Video Article

Establishing a Liquid-covered Culture of Polarized Human Airway Epithelial Calu-3 Cells to Study Host Cell Response to Respiratory Pathogens *In vitro*

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Abstract

The apical and basolateral surfaces of airway epithelial cells demonstrate directional responses to pathogen exposure *in vivo*. Thus, ideal *in vitro* models for examining cellular responses to respiratory pathogens polarize, forming apical and basolateral surfaces. One such model is differentiated normal human bronchial epithelial cells (NHBE). However, this system requires lung tissue samples, expertise isolating and culturing epithelial cells from tissue, and time to generate an air-liquid interface culture.

Calu-3 cells, derived from a human bronchial adenocarcinoma, are an alternative model for examining the response of proximal airway epithelial cells to respiratory insult[1], pharmacological compounds[2-6], and bacterial[7-9] and viral pathogens, including influenza virus, rhinovirus and severe acute respiratory syndrome - associated coronavirus[10-14]. Recently, we demonstrated that Calu-3 cells are susceptible to respiratory syncytial virus (RSV) infection in a manner consistent with NHBE[15,16]. Here, we detail the establishment of a polarized, liquid-covered culture (LCC) of Calu-3 cells, focusing on the technical details of growing and culturing Calu-3 cells, maintaining cells that have been cultured into LCC, and we present the method for performing respiratory virus infection of polarized Calu-3 cells.

To consistently obtain polarized Calu-3 LCC, Calu-3 cells must be carefully subcultured before culturing in Transwell inserts. Calu-3 monolayer cultures should remain below 90% confluence, should be subcultured fewer than 10 times from frozen stock, and should regularly be supplied with fresh medium. Once cultured in Transwells, Calu-3 LCC must be handled with care. Irregular media changes and mechanical or physical disruption of the cell layers or plates negatively impact polarization for several hours or days. Polarization is monitored by evaluating trans-epithelial electrical resistance (TEER) and is verified by evaluating the passive equilibration of sodium fluorescein between the apical and basolateral compartments[17,18]. Once TEER plateaus at or above 1,000 Ω×cm^2, Calu-3 LCC are ready to use to examine cellular responses to respiratory pathogens.

Video Link

The video component of this article can be found at https://www.jove.com/video/50157/

Protocol

1. Culturing Calu-3 Cells for Use in Transwell Cultures

Safety Measures: Perform all procedures in a biosafety cabinet using sterile culture technique.

1. To thaw cells from frozen storage:
    1. Prepare Eagle's Minimum Essential Medium containing 20% heat-inactivated fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 2 mM L-glutamine, and10 mM HEPES pH 7.4 (EMEM-20%+S). Sterile-filter the prepared medium using a 0.2 µm pore-size filter, and warm in a water bath to 37 °C. To be more reflective of the *in situ* airway epithelium environment, medium is not supplemented with antibiotics or antimycotics.
    2. Thaw a frozen cryovial of Calu-3 cells rapidly (< 1 min) in a 37 °C water bath.
    3. Transfer the thawed cells to a 50 ml sterile conical tube and add 30 ml of warmed EMEM-20%+S.
    4. Pellet the cells by centrifugation for 5 min at 1,000-1,200 × g, without refrigeration, and with low brake speed.
    5. Decant the supernatant gently and resuspend the cells in 5 ml of warmed EMEM-20%+S by gently pipetting up and down. Seed 2 to 5 × 10^6 cells per well into a 6-well tissue culture plate and incubate at 37 °C, 7% CO_2 in air atmosphere until the cells are 75% - 80% confluent. Check daily; up to 7 days may be required. Every 3-4 days aspirate the medium from the cells and replace it with fresh EMEM-20%+S.

2. To subculture cells:
    1. Warm 0.05% trypsin - 0.02% EDTA in a 37 °C water bath. Aspirate the medium from the cells, and wash cells once with warmed 0.05% trypsin - 0.02% EDTA to remove excess medium and serum. Remove the wash and add 1 ml of warmed trypsin per well to the cells.
    2. Incubate at 37 °C and 7% CO_2 and monitor under a microscope every 5 min until at least 75% of cells detach from the well(s) of the tissue culture plate. This may take between 5 and 30 min.
    3. Wash cells in EMEM-20%+S to remove excess trypsin. Transfer the trypsinized cells to a 50 ml sterile conical tube. Multiple wells of cells from one 6-well culture plate may be pooled into one 50 ml sterile conical tube. Add 30 ml of EMEM-20%+S and pellet the cells by centrifugation at 1,000-1,200 × g, without refrigeration, and with low brake speed.
    4. Decant the supernatant gently and resuspend the cells in fresh EMEM-20%+S by gently pipetting up and down. Using steps 1.2.1 through 1.2.3 above to trypsinize cells, continue to subculture cells into tissue culture dishes as described in steps 1.2.5 through 1.2.7 when they are between 75 - 80% confluent.
    5. Subculture from one well of a 6-well plate up to one T-25 cm^2 flask in a final volume of 5 ml of EMEM-20%+S, seeding between 0.5 to 1 × 10^6 cells/flask.
    6. Using 5 ml warmed trypsin per T-25 cm^2 flask to detach cells, subculture from one T-25 cm^2 flask up to one T-75 cm^2 flask in a final volume of 10 ml of EMEM-20%+S, seeding between 1 to 5 × 10^6 cells/flask.
    7. Using 8 ml warmed trypsin per T-75 cm^2 flask to detach cells, subculture from one T-75 cm^2 flask into 2 T-75 cm^2 flasks. For optimal cell growth, do not subculture cells into flasks larger than T-75 cm^2 or subculture at a ratio greater than 1 parent flask:3 new flasks.
3. Once cells have been subcultured, completely remove and replace medium every 2 to 3 days until they reach 75-90% confluence. Cells may take up to 3 weeks to reach 75-90% confluence. For optimal generation of polarized cultures, subculture cells before they grow beyond 90% confluence as a monolayer.
4. Continue to subculture cells until enough cells have grown to seed the desired number of Transwell inserts. Each Transwell insert requires 2 × 10^5 cells. For optimal performance generating polarized cultures, use cells that have undergone no more than 10 subcultures.

2. Growing Polarized Calu-3 Liquid-covered Cultures (LCC)

Safety Measures: Perform all procedures in a biosafety cabinet using sterile culture technique.

1. Prepare EMEM containing 10% FBS (EMEM-10%) and warm in a water bath to 37 °C.
2. Prepare a 24-well Transwell plate for seeding. Using sterile forceps, move Transwell inserts from the interior to the exterior rows of the plate without touching the insert membrane. Cells should only be subcultured into wells on the exterior rows of the plate. To prevent the introduction of air bubbles into the basolateral compartments, add 600 µl of EMEM-10% to each one by angling the pipette against the wall of each compartment and slowly releasing the medium into the well.
3. Detach cells from T-75 cm^2 tissue. Subculture flasks using warmed trypsin, and wash in 30 ml of EMEM-20%+S per every 2 flasks of trypsinized cells, as described in step 1.2.
4. Thoroughly resuspend the washed cells in 5 ml of EMEM-10% by gently pipetting up and down.
5. Determine viable and total cell counts by the trypan blue exclusion method. Proceed only if 80% - 90% are viable.
6. In a 50 ml sterile conical tube, dilute cells to a concentration of 2 × 10^6 viable cells/ml with EMEM-10%.
7. Add cells to the apical compartment of each Transwell. To wells A1 and D1, which will be cell-free control wells, that is, blanks, add 100 µl of EMEM-10% without cells. To each of the remaining wells, add 100 µl of resuspended Calu-3 cells, gently angling the pipette against the interior guide channel of the insert wall and slowly releasing the cells. Do not touch the pipette to the insert membrane. To maintain a homogeneous suspension of Calu-3 cells, gently agitate the cell suspension during this seeding step.
8. Incubate Transwell plate at 37 °C and 7% CO_2 in air atmosphere. Place plate in an incubator where physical disturbance will be minimal. To improve the efficiency of polarization, do not stack plates on top of each other.
9. To maintain cultures until polarized and ready to use in experiments, completely replace medium in Transwells, as described in steps 2.10 through 2.13 below. Medium should be completely replaced three days after being subcultured into Transwells, and then on a cycle alternating between the fourth and then third day after the previous feeding, until cells are fully polarized.
10. Warm EMEM-10% in a water bath to 37 °C.
11. Gently aspirate medium from Transwell inserts. With a capillary pipette attached to a vacuum trap with a gentle vacuum, or with a standard 1 ml pipette, aspirate medium from cell-free wells A1 and D1, then from seeded wells, first removing apical medium from all wells, and then removing basolateral medium from all wells. Do not touch insert membranes while aspirating medium.
12. Add 200 µl of EMEM-10% to the apical compartment of cell-free wells A1 and D1, then to the seeded wells, directing the medium into the apical compartment using the side of the insert to guide the pipette tip. Do not add medium directly onto cells, and do not touch the insert membrane.
13. Add 600 µl of EMEM-10% to the basolateral compartments. To prevent the introduction of air bubbles into the basolateral compartments, add medium to each one by angling the pipette against the wall of each compartment and slowly releasing the medium into the well.

3. Evaluating Resistance Development of Calu-3 LCC

Safety Measures: Perform all procedures in a biosafety cabinet using sterile culture technique.

1. Evaluate trans-epithelial electrical resistance (TEER) of Calu-3 LCC 30 min after medium changes; the evaluation can be performed after each medium change if desired. Perform measurements under sterile conditions. Begin measurements with the cell-free control wells A1 and D1 (the blanks) to obtain baseline measurements, and continue with measurements for each well.
    1. Under sterile conditions, transfer the STX2 electrode to a 50 ml centrifuge tube containing 70% ethanol in sterile water, and sterilize 15 min.
    2. Calibrate and test the voltohmmeter for use according to manufacturer's directions.
    3. Remove the electrode from the ethanol, air dry 5-10 sec, and rinse the electrode with sterile EMEM-10%.
    4. Set the mode switch of the voltohmmeter to the RESISTANCE setting, and turn power ON.

5. Gently place the electrode into one of the 3 ports that allow access into the basolateral compartment of one Transwell culture. Place electrode so that the longer lead just lightly touches the bottom of the outer well and remains vertical, and the shorter lead is in the tissue culture medium of the apical compartment, without touching the insert membrane.
6. Push "Measure R" button, and wait for the reading to stabilize. Repeat for the other 2 ports for each well and record the measurements from all three ports, for a total of three measurements per well. Continue to measure the resistance for all wells of cells.
7. Clean the electrode by soaking 5-10 min in 70% ethanol, rinsing in sterile dH_2O, and drying thoroughly. Store in the original container.
8. Calculate the resistance of each well, using Equation 1.
   $\Omega_{actual} = \Omega_{sample} - \Omega_{blank}$, *Equation 1*
   where Ω_{sample} is the average measurement from a seeded well and Ω_{blank} is the average measurement from the 2 wells, A1 and D1, containing inserts and medium, but no cells.
9. Calculate the unit area resistance, using Equation 2.
   $\Omega_{actual} \times$ effective membrane area = $\Omega \times cm^2$, *Equation 2*
   where the effective membrane area is 0.33 cm^2 for 24-well Transwell inserts.

2. Verify that polarization is complete by performing a secondary assay, measuring passive sodium fluorescein diffusion between the apical and basolateral compartments, on one to three Transwell cultures. The wells used for the sodium fluorescein assay should be discarded immediately after completion of the assay.
   1. Prepare non-fluorescent buffer (118 mM NaCl; 4.75 mM KCl; 2.53 mM $CaCl_2.2H_2O$; 2.44 mM $MgSO_4$; 1.19 mM KH_2PO_4; 25 mM $NaHCO_3$ in sterile water; sterile filter with 0.45 µm filtration device). Prepare sodium fluorescein at 1 mg/ml in non-fluorescent buffer, sterile filter, wrap in aluminum foil, and store at 4 °C, protected from light. Warm to room temperature before use.
   2. After completing resistance measurements, carefully remove medium from both the basolateral and apical compartments of one to three individual Transwell cultures.
   3. Rinse Transwell cultures. Gently add 600 µl room-temperature sterile Dulbecco's PBS (D-PBS) to the basolateral compartments and 100 µl room-temperature sterile D-PBS to the apical compartments.
   4. Gently remove D-PBS from wells. Add 600 µl sterile non-fluorescent buffer to the basolateral compartments. Add 100 µl sterile 1 mg/ml sodium fluorescein to the apical compartments.
   5. Incubate the plate at 37 °C for 1 hr. CO_2 is not required during this incubation.
   6. During incubation, prepare a sodium fluorescein standard curve ranging between 20 µg/ml and 0 µg/ml, using non-fluorescent buffer to dilute sodium fluorescein. Prepare at least 8 different concentrations of sodium fluorescein, each in a final volume of at least 600 µl. Keep standard curve dilutions protected from light until ready to measure absorbance.
   7. Transfer the non-fluorescent buffer sample from the basolateral compartment of each test Transwell to a separate clean tube for analysis. Remove the test Transwells used to examine passive sodium fluorescein diffusion from the plate and discard. Return the plate with remaining Transwells to the incubator.
   8. Place 100 µl of each standard solution in triplicate into a 96-well flat-bottomed plate.
   9. Prepare three dilutions of each basolateral sample (1:2, 1:20, and 1:50) in non-fluorescent buffer, and add 100 µl of each undiluted sample and of each dilution, in duplicate, into a 96-well flat-bottom plate.
   10. Measure sample absorbance on an ELISA plate reader at 486 nm or 490 nm.
   11. Determine the concentration of sodium fluorescein in the basolateral compartment by comparing the absorbance of samples against the absorbance values for the sodium fluorescein standard curve.

4. Infecting Polarized Calu-3 LCC with Respiratory Virus

Safety Measures: Perform all procedures in a biosafety cabinet using sterile culture technique, at a biosafety level appropriate for the virus being used.

1. Dilute virus in serum-free EMEM so that desired inocula would be in 100 µl.
2. Wash cells in serum-free EMEM. Gently aspirate medium from all wells, removing the apical medium then the basolateral medium. With a capillary pipette attached to a vacuum trap with a gentle vacuum, or with a standard 1 ml pipette, aspirate medium from cell-free wells A1 and D1, then from seeded wells, first removing the apical medium from all wells, and then removing the basolateral medium from all wells. Do not touch insert membranes while aspirating medium.
   1. Add 100 µl of serum-free EMEM to the apical compartment of cell-free wells A1 and D1, and then to the seeded wells, directing medium into the apical compartment using the side of the insert to guide the pipette tip. Do not add medium directly on cells, and do not touch the insert membrane.
   2. Add 600 µl of serum-free EMEM to the basolateral compartments. Add medium to each Transwell by angling the pipette against the wall of the compartment and slowly releasing the medium into the well.
   3. Gently aspirate serum-free medium from wells.

3. Beginning with uninfected or mock-infected wells, add appropriate virus dilutions to the apical compartment of Transwells. For uninfected wells, add 100 µl of serum-free EMEM to the apical compartment of appropriate Transwells, for mock-infected wells, add 100 µl of virus-free preparation diluted in serum-free EMEM to the apical compartment of appropriate Transwells, and for virus infection, dilute virus in serum-free EMEM and add 100 µl to the apical compartments of appropriate Transwells.
4. Add 600 µl of serum-free EMEM to all basolateral compartments.
5. Incubate at 37 °C and 7% CO_2 for 2 hr.
6. Aspirate medium from wells, first from uninfected and mock-infected wells, then from infected wells. Remove apical supernatants first, followed by basolateral supernatants.
7. Replace medium with 200 µl of EMEM-10% in the apical compartments and 600 µl of EMEM-10% in the basolateral compartments.

Representative Results

When grown as liquid-covered cultures (LCC) in Transwell culture systems, as illustrated in **Figure 1**, Calu-3 cells polarize, developing distinct apical and basolateral surfaces. Following the method described here, the trans-epithelial electrical resistance (TEER) of Calu-3 LCC reaches a plateau at or above 1,000 Ω×cm^2 within 3 weeks after seeding, an example of which is shown in **Figure 2**. The tight junctions formed between polarized cells prevent passive equilibration of small molecules between the apical and basolateral compartments. Thus, a modified sodium fluorescein equilibration assay is used to confirm polarization of Calu-3 LCC [15,17,18] . As the TEER of Calu-3 cell monolayers in LCC increases, the amount of fluorescein that passively equilibrates into the basolateral compartment decreases. Once the TEER is 1,000 Ω×cm^2, the amount of fluorescein that equilibrates into the basolateral compartment is ≤ 1%, as shown in **Figure 3**; therefore, Calu-3 LCC are considered to be fully polarized when the TEER is ≥ 1,000 Ω×cm^2. The peak TEER measurement of Calu-3 LCC may vary from experiment to experiment. However, once TEER values plateau for any given experiment, a fully polarized, uninfected Calu-3 LCC may be stable for 5 through 12 weeks post-seeding. Absence of resistance development in Transwell-cultured Calu-3 may be caused by several factors as outlined in **Table 1.** Once the TEER of Calu-3 LCC plateaus at or above 1,000 Ω×cm^2, the model is ready to be used to examine airway epithelial cell responses to respiratory pathogens, including respiratory syncytial virus (RSV). Exposure to RSV results in a more rapid decline in polarized culture integrity compared to a mock-infection of cells (**Figure 4**).

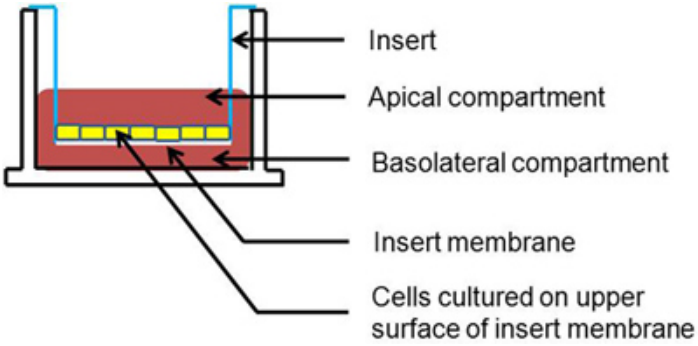

Figure 1. Cross-section representation of Transwell-cultured cells. Cells are grown on the apical surface of the insert membrane.

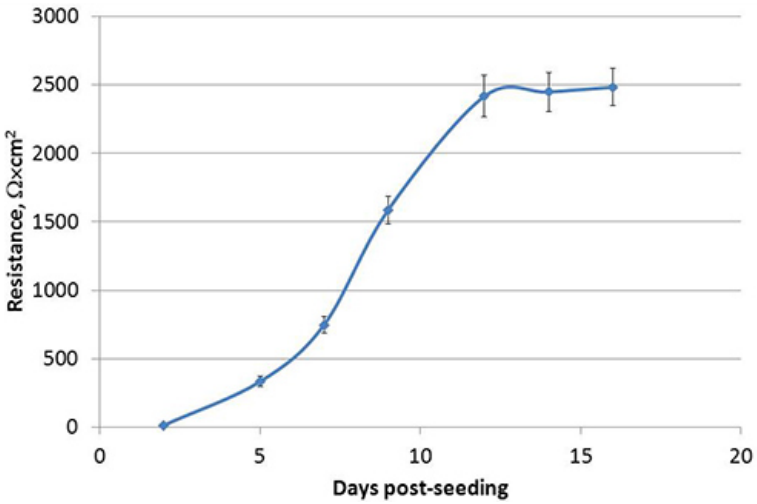

Figure 2. Development of trans-epithelial electrical resistance (TEER) and polarization of Calu-3 cells after seeding in Transwell inserts. At each time point, TEER is presented as median Ω x cm^2 ± SEM of 32 independent wells from one representative experiment.

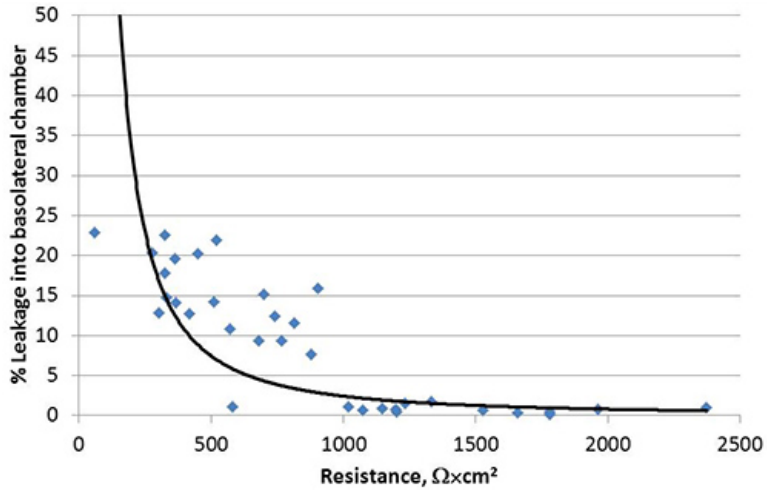

Figure 3. Passive equilibration of sodium fluorescein into the basolateral compartment of Calu-3 cell monolayers is inhibited as cells become polarized. Cumulative data from four independent experiments is presented. Each data point represents an individual measurement.

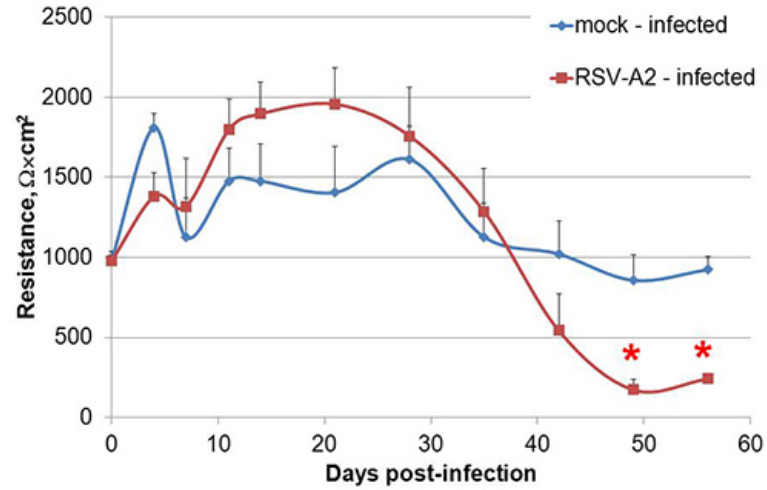

Figure 4. RSV infection of polarized Calu-3 LCC accelerates a decline in monolayer integrity. Polarized Calu-3 cells were infected with RSV-A2 at an MOI=1 on day 0, and polarity was monitored for 8 weeks post-infection. One representative experiment is shown. Data are presented as median resistance of 8 independent wells per infection ± SEM per time point. *p<0.05 between mock- and RSV-A2-infected cultures, as determined by Student's t-test.

Issue	Resolution(s)
Resistance is not measurable	• Remove any air bubbles in the wells • Use Calu-3 cells that have been subcultured from frozen stock less than 10 times • Do not allow Calu-3 cells to reach 100% confluence in monolayer subculture • Check that cells are not growing on the plastic well below the insert (indicating that cells may have grown through the pores of the insert) • Allow 2-3 weeks post-seeding for full resistance development • Check the composition and pore size of inserts used, change if necessary (recommended polyester insert, 0.3 µm pore size, no coatings, see materials for details) • Check the quality of the electrode, sanding gently to remove accumulated proteins from the tip, and replacing if necessary • Check medium for bacterial growth
Resistance develops, but full polarization (≥1,000 Ω×cm^2) is not attained	• Allow additional time for polarization to develop (may occasionally take up to 4 weeks) • Use Calu-3 cells that have been subcultured from frozen stock less than 10 times • Do not allow Calu-3 cells to reach 100% confluence in monolayer subculture • Consistently replace medium in Transwell cultures, alternating between the third and then fourth day after previous feeding • Only culture cells on inserts that are placed in the exterior rows of plates • Use a different lot of Transwell inserts
Cells fully polarize, but resistance is not consistent from one reading to the next	• Do not disrupt or stack plates in the incubator • Do not cause vibration in the biosafety cabinet while Transwell plates are being handled • Remove any air bubbles in the wells • Do not disturb the cell layer with pipette tips when changing media or performing TEER readings • Use 200 µl medium in the apical compartment; lower volume may result in unstable TEER readings • Check the quality of the electrode, sanding gently to remove accumulated proteins from the tip, and replace if necessary

Table 1. Trouble-shooting problems that may arise when culturing Calu-3 cells in Transwells. Multiple factors contribute to full polarization of Calu-3 cells in liquid-covered cultures, the most likely of which are highlighted in this table.

Discussion

When establishing Calu-3 LCC in Transwell inserts, cells may not polarize at all, or may not fully polarize, as defined by a TEER ≥ 1,000 Ω×cm^2 and ≤ 1% sodium fluorescein dye equilibration between the apical and basolateral compartments. In addition, Calu-3 cells in LCC may fully polarize, but TEER may be inconsistent between measurements. Although fluctuations in TEER measurements of Calu-3 LCC are normal from day to day, once fully polarized, dramatic swings in TEER are not expected until the culture naturally declines with age, which may be as little as 5 weeks or as long as 12 weeks after seeding.

The ability of Calu-3 LCC to polarize depends in part on how cells are maintained and subcultured before use in the Transwell system. Cells that have grown beyond 90% confluence as a monolayer during subculturing, that have been subcultured more than 10 times from frozen stock, or that have not been supplied with fresh medium on a regular schedule are less likely to fully polarize, and any polarization is likely to decline rapidly. Incomplete or total lack of polarization may also be attributed to variation in the material and pore size of Transwells used for Calu-3 LCC, and lot-to-lot variation in Transwells of similar composition and pore size may also affect polarization. Larger pore sizes can allow Calu-3 to grow through the Transwell membrane into the basolateral compartment, preventing the culture from polarizing. An absence of polarization may also be due to bacterial growth, indicated by clouded culture medium, which leads to subsequent breakdown of the tight junctions between Calu-3 cells.

Variable TEER measurements of Calu-3 LCC may be caused by mechanical disruptions of the Calu-3 LCC cell monolayers, the inserts, or the plates themselves. Medium changes and TEER measurements should be performed without pipette tips or electrode leads touching the cells. While performing these operations, care should be taken to avoid introducing air bubbles into the apical and basolateral compartments, which will disrupt the ability of the voltohmmeter to detect resistance. The ability of the voltohmmeter to detect resistance in a culture that is actually polarized may also limited by protein buildup on the electrode leads. This build-up may be removed with gentle sanding, or may be corrected by replacing the electrode.

Once Calu-3 LCC completely polarize and the TEER is no longer increasing, Calu-3 LCC are ready for use as an *in vitro* model for characterizing host lung epithelial cell responses to respiratory infection. This system permits better characterization of directional responses to pathogens compared to monolayer-cultured lung cell lines traditionally used to study respiratory pathogens, such as A549 and HEp-2 cells, with the additional advantages of Calu-3 LCC being more rapid to develop, more easily obtained, and less expensive to generate than primary, polarized, differentiated NHBE. Similar to NHBE, polarized Calu-3 demonstrate tight junction formation, and produce mucins. However, unlike NHBE, polarized Calu-3 cells do not differentiate into layers of basal cells and ciliated columnar epithelial cells, and few polarized Calu-3 cells develop cilia-like projections[19]. Thus, although useful for examining polarized responses of airway epithelial cells to respiratory insult, polarized Calu-3 LCC are not an ideal model to examine airway development or remodeling in response to respiratory insult or injury. Mucus production by cultured cells *in vitro* may impact cellular infectivity, as well as release of infectious virus and virus spread in a polarized model, and a direct comparison of the mucus production between polarized Calu-3 LCC and polarized, differentiated NHBE has not been reported. A549 and HEp-2 cells are easier to culture than Calu-3 cells, however, unlike Calu-3 LCC, they do not form polarized cultures when grown on Transwell inserts, and are thus not ideal models for examining *in vitro* the responses of polarized epithelial cells to respiratory virus infection.

Disclosures

No conflicts of interest declared.

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