 50% glycerol/PBS. Coverslips and culture inserts, after being cut from their supports, were mounted in Pro-Long Gold (Invitrogen). Cytospin samples were prepared by centrifuging suspended cells from each culture condition at 1000 rpm for 5 minutes in the Shandon EZ Cytospin (Thermo Electron). Briefly, cell samples were trypsinized in .05% trypsin-EDTA (Invitrogen), neutralized using 1 mg/mL soybean trypsin inhibitor (Sigma), centrifuged to pellet cells, and then gently resuspended in HITES medium prior to being loaded into the cytofunnel. A polyclonal goat antibody for proSP-B (pro-surfactant protein B; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal rabbit antibodies for SP-B (which recognizes all forms of SP-B, especially mature dimeric SP-B; Chemicon, Billerica, MA, USA), and proSP-C (Chemicon) were used to stain for surfactant protein expression in cytospin preparations. Fluorescein isothiocyanate (FITC) donkey anti-goat secondary (Jackson Laboratories, West Grove, PA, USA) and Alexa Fluor 546 donkey anti-rabbit secondary antibodies (Invitrogen) were used for immunofluorescence detection. Preparations were imaged using an upright MDMRE-7 Leica TCS SPA Acousto-Optical Beam Splitter Confocal Microscope System (Leica, Bannockburn, IL, USA). Immunofluorescence intensity analyses were conducted based upon the mean pixel intensity of positively staining pixels of 10 random images acquired for each culturing condition under fixed acquisition settings. Intensities were normalized to the mean pixel intensity of TO-PRO-3–stained nuclei. Differential interference contrast (DIC) images were acquired using an Axiovert 200M microscope equipped with a 100 $\times$ oil immersion objective (Carl Zeiss Microimaging, Thornwood, NY) and Openlab 5.0.1 software (Improvision, UK).

Transmission and Scanning Electron Microscopy

According to standard transmission electron microscopy (TEM) protocols, samples were fixed in 2% glutaraldehyde, postfixed in buffered osmium tetroxide, dehydrated in a series of graded alcohols, and then embedded in resin, sectioned, and imaged. Sections were viewed and TEM images were acquired with a Hitachi H7000 high-resolution transmission electron microscope (Hitachi, Pleasanton, CA, USA). Morphological analysis of the hollow fiber membranes was conducted with the use of scanning electron microscopy (SEM). Membranes were allowed to air dry in a laminar flow hood prior to SEM analysis. Samples were then carefully fixed on SEM sample holders with a conductive tape before being

sputter coated with gold at a thickness of 50 to 70 nm with the use of a Cressington 108 AUTO sputter coater (30 mA for 2 minutes). SEM images were acquired using a JEOL 5410 with an acceleration voltage of 5 to 10 kV. TEM and SEM preparations were performed by the core electron microscopy facility (Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC).

Immunoblotting

Cells were rinsed with ice-cold PBS and then gently trypsinized using .05% trypsin-EDTA. Cell suspension was collected into serum-containing HITES medium to inactivate trypsin and centrifuged for 5 minutes at $100 \times g$. Cell pellet was washed in PBS and then resuspended in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% tert-octylphenoxy poly(oxyethylene)ethanol (Igepal CA-630), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and a cocktail of additional protease inhibitors (Sigma product number P8340). Lysis of cells within the hollow fiber membranes fibers was performed by similarly filling the fiber lumen with 0.05% trypsin-EDTA and collecting the resulting cell suspension. After a 20-minute incubation on ice with occasional vortexing, samples were centrifuged at $16000 \times g$ for 10 minutes at 4°C . Protein concentrations of the supernatants were determined using a micro-bicinchoninic acid quantification kit (Pierce Biotechnology, Rockford, IL, USA). For secretion experiments, attached cells were washed twice with serum-free medium to remove extracellular surfactant and then incubated for an additional 24 hours in fresh serum-free medium. Medium was collected and clarified by centrifugation at $16,100 \times g$ for 10 m. Secreted proteins in clarified medium were separated by SDS polyacrylamide gel electrophoresis on 12% Bis-Tris minigels (NuPAGE, Invitrogen) and transferred to nitrocellulose membranes using 25 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) transfer buffer (pH 11). Membranes were blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 and probed with antibodies against human proSP-C (Chemicon, Billerica, MA, USA) and/or SP- B (Chemicon), followed by peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody (Pierce) and detection using the ECL Plus Western Blotting Detection kit (GE LifeSciences, Piscataway, NJ, USA).

RESULTS

Development of Hollow Fiber Model System

A schematic of our model system involving hollow porous polyurethane fibers sealed within a Plexiglas tube is depicted in Figure 1A. Also indicated are connectors at the hollow fiber ends serving as air inlet and outlet, in addition to connectors in the Plexiglas tube body for modulation of the environment surrounding the fiber. Scanning electron micrographs show porous surfaces that give the fiber semipermeable characteristics (Figure 1B). This design allows for control of gas or liquid flow rate and environment directly within the hollow fiber membrane, as well as that surrounding the microfiber. As cells are directly cultured on the internal surface of the fibers, air passage through the lumen in conjunction with medium surrounding the outside of the hollow fiber provide for the formation of an air-liquid interface.

Cellular Phenotype of MLE-15 Cells Grown Within Hollow Microfibers

The murine lung epithelial cell line MLE-15 is representative of the distal bronchiolar and alveolar epithelial cell and maintains a number of ATII-associated characteristics, including phospholipid secretion and surfactant protein synthesis and secretion [9]. To test our hypothesis that ATII-like cells grown within hollow porous microfibers would more accurately mimic ATII cells in a normal alveolar epithelium in vivo, MLE-15 cells were

seeded via direct injection into collagen-coated hollow fibers, and resulting phenotypic characteristics were compared with those of conventional cultures. MLE-15 cells grown within the hollow fiber membranes under conditions that provided a curved surface on which to grow and that allowed for the development of an air-liquid interface via the induction of air flow through the hollow fiber lumen maintained a morphological phenotype that more closely resembles the physiological milieu of ATII cells in vivo (Figure 2*D* to *F*). Results of immunofluorescence imaging using confocal microscopy and anti-F-actin antibody indicate that cells grown within the hollow microfibers grew to confluence without cellular overlap, maintained a cuboidal shape, and formed a solid monolayer consistent with the formation of cell-cell contact. Conversely, MLE-15 cells that were grown in standard cell culture conditions on collagen coated coverslips (Figure 2*A* to *C*) or on Transwell inserts (Figure 2*G* to *J*) generally lost their cuboidal shape and developed the elongated squamous appearance and cytoplasmic extensions more characteristic of ATI cells. Furthermore, cells in standard or Transwell culture conditions appeared to preferentially grow on top of one another rather than forming a continuous monolayer.

To further validate these observations, the expression of structural markers for epithelial tight junctions was measured by immunostaining for the tight junction protein ZO-1, which is found in polarized airway epithelia. Staining of cell cultures grown within the hollow fiber membranes revealed distinct junctional ZO-1 localization characterized by a sharp continuous band surrounding each cell at its apical border (Figure 3*B*). Immunofluorescence on control MLE-15 cells grown on collagen coated Transwell membrane inserts not only showed overlapping nuclei, indicating the cells were growing vertically on top of one another, but additionally showed discontinuous and enhanced cytoplasmic ZO-1 staining (Figure 3*A*). Similar results were seen with MLE-15 cell cultures that were grown on collagen-coated coverslips (data not shown). Thus, ZO-1 labeling demonstrated both proper cell-cell interactions and cell polarity only within the hollow fiber cultures.

Electron Microscopy and Differential Interference Contrast of MLE-15 Cells Grown Within Hollow Microfibers

For analyses of the ultrastructure of MLE cells grown within the hollow fiber membranes or in standard culture, transmission and scanning electron microscopy was performed. Transmission electron microscopic images revealed that MLE-15 cells grown within the hollow fibers had typical ATII cell characteristics, including well-defined cellular contact areas consistent with the ability to form tight junctions, a high density of cellular organelles representative of lamellar bodies and multivesicular bodies, as well as the presence of numerous fine microvilli (Figure 4*A* to *F*). Conversely, control MLE-15 cells grown on coverslips in standard cell culture conditions showed the presence of flattened nuclei and long cytoplasmic extensions, as well as the pronounced deficiency of microvilli in comparison to those grown within the hollow microfibers (Figure 4*G* to *J*). Due to a predominantly lipid composition with limited intralamellar space, lamellar bodies can be easily identified using phase-contrast and differential interference contrast (DIC) microscopies [19, 30]. DIC images of cytospin preparations confirmed EM results in indicating numerous optically dense particles representative of lamellar bodies within the cytoplasm of MLE-15 cells grown within hollow fibers with airflow as compared to cells grown on coverslips of Transwells (Figure 5). In addition, the cytoplasm appears to be profoundly condensed (less ATII-like) for MLE cells grown under conventional conditions versus cells cultured in hollow fibers, whereas that of cells grown on Transwells was moderately reduced versus that of cells grown in fibers (Figure 5).

Surfactant Protein Production and Secretion by MLE-15 Cells Grown Within Hollow Fibers

The primary function of ATII cells within the lung alveoli is to produce and recycle surfactant lipids and proteins; however, cultures of primary cells or alveolar cell lines often lose these capabilities [6, 12, 31]. To overcome this phenotypic loss, we investigated whether the MLE-15 cells produced and secreted surfactant when grown within the hollow fibers. Western blot analysis was performed on whole cell lysates collected from cells that were grown within hollow fiber membranes, on Transwell inserts or on coverslips for a total of 3 or 4 days (the final 24 or 48 hours of which the cells were exposed to constant airflow), using SP-A, proSP-B, mature SP-B, and the proSP-C as markers of surfactant synthesis. Immunocytochemistry analyses were performed on MLE-15 cells cultured on coverslips, Transwells, and hollow fibers and then stained for surfactant proteins. Cytospin preparations of each culture condition were used in order to maintain identical processing conditions and allow for direct comparison between samples. Immunofluorescence analyses indicated increased levels of SP-B staining as well as moderate increases in proSP-C staining. However, proSP-B staining levels remained consistent between culturing conditions (Figure 6). Although the anti-mature SP-B antibody used for immunofluorescence preferentially recognizes mature SP-B, it should be noted that it also recognizes proSP-B to a much lesser extent as well.

Western blot analyses further confirmed proSP-C and mature SP-B protein expression in hollow fiber, Transwell, and coverslip culturing conditions (Figure 7A). Although results presented in Figure 7 indicated similar levels of proSP-C expression in MLE cells under all culturing conditions, a 2- to 3-fold increase in the production of mature SP-B was routinely observed for cells cultured in hollow fibers compared with that in cells grown in standard cell culturing conditions. Conversely, quantitative real-time polymerase chain reaction (PCR) analyses of MLE-15 cells cultured on coverslips, Transwells, and fibers indicated no significant difference in either SP-B or SP-C mRNA levels between conditions (data not shown). Therefore, increased levels of surfactant protein for MLE cells grown in the fibers (versus other culture conditions) are most likely due to changes in translational regulation, enhanced protein and/or protein processing.

The molecular and cellular mechanisms that regulate surfactant secretion are numerous and complex and involve a number of hormones, growth factors, and signal transduction pathways, as well as post-transcriptional and post-translational control [32]. Due to the fact that protein production does not necessarily indicate that the MLE-15 cells grown within our hollow fiber membranes are correctly processing and secreting surfactant proteins, we analyzed SP-A and SP-B secretion into the surrounding media from MLE-15 cultures in standard epithelial cell culture conditions on Transwell membranes as well as that from our hollow fiber membranes. SP-A is the most abundant surfactant protein and thus has traditionally been used to monitor surfactant synthesis in tissue culture systems. Additionally, to our knowledge, SP-B secretion from MLE-15 cells has not been reported to date. Western blot analyses on the collected serum-free HITES medium wash of the air/cell "surface fluid" showed both cultures were secreting SP-A, but only the cultures grown within our hollow fiber membranes appeared to be functionally secreting the 16 k-Da mature SP-B dimer (Figure 7B, lanes 1 and 4, respectively; due to lack of detection, data not shown for cells grown in Transwells or on coverslips). Furthermore, when airflow within the hollow fiber membrane was allowed to proceed for an additional 24 hours (48 hours total), secretion levels of surfactant proteins A and B were maintained and slightly increased (Figure 7B, lanes 2, 5). It should be noted that no additional hormones or supplements, other than those typically found in HITES medium, were added in these experiments. Due to the lack of a commercially available antibody for mature SP-C, secretion of mature SP-C could not be measured. Taken together, these results indicate that surfactant proteins are successfully synthesized, post-translationally processed, and secreted by MLE-15 cells

grown within the hollow fiber membranes and confirm that this characteristic is maintained over time. This further supports the hypothesis that our model system accurately represents the appropriate cellular microenvironment required to maintain an *in vivo*-like phenotype required in studies of the alveolar epithelial cell types and surfactant regulation.

DISCUSSION

The complex design and function of the lung exposes alveolar epithelial cells to unique surface forces and higher oxygen tensions than any other cell type in the body. Due to the difficulties of maintaining primary cell cultures or developing alveolar epithelial cell lines that exhibit both the phenotypic and gene expression patterns of *in vivo* ATII cells, we developed a semipermeable microfiber system designed to mimic the 3-dimensional nature and air-liquid interface characteristics of intact native lung tissue that are critical for normal gene expression and cellular function. When the immortalized, ATII-like murine cell line MLE-15 was cultured within our system, the ability of these cells to maintain an *in vivo*-like cell phenotype was significantly improved in comparison to control cells grown under conventional epithelial cell culture conditions. This culture system is likely to aid studies in further defining the cellular and molecular mechanisms that are involved in ATII cell—specific gene regulation, surfactant lipid and protein synthesis, processing, secretion, and recycling.

The physiological microenvironment directly impacts the expression of ATII cell morphology, proliferation, differentiation, secretion, movement, signal transduction, and gene expression pathways. In studies that have used primary mouse and rat ATII cells, the lack of a sufficiently representative microenvironment results in the direct loss of a native cuboidal cellular morphology, a tight polarized monolayer with representative tight junction formation, functional organelle lamellar body formation, apical microvilli, and the production and secretion of surfactant lipids and proteins [4, 5, 8]. Primary cultures of ATII cells maintained on standard cell culture dishes become flattened and squamous and do not maintain the *in vivo* phenotype [6, 8, 12, 31]. However, cells cultured in our model system conserved a cuboidal shape, a characteristic that is critical for maintenance of ATII differentiation as well as surfactant protein mRNA stability and protein processing [6, 33].

Previous attempts to develop culture techniques that encourage the retention of an ATII phenotype have required growing the cells in an air-liquid interface in association with specific substrata (e.g., collagen or Matrigel) and the addition of a number of soluble factors, such as keratinocyte growth factor (KGF), species-specific serum, and dexamethasone [14, 19, 34]. Alcorn and colleagues directly examined the effects of coordinate medium placement on type II cell characteristics, confirming the now well-established observation that exposure of the apical surface of epithelial cells to air and the development of an air-liquid interface increase oxygen availability and are crucial for promotion and maintenance of type II cell differentiation with regard to cellular morphology, surfactant expression, and the presence of cellular organelles, lamellar bodies, and apical microvilli [35]. Because lamellar bodies play a critical role in surfactant regulation, the presence of a high number of functioning lamellar bodies is commonly used as an indicator for the identification of *in vivo*-like ATII. Interestingly, the cells grown within our hollow fiber membranes in addition to maintaining a cuboidal shape showed numerous microvilli and optically dense organelles representative of lamellar and multivesicular bodies without the use of additional growth factors. This could partially be due to the fact that our system directly promotes the formation of an air-liquid interface via efficient modulation of gas exchange and flow rate over the apical surface of cell monolayers. In addition, the curvature of the fibers themselves may enhance growth characteristics of the ATII-like cells. Such an environment would allow for increased oxygen availability and perhaps an increased ability to maintain cell

polarity. Functional ZO-1 staining, such as that observed in the hollow fiber membrane culture, is crucial to maintain the polarized phenotype of epithelial cells [36, 37]. Maintenance of signaling pathways associated with cell polarity are likely contributors to enhanced ATII cell phenotype and proper surfactant protein synthesis and export.

In order to function effectively, the alveolar surface must form a selectively permeable monolayer where cell-cell contact provides important spatial cues that are required to generate cell polarity, as well as inter- and intracellular communications [20, 21, 37]. The establishment and maintenance of such a barrier occurs via the interactions of tight junctions, gap junctions, and adherens junctions and confers rate-limiting vectorial transport properties that restrict the passage of lipid-insoluble molecules between alveolar and interstitial spaces. In previous studies, investigators have used the human cell line A549 as a model of the ATII cell [26, 34, 38–41]. However, it has been routinely observed that these cells fail to form a sufficiently restrictive paracellular barrier, in addition to displaying insufficient differentiation with respect to formation of a tight epithelial barrier with intact cell-cell junctions [34, 39]. Although MLE-15 are representative of normal ATII cells in terms of surfactant protein expression, few studies, if any, have reported the successful establishment of a polarized monolayer without the addition of growth factors or hormones. Immunostaining with ZO-1 as well as TEM analyses indicate that our culture system provides the appropriate microenvironment that specifically enables MLE cells to form a tight polarized monolayer without the need for additional supplements.

Studies of surfactant secretion by primary or model ATII cells have been greatly hampered by the fact that synthesis and secretion of surfactant phospholipid and protein decrease rapidly during culture and that immortalized lines do not appear to express all surfactant mRNAs [6, 12, 13]. Secretion of the surfactant proteins SP-B and SP-C is lamellar body dependent, initially involving packaging surfactant components from the endoplasmic reticulum (ER) and the Golgi apparatus to the trans-Golgi network and eventually to multivesicular bodies where proSP-B and proSP-C are processed, and apparently leads to the formation of surfactant-rich lamellar bodies. Past studies have shown that isolated ATII cells and immortalized ATII-like cell lines appear unable to traffic transfected surfactant proteins to lamellar bodies and become unresponsive to additives used to stimulate surfactant secretion [42, 43]. This suggests that the functional secretory pathway within these cells becomes compromised in culture. Although permitting the maintenance of surfactant gene expression and the cuboidal phenotype ATII, cells that are grown on Matrigel-coated Transwell plates promote the formation of cells aggregates, resulting in the inward orientation of apical surfaces [44]. Successful *in vivo*-like surfactant protein secretion thus involves a number of mechanisms that go beyond the ability for the cell to successfully produce surfactant protein mRNAs. Although the precise pathway(s) of secretion of SP-A have not been rigorously defined, it has been routinely used to as a marker for surfactant protein secretion from ATII cells [6].

Our data indicate that MLE alveolar epithelial cells grown in our hollow fiber membrane culture system actively secrete both SP-A and SP-B. SP-B secretion by immortalized ATII cell lines has not been previously reported. In our studies, both control and experimental cultures showed similar SP-A secretion, which is consistent with an ATII phenotype. Although there was an observed relative decrease of organelles representative of lamellar bodies in the control cultures, previous studies have found that the amount of SP-A found in lamellar bodies is low when compared to overall SP-A secretion, indicating that SP-A secretion occurs via other lamellar body-independent means [45]. Furthermore, both SP-A and SP-B secretion appeared to increase when the cells were allowed to culture with airflow for an additional 24 hours. Though MLE cells lysates from MLEs cultured in either standard culture or in the hollow fibers demonstrated characteristic ATII SP-A secretion and SP-B

and pro SP-C expression, Western blot and immunocytochemistry microscopic results indicated that cells grown within the hollow fiber membranes appeared to routinely produce approximately 2 to 3 times more mature SP-B based on total cellular protein when compared to those in standard cell culture conditions. Immunocytochemistry staining for proSP-B showed consistent staining levels regardless of culture conditions and suggests that this increase in SP-B levels may be due to enhanced proSP-B processing. This is supported by the presence of numerous organelles representative of multivesicular bodies and lamellar bodies within MLE-15 cells grown in hollow fibers. Furthermore, our quantitative real-time PCR analyses results indicated no significant change in mRNA levels of surfactant protein B or C in MLE cells as a function of culturing conditions, an observation that is consistent with the concept that observed increases in surfactant protein levels are the result of the fiber microenvironment enhancing translation, increased protein processing, and/or increased protein stability. Taken together, these novel findings further validate the use of our hollow fiber membranes to maintain a representative *in vivo* phenotype in ATII cells.

In summary, the hollow fiber culture model described here presents an initial step towards the development of a system that has a number of important advantages as compared with previously applied methodologies. Most notably, it allows for the culture of alveolar epithelial cells within a microenvironment that accurately mimics the physiology that exists in the alveolus and allows for the normal phenotypic gene expression and functioning of ATII cells without the need for additional hormones or growth factors. This is important for developing and maintaining a cuboidal shape and a polarized monolayer with the formation of tight junctions as well in terms of maintaining abundant functioning lamellar bodies and expressing and secreting surfactant proteins. In addition to these advantages, this hollow fiber culture system would also provide a useful platform for manipulation of experimental parameters, including studies in which ATII cells are co-cultured with fibroblasts or endothelial cells in an attempt to improve type II differentiation and/or more accurately represent *in vivo* events. These endothelial-epithelial coculture systems would act to mimic *in vivo* alveolo-capillaries, whereas fibroblast coculture has been previously shown to improve ATII phenotype, as well as phospholipid, SP-A, SP-B, and SP-C biosynthesis [8, 33]. Also, this hollow fiber culture system would allow for a highly controlled basolateral or apical introduction of factors such as cytokines or toxins and/or variation that may elucidate particular genes and proteins involved in maintaining lung function during stress.

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FIGURE 1. Schematic of selectively permeable, hollow fiber membranes for air-liquid culture of cells (A) and scanning electron micrographs of polyurethane hollow fiber membranes (B).

FIGURE 2.

MLE-15 cells maintain an ATII-like phenotype when grown in hollow fibers. MLE-15 cells grown in (A–C) standard cell culture conditions on coverslips, (D–F) hollow polyurethane microfibers, and (G–I) standard cell culture conditions on Transwell inserts. Cells were stained with ToPro-3 nuclear stain (*blue*) and actin-specific Alexa 488 phalloidin stain (*green*). Merged images are depicted on far right. Cells were grown in standard HITES medium for a total timecourse of 72 hours.

FIGURE 3.

MLE-15 cells form tight junctions when grown in hollow fibers. MLE-15 cells grown in (A) standard cell culture conditions or (B) hollow fiber membranes. Stains: ToPro-3 nuclear stain (*blue*) and anti-zona occludins (ZO-1) antibody (*red*).

FIGURE 4.

MLE-15 cells form an ATII cell-like phenotype when grown within semipermeable hollow microfibers. (A–F) TEM images of MLE-15 cells grown and fixed within hollow fiber. (G–I) TEM images of MLE-15 cells grown and fixed on collagen-coated coverslips. Arrows indicate cell-cell contact sites consistent with the appearance of tight junctions. All scale bars = 500 nm.

FIGURE 5.

Culture condition–dependent differences in optically dense inclusions representative of lamellar bodies. (A) DIC images of MLE-15 cells grown on coverslips; (B) Transwell membranes and (C) within hollow fiber membranes. Cytospin protocol was used to generate preparations as described in Materials and Methods. Scale bars = 5 μm .

FIGURE 6.

Enhanced production of surfactant proteins by MLE-15 cells grown in hollow fiber membranes. Immunofluorescence analyses of MLE-15 cells grown on coverslips, Transwell membranes, and within hollow fibers. Cytopsin protocol was used to generate preparations. SP-B (red; upper panel), proSP-B (green; upper and lower panels), and proSP-C (red; lower panel) polyclonal antibodies were used to stain for surfactant proteins. Nuclei are stained with ToPro-3 (blue). Scale bars = 10 μm. Mean intensity measurements of positive staining pixels indicate increased levels of mature SP-B and proSP-C in hollow fiber cultures (values normalized using nuclear stain pixel density).

FIGURE 7.

Surfactant proteins are produced and secreted by MLE-15 cells grown within semipermeable hollow fibers. (A) Western blot analysis of surfactant proteins in lysates from MLE-15 cells grown in hollow fiber membranes, on Transwell membranes, or on coverslips using proSP-C and mature SP-B polyclonal antibodies (15 μ g total cellular protein were loaded to each lane, with the exception that 10 μ g total protein loaded for lanes for proSP-C detection). (B) Western blot analysis of mature SP-B secretion using equal volumes of serum-free medium collected after growth in fibers with air flow for 24 hours (lanes 1 and 4) or growth in fibers with air flow for 48 hours (lanes 2 and 5); lane 3, purified mature SP-B standard. Western blot analyses of surfactant proteins using SP-A and mature SP-B polyclonal antibodies.