ORIGINAL ARTICLE

A Three-Dimensional Human Tissue-Engineered Lung Model to Study Influenza A Infection

Rudra Bhowmick, PhD,¹ Tahereh Derakhshan, MS,¹ Yurong Liang, PhD,² Jerry Ritchey, DVM, PhD,³ Lin Liu, PhD,² and Heather Gappa-Fahlenkamp, PhD¹

Influenza A virus (IAV) claims ~250,000–500,000 lives annually worldwide. Currently, there are a few *in vitro* models available to study IAV immunopathology. Monolayer cultures of cell lines and primary lung cells (two-dimensional [2D] cell culture) is the most commonly used tool, however, this system does not have the *in vivo*-like structure of the lung and immune responses to IAV as it lacks the three-dimensional (3D) tissue structure. To recapitulate the lung physiology *in vitro*, a system that contains multiple cell types within a 3D environment that allows cell movement and interaction would provide a critical tool. In this study, as a first step in designing a 3D-Human Tissue-Engineered Lung Model (3D-HTLM), we describe the 3D culture of primary human small airway epithelial cells (HSAEpCs) and determined the immunophenotype of this system in response to IAV infections. We constructed a 3D chitosan-collagen scaffold and cultured HSAEpCs on these scaffolds at air–liquid interface (ALI). These 3D cultures were compared with 2D-cultured HSAEpCs for viability, morphology, marker protein expression, and cell differentiation. Results showed that the 3D-cultured HSAEpCs at ALI yielded maximum viable cells and morphologically resembled the *in vivo* lower airway epithelium. There were also significant increases in aquaporin-5 and cytokeratin-14 expression for HSAEpCs cultured in 3D compared to 2D. The 3D culture system was used to study the infection of HSAEpCs with two major IAV strains, H1N1 and H3N2. The HSAEpCs showed distinct changes in marker protein expression, both at mRNA and protein levels, and the release of proinflammatory cytokines. This study is the first step in the development of the 3D-HTLM, which will have wide applicability in studying pulmonary pathophysiology and therapeutics development.

Keywords: tissue-engineered lung model, human small airway epithelial cells, influenza A virus, 3D cell culture, 2D cell culture, air–liquid interface

Introduction

INFLUENZA INFECTION is a major cause of morbidity and mortality worldwide.¹ In the United States, ~5–20% of the population develop influenza-associated respiratory disease with more than 200,000 hospitalization cases every year.² The influenza A virus (IAV) is classified based on the antigenicity of two transmembrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA). IAV with HA types H1–H3 and NA types N1 and N2 are found in humans.³ The pandemic IAV strain H1N1, which emerged in 2009, resulted in the first “flu” pandemic of the 21st century, during which 61 million people became ill and more than 12,000 people died in the United States within a year.^{4,5} Pandemic H1N1 shows higher pathogenicity and infectivity compared to seasonal strains, such as H3N2.^{6,7}

The hallmark of IAV infection is the “cytokine storm,” a combination of robust and uncontrolled pro- and anti-inflammatory responses.⁸ Airway epithelial cells (AECs) are the primary site of IAV replication, and IAV alters cytokine/chemokine production of the AECs and alveolar macrophages.^{3,9} This leads to the increased migration and differentiation of activated macrophages that drive an excessive inflammatory response.^{10,11}

Several animal models are currently available to study IAV immunopathology, however, these models do not represent the human lung. Although IAV is highly zoonotic, and some animals are susceptible to human IAV strains, they are either expensive (e.g., ferrets) or do not exhibit symptoms (e.g., guinea pigs).^{12,13} Mice, although widely used, are not natural hosts for IAV, and their immune system is significantly different from that of humans.¹⁴

¹School of Chemical Engineering, Oklahoma State University, Stillwater, Oklahoma.

Departments of ²Physiological Sciences and ³Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma.

Human airway cells, *ex vivo* samples, cell lines, or primary cells are better alternatives to study IAV pathogenesis. Autopsy samples and *ex vivo* lung tissue have been used to identify target cells for IAV.^{15,16} However, the utility of using human tissue is limited by its empirical nature and trial and error approaches.¹⁷ Therefore, culture of primary cells and cell lines are widely used to study IAV infection *in vitro*.^{18–21} Unfortunately, culturing these cells as monolayers (the so-called “Two-dimensional [2D] cell culture”) poorly model the *in vivo* lung, as in 2D cultures the cells lack signals from their physiological three-dimensional (3D) environment. Furthermore, the 3D environment affects intracellular signaling, solute diffusion, and interaction with growth factors, resulting in formation of functional tissue structures.^{22,23} We and others have shown that there are differences in cellular responses between 2D and 3D cultures.^{24,25} Therefore, to approximate the *in vivo* lung within an *in vitro* model, a system that contains multiple cell types within a 3D environment that allows cell movement and interaction would provide a critical tool to investigate IAV pathogenesis. Our present study is the first step in developing this *in vitro* model.

In this study, we described the culture of primary human small airway epithelial cells (HSAEpCs) on a 3D chitosan-collagen scaffold, compared this model with 2D-cultured HSAEpCs, and determined the immunophenotype of the 3D-cultured HSAEpCs in response to two major IAV strains, H1N1 and H3N2. This study is a major step in the development of a 3D Human Tissue-Engineered Lung Model (3D-HTLM), which would be useful in immunopathology and therapeutics testing for pulmonary pathogens.

Materials and Methods

Scaffold preparation and HSAEpC culture

The chitosan-collagen scaffolds for seeding HSAEpCs were prepared by phase-separation and freeze-drying, following our recent publication.²⁶ The scaffolds were examined by scanning electron microscopy (SEM) as described before.²⁶ Briefly, dried scaffolds were detached from the membrane inserts and mounted on aluminum stubs. The stubs were then sputter coated with gold and images were captured with SEM (JOEL JSM 6360; Tokyo, Japan), at 15 kV with 50× or 70× magnification. Images were analyzed with ImageJ software to determine the pore size and thickness of the scaffolds.

Small airway epithelial cell (SAEC) media and HSAEpCs were purchased from PromoCell (Heidelberg, Germany). Culture flasks were coated with type 1 collagen (75 µg/mL; Advanced BioMatrix, San Diego, CA) and fibronectin (25 µg/mL; Alfa Aesar, Tewksbury, MA), before seeding. Media were changed every 72 h.

To culture HSAEpCs in 3D (Fig. 1A), scaffolds were coated with collagen-fibronectin, and incubated in SAEC media for 1 h. HSAEpCs were seeded (~75,000 cells/scaffold) and placed in 24-well culture plates (Fisher Scientific, Hampton, NH). Media (700 µL to the outer well, 100 µL to the inner well) were added and incubated at 37°C, 5% CO₂ (standard conditions) for 7 days without media change. On the seventh day, media was removed from the top of the HSAEpCs to expose them to air-liquid interface (ALI), and incubated for seven additional days, before use.

When required, scaffolds were digested to harvest HSAEpCs. For this, scaffolds were removed from the inserts and shaken in a 40:1 (v/v) mix of collagenase D (Roche Diagnostics, Mannheim, Germany) and chitosanase (EMD Millipore, Billerica, MA) at 37°C for at least 2 h. When the solution appeared visually clear, it was centrifuged (2 g, 10 min) and the pellet was discarded. The supernatant was further centrifuged (45 g, 5 min) and the pellet containing HSAEpCs was used for further analyses.

To culture HSAEpCs in 2D (Fig. 1B), 24-well membrane inserts were coated with collagen-fibronectin, seeded with HSAEpCs (~75,000 cells/scaffold), and cultured for 7 days without media change. On the seventh day, the HSAEpCs were exposed to ALI, and incubated for seven additional days. Whenever required, cells were lifted using trypsin/ethylenediaminetetraacetic acid.

IAV culture and infection

IAV strains A/PR/8/34 (H1N1; PR8) and A/Hong Kong/01/68 H3N2 (H3N2) were grown from laboratory stocks in embryonated chicken eggs. The chorioallantoic fluid was harvested, aliquoted, and titered in Madin-Darby Canine Kidney Epithelial (MDCK) cells using standard procedures.²⁷ For infection, HSAEpCs were washed with sterile phosphate buffered saline (PBS) and the required plaque forming units of either H1N1 or H3N2 were added in media at multiplicity of infections (MOIs) 0.1 or 1.0 to the top of the cells. Following 24 h infection under standard conditions, supernatants were collected and stored at –80°C. Cells and scaffolds were either fixed or lysed as described elsewhere.

Viability assay

Viability of 2D- and 3D-cultured HSAEpCs either at ALI or with media on top of the cells (“media submerged”) was measured using CellTiter Blue (Promega Corporation, Madison, WI) assay, following manufacturer’s protocol. HSAEpCs cultured at 2D or 3D, either at ALI or media submerged, were treated with 1% Triton X-100 (Fisher Scientific) for 20 min and taken as respective negative controls for cell viability.

Histology and immunohistochemistry

Morphology of the 3D-cultured naïve or IAV-infected HSAEpCs was studied by hematoxylin and eosin (H&E) staining. Three-dimensional-cultured HSAEpCs were infected as described above. Scaffolds were peeled from the inserts and fixed in 4% paraformaldehyde (v/v, in PBS). Scaffolds were processed, paraffin-embedded, sectioned at 5 µm, and stained with H&E. Stained sections were observed under an Olympus BX50 microscope. Images were captured with an Olympus DP26 digital camera.

For immunohistochemical (IHC) staining of 3D-cultured HSAEpCs, paraffin sections (5 µm) were mounted on positively charged glass slides, dried, and deparaffinized. Sections were labeled with primary antibodies against aquaporin-4 (Aqp4, 1:100; Abcam, Cambridge, MA), club cell-10 (CC-10, 1:500; Abcam), cytokeratin-14 (CK-14, 1:100; Abcam), and pro-surfactant protein-C (proSP-C, 1:200; Abcam). Isotype control antibodies (murine IgG3 and rabbit polyclonal IgG; Abcam) were used at the same dilutions and conditions. Primary antibody and isotype-

FIG. 1. 3D and 2D cultures of HSAEpCs and comparative viability of cells. (A) The HSAEpCs were cultured on chitosan-collagen scaffolds (3D) or (B), on membrane inserts (2D) as outlined in section “Materials and Methods”. (C) Scanning electron micrographs of the chitosan-collagen scaffolds depicting the (i) thickness and (ii) pores. Shown is a representative of three independent experiments. (D) Comparison of cell viability between 2D and 3D-cultured HSAEpCs, media submerged and ALI-exposed. As described in section “Materials and Methods”, HSAEpCs were cultured in 2D or 3D. The cells were either cultured media submerged for 14 days (labeled “submerged”) or exposed to ALI for 7 days (labeled “ALI”), following 7 days of submerged culture. Viability of the HSAEpCs was measured using CellTiter Blue assay. Shown is the relative fluorescence units at excitation/emission of 560/590 nm of a representative of three independent experiments (**p*-value < 0.05). 3D, three-dimensional; 2D, two-dimensional; HSAEpC, human small airway epithelial cell; ALI, air-liquid interface. Color images available online at www.liebertpub.com/tea

matched control antibody were incubated overnight at 4°C in a humidified chamber. Following incubation, a secondary antibody (Ultra High Def Polymer; Statlab Medical Products, McKinney, TX) was applied followed by washing and application of a peroxidase substrate (ImmPact DAB; Vector Labs, Burlingame, CA) according to manufacturers’ directions. Following staining, sections were counterstained with Mayer’s hematoxylin.

Cell marker protein expression

Expression of cell marker proteins was determined by enzyme-linked immunosorbent assay (ELISA) using a modification of a previously described protocol²⁸ and flow cytometry. For ELISA, cells cultured in 2D or 3D and exposed to ALI were harvested and transferred into cold lysis buffer.²⁹ Cells were lysed by freeze-thaw, followed by two rounds of sonication (100% power, 130 W). Samples were centrifuged (14,000 *g*) and the supernatant was used for ELISA. Equal amounts of total protein were precoated on 96-well microtiter plates (Costar; Fisher Scientific) in triplicate, and incubated overnight at 4°C. Next day, the wells were washed, and probed with primary antibodies against Aqp5, SP-C, CK-14, and CC-10 (all from Santa Cruz Biotechnology, Dallas, TX). After washing, horseradish

peroxidase-conjugated secondary antibodies were added, and color was developed using *o*-phenylenediamine dihydrochloride (ThermoFisher Scientific, Waltham, MA). Absorbance was measured at 490 nm (Packard SpectraCount; Cole-Parmer, Vernon Hills, IL). Naïve cells were used as controls.

For flow cytometry, cells cultured in 2D or 3D and exposed to ALI were harvested. Cells were stained on ice with relevant primary antibodies against Aqp5, SP-C, CK-14, and CC-10, at manufacturer-instructed dilutions following standard procedures. Cells were analyzed with a BD Accuri C6 flow cytometer (BD Biosciences). Data analysis was performed using BD Accuri C6 software to determine the percentage of HSAEpCs that were positive for Aqp5, SP-C, CC-10, and CK-14.

Cytokine release

To study cytokine release from 3D-cultured HSAEpCs in response to IAV, supernatants were collected following infection, and probed with the following commercial ELISA kits: IL-1 β , TNF- α , MCP-1 (all from ThermoFisher Scientific), IL-6, IL-8, and IL-10 (all from Abcam) following manufacturers’ protocol. Absorbance was measured at 492 nm using a VMax microplate reader (Molecular

Devices, Sunnyvale, CA). Supernatants from naïve HSAEpCs were used as controls.

Real-time polymerase chain reaction

For gene expression in response to infection, HSAEpCs were harvested and total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) following manufacturer's protocol. UltraPure™ Glycogen (0.5 µL; ThermoFisher Scientific) was included as a carrier for nucleic acid precipitation in ethanol. After DNase I treatment, 1 µg of total RNA was used to synthesize complementary DNA (cDNA) using a cDNA synthesis kit (Thermo Fisher Scientific). Real-time PCR was performed using an ABI 7500 fast Real-time PCR System (Applied Biosystems, Foster City, CA) and a reaction mixture qPCR MasterMix Plus (SYBR Green 2×PCR Master Mix, a cDNA template, and forward and reverse primers). Primer sequences were as follows: (Aqp5F: ACCCTCCCCAGAG CTCCTTA, Aqp5R: GCATGCATCCTCACCAGAAA; SP-CF:CCCCAGTCTTGAGGCTCTCA, SP-CR: TGCCTGG CCCAGCTTAGA; CC-10F: CCACCATGAAACTCGGTG TC, CC-10R: GTGGGCGTGGACTCAAAGC; CK-14F: TGAGAGGCCAGGTGGGTGGAGAT, CK-14R: CCAGC TGCATGCAGTAGCGACCTTTG; β-actinF: GCCGGA CCTGACTGACTAC, β-actinR: TTCTCCTTAATGTCAC GCACGAT). Primers were synthesized by Sigma-Aldrich. Relative expression levels of *Aqp5*, *SP-C*, *CC-10*, and *CK-14* were normalized to that of β-actin (housekeeping gene) expression using the $2^{-\Delta\Delta CT}$ method.³⁰

Statistical analyses

Experimental results are expressed as mean ± standard deviation of replicate samples. For testing multiple groups, analysis of variance was used to determine whether there is a significant difference between any of the groups (p -value <0.05). Differences between two groups were determined using Student's *t*-test, with a p -value <0.5 considered significant. Statistical analyses were done using GraphPad Prism (GraphPad Software, San Diego, CA). Microscopic images shown are representative of analyzed samples.

Results and Discussion

Effect of culture conditions on HSAEpC viability

We have captured SEM images of the chitosan-collagen scaffolds before seeding the HSAEpCs, and analyzed the images to understand the thickness and pore size of the scaffolds using our previously published method.²⁶ Representative SEM images are shown in Figure 1C. The scaffolds had a mean major pore size of 91.61 ± 7.64 µm, mean minor pore size of 54.33 ± 7.11 µm, and average thickness of 364 ± 77.17 µm. The porosity of the scaffolds was $77.52 \pm 7.18\%$. As the first step in establishing the 3D-HTLM, 3D-cultured HSAEpCs were compared with 2D cultures. To mimic the *in vivo* lung, both systems were exposed to ALI; and the number of viable HSAEpCs in the ALI-exposed 2D and 3D cultures were compared to the respective media-submerged cultures. For 2D, ALI-exposure had no statistically significant effect on viable cells (Fig. 1D, $p=0.33$). However, for 3D cultures, ALI-exposure resulted in 32% higher number of viable cells, compared to

media-submerged 3D cultures (Fig. 1D, $p<0.05$). The number of viable cells in 2D and 3D models (both upon ALI-exposure) were $13.62 \pm 2.32 \times 10^3$ per well and $17.11 \pm 2.45 \times 10^3$ per scaffold, respectively. The number of viable cells was 25.6% higher ($p<0.05$) in 3D compared to the 2D cultures, upon ALI-exposure. Among the culture conditions tested, the 3D, ALI-exposed cultures yielded the maximum number of viable HSAEpCs, making such conditions suitable for the development of the 3D-HTLM. The 2D and 3D-cultured HSAEpCs, either at ALI or media submerged, treated with 1% Triton X-100 for 20 min were taken as negative controls for cell viability.

Such differences in viable cells between these systems were not unexpected, as there are several reports showing the differences between 2D- and 3D-cultured lung cells.¹⁷ Cell lines such as A549 and H1650s also result in higher cell viability when grown in 3D, spheroid cultures.³¹ Our observation that the ALI-exposed 3D cultures yield the maximum number of viable cells is also supported by a study where ALI-cultured Calu-3 cells showed higher viability compared to their submerged cultures.³² However, we cannot rule out the possibility that the high number of viable cells in our 3D system is due to the higher available surface area in 3D resulting in better cell proliferation.

Effect of culture conditions on HSAEpC morphology and phenotype

Few studies have compared the cellular and immunological phenotypes of 2D- and 3D-cultured AECs, with most studies using the A549 cell line. However, A549 cells are not ideal to study the lower airway epithelium, as these are carcinoma lines that are distinct from primary alveolar type II (ATII) cells, and do not transform into alveolar type I (ATI) cells.^{33–35} Since HSAEpCs are the primary cells of the lower airway epithelium,³⁶ we first studied the morphology of their 2D and 3D cultures, both ALI-exposed, using H&E staining. We found that the 2D-cultured HSAEpCs formed a non-uniform layer, with occasional cell clustering (Fig. 2A[i], [ii], respectively). In contrast, the 3D-cultured HSAEpCs formed a more continuous layer on the scaffold (Fig. 2B[i]), and were also sometimes found embedded within the scaffold (Fig. 2B[ii]). The 3D-cultured HSAEpCs also formed clusters (Fig. 2B[iii], [iv]), were spherical, with occasional occurrence of elongated/squamous cells (Fig. 2B[iii–v]). In addition, the 3D-cultured HSAEpCs showed an apical, uniform layer of mucus or surfactant (Fig. 2B[i]).

ALI-differentiated AECs have been shown to be *in vivo*-like, where the cells first divide and then differentiate to form polarized structures.^{37,38} Studies have shown that ALI-exposed A549s formed a defined barrier as evidenced by time-dependent increase in transepithelial electrical resistance (TEER).³⁹ However, we did not see a similar phenotype for 2D-cultured, ALI-exposed HSAEpCs. This could be due to the inherent differences between cell lines (such as A549) and primary cells (such as HSAEpCs). We also cannot rule out the effect of cell sources, as others have shown that ATII cells isolated from rats can form monolayers in 2D.⁴⁰

Our observation that the ALI-exposed, 3D-cultured HSAEpCs form a uniform cell layer is a close representation of the *in vivo* small airway epithelium that forms a barrier

FIG. 2. Morphology of HSAEpCs cultured in 2D and 3D. The HSAEpCs were cultured in 2D or 3D and ALI-exposed. Constructs were fixed and stained with H&E, as described in section “Materials and Methods”. Representative micrographs of (A), HSAEpCs cultured in 2D. (i) Disrupted monolayers of HSAEpCs; (ii) isolated cell clusters, *arrow* indicates a cell cluster. (B) HSAEpCs cultured on the 3D scaffolds. (i) Continuous monolayer of HSAEpCs (*arrows*) and overlying layer of mucus/surfactant (*arrowheads*); (ii) monolayer of HSAEpCs on the scaffold (*arrowheads*) and HSAEpCs growing within the scaffolds (*arrows*); (iii) cell clusters of HSAEpCs, low magnification; (iv) cell clusters of HSAEpCs, high magnification; (v) HSAEpCs of different morphology on the 3D scaffold. Elongated/squamous (*black arrow*) and spherical (*blue arrow*) HSAEpCs. H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea

between the circulation and the external environment.¹⁷ We also observed few HSAEpCs within the scaffolds, possibly due to the porosity and increased surface area of the scaffold. The 3D-cultured HSAEpCs also presented a combination of elongated/squamous and spherical cells, similar to the *in vivo* alveolar epithelium, which is composed of squamous ATI and cuboidal ATII cells.⁴¹ Interestingly, we saw low numbers of squamous cells in our system, which may be due to the transdifferentiation process observed in 3D cultures.^{41,42}

Presence of an overlying layer of surfactant or mucus on the 3D-cultured HSAEpCs was similar to the *in vivo* airway, which is covered by layers of preciliary fluid, surfactant, and mucus.⁴³ Surfactants, secreted by the ATII cells, are an integral part of the epithelial fluid and critical for immune responses.^{44,45} Similar observations were made in 3D-cultured human bronchial epithelial cell line VA-10, which expressed pro-SP-C when cocultured with human umbilical vein endothelial cells.⁴⁶ Taken together, our observations demonstrate that the ALI-exposed, 3D-cultured HSAEpCs closely resemble the *in vivo* airway epithelium, making it ideal for the development of the 3D-HTLM. As the work presented here is the first step in developing the 3D-HTLM, it was imperative that we grow the HSAEpCs on the scaffold

surface, similar to the lower airway epithelium. As shown in Figure 2, we were successful in growing the HSAEpCs on the scaffold surfaces.

Effect of culture conditions on HSAEpC cell marker protein expression

The lower and middle airway epithelium consists of ciliated cells (50–70%), club cells (11%), goblet cells (up to 25%), and basal cells (up to 30%); while the alveolar epithelium consists of ATI (covering 98% of the alveolar surface) and ATII cells.^{47–49} Each of these cells express specific marker proteins. Aquaporins are small water channel proteins, and AQP5, located on the apical surface of ATI cells is considered an ATI marker protein.^{50,51} ATII cells secrete SPs-A, B, C, and D, and of these, SP-C is considered an ATII marker protein.^{51,52} Club cells of the distal bronchioles selectively express CC10, and perform anti-inflammatory roles,⁵³ while basal cells are located throughout the conducting airways, and express cytokeratin 5 and 14 (CK5 and CK14).^{47,54}

To further compare 2D- and 3D-cultured HSAEpCs, we analyzed the marker protein expression of these cells. ELISA data showed that Aqp5, CC10, and CK14 in 3D-cultured HSAEpCs was 1.7-fold ($p < 0.0001$), 2.3-fold ($p < 0.0001$),

FIG. 3. Comparison of marker protein expression by 2D- and 3D-cultured, ALI-exposed HSAEpCs. HSAEpCs were cultured in 2D or 3D, ALI-exposed and harvested as mentioned in section “Materials and Methods”. (A) Expression of Aqp5, CC-10, CK14, and SP-C was measured by ELISA. Cells were lysed and equal amounts of proteins were used to coat ELISA plates. (B) Percentage of cells positive for Aqp5, CC-10, CK14, and SP-C as analyzed by flow cytometry. Intact cells were stained with primary antibodies against these proteins. Shown is a combination of five independent experiments (* p -value <0.05; ** p -value <0.01; ns, not significant). ELISA, enzyme-linked immunosorbent assay. Aqp5, aquaporin-5; CC-10, club cell-10; CK-14, cytokeratin-14; SP-C, surfactant protein-C.

and 1.73-fold ($p < 0.005$) higher than the 2D cultures (Fig. 3A). Expression of SP-C, however, remained unchanged between the culture systems ($p = 0.06$). Flow cytometry showed a similar trend, with the percentage of cells expressing Aqp5 and CK14 2.22-fold and 1.87-fold ($p < 0.05$ in both cases) higher respectively, in the 3D-cultured HSAEpCs, compared to the 2D cultures (Fig. 3B). The percentage of CC-10-positive cells was higher in 3D-cultured HSAEpCs, although not at statistically significant levels ($p = 0.0512$). The percentage of SP-C-positive cells was comparable between the culture systems ($p = 0.217$). Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/tea) shows the representative flow cytometry histograms. As 2D- and 3D-cultured AECs express significantly different protein profiles (reviewed in Bhowmick and Gappa-Fahlenkamp¹⁷), our results were not unexpected. Interestingly, as suggested by flow cytometry, the ALI-exposed HSAEpCs in both 2D and 3D appeared to differentiate dynamically, expressing multiple marker proteins (Fig. 3B). Since these cells were not terminally differentiated, their expression of multiple marker proteins suggested an active state of differentiation. A recent report has made similar observations where ALI-exposed ovine AECs showed a similar, time-dependent differentiation.⁵⁵ The undifferentiated airway basal cells are known to possess stem cell properties, and may differentiate into other cell types when required.^{56,57} Taken together, our data show that the ALI-exposed, 3D-cultured HSAEpCs display characteristics of the *in vivo* airway epithelium, as evidenced by their differentiation and marker protein expression.

Effects of H1N1 and H3N2 infection on 3D-cultured HSAEpCs

To understand the responses of the 3D-cultured HSAEpCs to IAV, we infected the cells with the highly infectious H1N1 strain PR8 for 24 h at MOI 1.0 and 0.1. In addition, to study the effect of IAV on 2D-cultured HSAEpCs, we infected the cells with H1N1 at the same MOI and time period. At this time point and these MOIs, AECs remain viable and immune-responsive.^{58,59} It has

been reported that H1N1 infection for 24 h, even at MOI of 10, does not result in a significant change in viability and barrier function in Calu-3 cells.⁶⁰

Changes in marker protein expression. The correlation between viral infection and protein expression in AECs is well established. For example, human rhinovirus-infection of the bronchial epithelial cell line BEAS-2B resulted in a significant reduction in E-cadherin and CK-18, leading to an epithelial-mesenchymal transition.⁶¹ Our ELISA data showed that upon H1N1 infection in the 2D-cultured HSAEpCs, the Aqp5 and SP-C levels remained unchanged ($p = 0.11$ and 0.41 , respectively), when compared to the naïve cells (Fig. 4A). Similar results were obtained from flow cytometry, with the percentage of cells positive for Aqp5 and SP-C remaining unchanged ($p = 0.34$ and 0.96 , respectively), when compared to the naïve cells (Fig. 4C). Supplementary Figure S2 shows the representative flow cytometry histograms. This corresponds to the previous observations that 2D-cultured cells are less responsive to infection.⁶² To understand whether the 2D-cultured HSAEpCs were responsive to other IAV strains, we studied its responses to the other major circulating IAV strain, H3N2. H3N2 is a major public health concern, as it is difficult to raise vaccines against this strain.^{3,63} ELISA data showed that there was no significant difference in Aqp5 levels between the H3N2-infected and naïve cells (Fig. 4B, $p = 0.1$), however, SP-C levels were 1.8-fold lower in the infected, 2D-cultured HSAEpCs (Fig. 4B, $p < 0.0001$). Flow cytometry also showed that the percentage of cells positive for Aqp5 did not differ between the H3N2-infected and naïve 2D-cultured cells (Fig. 4D, $p = 0.19$), however, the number of cells positive for SP-C was 1.8-fold lower in the H3N2-infected, 2D-cultured HSAEpCs when compared with the naïve cells (Fig. 4D, $p < 0.005$). Supplementary Figure S2 shows the representative flow cytometry histograms.

On the other hand, ELISA data with the 3D-cultured HSAEpCs showed that upon H1N1 infection, the Aqp5 and SP-C levels were 2.75-fold and 4.17-fold lower ($p < 0.0001$ in both cases), while CK-14 was 2.1-fold higher ($p < 0.005$)

FIG. 4. Comparison of marker protein expression by 2D-cultured, ALI-exposed HSAEpCs infected with H1N1 or H3N2. The HSAEpCs were cultured in 2D, ALI-exposed, and infected with H1N1 or H3N2, MOI 1.0, 24 h. Cells were harvested as mentioned in section “Materials and Methods”, and expression of Aqp5, CC-10, CK14, and SP-C was measured by ELISA using equal amounts of proteins from (A), H1N1-infected HSAEpCs, and (B), H3N2-infected HSAEpCs. Percentage of cells positive for Aqp5, CC-10, CK14, and SP-C was analyzed by flow cytometry for (C), H1N1-infected HSAEpCs; and (D), H3N2-infected HSAEpCs, using primary antibodies against these proteins. Shown is a representative of three independent experiments. Naïve HSAEpCs were used as negative controls (* p -value < 0.05; ** p -value < 0.01). MOI, multiplicity of infection.

when compared to the naïve cells (Fig. 5A). Interestingly, CC-10 levels remained unaltered ($p=0.7$, Fig. 5A). Flow cytometry showed a similar trend, with the percentage of cells positive for Aqp5 and SP-C 1.45-fold and 1.6-fold lower ($p<0.05$ in both cases) respectively (Fig. 5C). Supplementary Figure S3 shows the representative flow cytometry histograms. We also investigated the responses of the 3D-cultured HSAEpCs to H3N2. ELISA data showed that there was no significant difference in marker protein levels between the H3N2-infected and naïve cells (Fig. 5B, p -value range 0.72–0.95). Flow cytometry also showed that the percentage of cells positive for the marker proteins did not differ between the H3N2-infected and naïve cells (Fig. 5D, p -value range 0.27–0.91). Supplementary Figure S3 shows the representative flow cytometry histograms.

Aqp5 and SP-C are marker proteins for ATI and ATII cells, which make up the entire alveolar surface, and are attachment sites for IAV.⁶⁴ Our data demonstrated that the 3D-cultured HSAEpCs suffered significant reduction in the expression of both Aqp5 and SP-C in response to the H1N1, while the levels of these two marker proteins remained unchanged in response to H3N2. This result is in accordance with previous reports, which show that while H1N1 and H3N2 can similarly infect and replicate in pneumocytes, H1N1 is more immunogenic than H3N2.^{3,65} However, the 2D-cultured HSAEpCs did not show any changes in the expression of these marker proteins in response to H1N1, and showed decreased levels of only SP-C in response to H3N2.

Therefore, the IAV strains could affect the 3D-cultured HSAEpCs in a more physiological manner. Moreover, there are several reports that show 3D cultures respond to infections in a more physiological manner compared to 2D systems.^{62,66} For these reasons, we continued further characterization of the 3D-cultured HSAEpCs.

Morphological changes. Following H1N1 infection, the 3D-cultured HSAEpCs exhibited enlarged nucleus and structures resembling inclusion bodies, characteristic of influenza infection (compare Fig. 6A[i] and [ii]). Such structures have also been observed in H1N1-infected MDCK cells.⁵⁹ Our observation is also supported by similar findings in lung autopsies from H1N1 patients, where viral nucleoproteins were detected within ATII cells.⁶⁷ IHC observations showed the levels of aquaporin and CC-10 remained unchanged (compare Fig. 6B[i] and [ii]; C[i] and [ii], respectively); while CK-14 expression increased (compare Fig. 6D[i] and [ii]) and SP-C was decreased in the H1N1-infected HSAEpCs (compare Fig. 6E[i] and [ii]), compared to their naïve counterparts. The reduced SP-C levels observed in H1N1-infected HSAEpCs is similar to the lower SP-C levels seen in ATII cells of H1N1-infected C57BL/6-congenic SP-C/GFP mice, which contribute to their reduced lung compliance.⁶⁸ The increased CK14 levels in H1N1-infected HSAEpCs is also supported by previous studies. CK14 is a type I cytokeratin known to interact with IAV, and H1N1 infections prompted an increase in type I

FIG. 5. Comparison of marker protein expression by 3D-cultured, ALI-exposed HSAEpCs infected with H1N1 or H3N2. The HSAEpCs were cultured in 3D, ALI-exposed, and infected with H1N1 or H3N2, MOI 1.0, 24 h. Cells were harvested as mentioned in section “Materials and Methods”, and expression of Aqp5, CC-10, CK14, and SP-C was measured by ELISA using equal amounts of proteins from (A), H1N1-infected HSAEpCs, and (B), H3N2-infected HSAEpCs. Percentage of cells positive for Aqp5, CC-10, CK14, and SP-C was analyzed by flow cytometry for (C), H1N1-infected HSAEpCs; and (D), H3N2-infected HSAEpCs, using primary antibodies against these proteins. Shown is a combination of five independent experiments. Naive HSAEpCs were used as negative controls (* p -value <0.05; ** p -value <0.01).

FIG. 6. Morphology and immunohistochemistry of 3D-cultured, ALI-exposed HSAEpCs infected with H1N1. The HSAEpCs were cultured in 3D, ALI-exposed, and infected with H1N1, MOI 1.0, for 24 h. Cells were then fixed and prepared for microscopy (either using H&E or IHC) as described in section “Materials and Methods”. (A) Representative micrograph of H&E stained HSAEpCs, (i) naive, (ii) H1N1-infected, showing dense, enlarged nucleus (arrowhead) and vacuole with inclusion bodies (arrow). (B) Representative IHC micrograph of HSAEpCs with anti-Aqp4 antibody, (i) naive, (ii) H1N1-infected. (C) Representative IHC micrograph of HSAEpCs with anti-CC10 antibody, (i) naive, (ii) H1N1-infected. (D) Representative IHC micrograph of HSAEpCs with anti-CK14 antibody, (i) naive, (ii) H1N1-infected. (E) Representative IHC micrograph of HSAEpCs with anti-proSP-C antibody, (i) naive, (ii) H1N1-infected. IHC, immunohistochemical. Color images available online at www.liebertpub.com/tea

cytokeratin levels in A549.^{69–71} The increase in CK14 suggests a possible role of the cytoskeleton in IAV infection, as suggested previously.⁷²

Changes in mRNA expression of cellular markers. To understand whether the changes in marker proteins in 3D-cultured HSAEpCs occurred transcriptionally, we studied the effect of infection on the mRNA expression of *Aqp5*, *CC-10*, *CK-14*, and *SP-C* genes. Real-time PCR showed that the mRNA expression of *Aqp5* was three-fold lower ($p < 0.05$) while that of *CK14* was 2.2-fold higher ($p < 0.005$) in H1N1-infected HSAEpCs at MOI=1.0, when compared to naïve cells (Fig. 7A, C, respectively). *SP-C* levels remained unchanged upon infection ($p = 0.169$, Fig. 7D). Interestingly, in contrast to the CC-10 protein levels, the expression of *CC10* was 2.7-fold lower in the H1N1-infected HSAEpCs ($p < 0.05$) (Fig. 7B). Such discrepancy can be explained by the fact that RNA levels do not always accurately reflect protein levels, and several post-translational mechanisms maybe involved in converting mRNA into protein.⁷³ Some proteins, such as IL-4, are regulated transcriptionally, while some, such as IL-1, are modified post-translationally.^{74–76} In addition, mammalian cells produce RNA at a significantly lower rate than proteins, with two copies of mRNA and dozens of copies of the corresponding protein produced per hour.⁷⁷

Interestingly, the mRNA induction in H3N2-infected cells was distinct from the H1N1-infected cells. Compared to naïve cells, the expression of *Aqp5* was 3.3-fold ($p < 0.05$), *CK14* was 4.1-fold ($p < 0.0001$), and *SP-C* was 13.6-fold ($p < 0.05$) higher in the H3N2-infected HSAEpCs (Fig. 6A, C, and D, respectively). The expression of *CC10* was

3.2-fold lower ($p < 0.005$) in the H3N2-infected HSAEpCs (Fig. 6B).

Our observation that H1N1 infection downregulated *Aqp5* and upregulated *CK14* in HSAEpCs is supported by data from mice, as these two genes were down- and upregulated in adenovirus and *Mycoplasma pulmonis*-infected murine lungs, respectively.^{78,79} The decrease in *CC10* expression in infected HSAEpCs was also supported by similar observations in gammaherpesvirus-infected murine bronchioles.⁸⁰ Taken together, our data show that the H1N1-infected 3D-cultured HSAEpCs closely recapitulate the *in vivo* immunological phenotype.

Cytokine expression. To understand the immune responses of 3D-cultured HSAEpCs to H1N1, we studied several cytokines/chemokines by ELISA. Results showed that IL-1 β , IL-6, and IL-8 were highly increased in the infected HSAEpCs (MOI=0.1) when compared to the naïve cells (Fig. 8A–C, $p < 0.05$ in all cases). MCP-1 was 20-fold lower in the H1N1-infected HSAEpCs at MOI=0.1 ($p < 0.005$), while at MOI=1.0, the MCP-1 levels were not significantly different from naïve cells (Fig. 8E, $p = 0.09$). Thus, the 3D-cultured HSAEpCs exhibited broad-range immune responses. Similar observations have been made in *ex vivo* human lung slices, where a 2 to 10-fold increase in mRNA and 2–16-fold increase in protein levels of IL-6, IL-8, and MCP-1 were observed in response to H1N1.³ Studies have also shown that IAV H5N1 induced IL-1 β in avian and murine lung.^{81,82} Our results showed only marginal increase in the anti-inflammatory IL-10 levels (Fig. 8D), similar to results observed in H1N1-infected AECs.⁸³ Taken together, these results demonstrate that the 3D-cultured HSAEpCs

FIG. 7. Transcript levels of marker protein expression by 3D-cultured, ALI-exposed HSAEpCs infected with H1N1 or H3N2. The HSAEpCs were cultured in 3D, ALI-exposed, and infected with H1N1 or H3N2, MOI 0.1 or 1.0 for 24 h. RNA was harvested from the cells as mentioned in section “Materials and Methods”, and mRNA expression levels of (A), *Aqp5*, (B), *CC-10*, (C), *CK14*, and (D), *SP-C* were measured by quantitative PCR. Expression levels of the abovementioned genes were normalized against the expression of the housekeeping gene β -actin. Naïve HSAEpCs were used as controls. Shown is a combination of three independent experiments (* p -value < 0.05 ; ** p -value < 0.01).

FIG. 8. Cytokine and chemokine expression by 3D-cultured, ALI-exposed HSAEpCs infected with H1N1 or H3N2. The HSAEpCs were cultured in 3D, ALI-exposed, and infected with H1N1 or H3N2, MOI 0.1 or 1.0 for 24 h. Levels of (A), IL-1 β ; (B), IL-6; (C), IL-8; (D), IL-10; and (E), MCP-1 in the culture supernatants were determined by ELISA, using commercial ELISA kits and manufacturers' instructions. Naive HSAEpCs were used as negative controls. Shown is a representative of three independent experiments (* p -value <0.05; ** p -value <0.01).

closely recapitulate the physiological immune responses to H1N1, making it an ideal component for the 3D-HTLM.

H3N2 infection of the 3D-cultured HSAEpCs also resulted in a distinct, broad-range immune response. ELISA data showed that at 24 h postinfection, IL-1 β , IL-6, and IL-8 levels were significantly increased, compared to the naive cells (Fig. 8A–C, p < 0.005, p < 0.05, p < 0.005, respectively). In contrast, MCP-1 levels were 800-fold lower in the H3N2-infected HSAEpCs (Fig. 8E, p < 0.005). The observed differences in immune responses between H1N1 and H3N2 are in line with previous studies.^{3,65} These strains also differ in the cytokine mRNA induction kinetics, with H3N2 inducing early responses.³ Clinical manifestations of these strains are also distinct: while H1N1 infections result in dry cough and fever, they are less likely to show other symptoms such as chest pain, which are seen with H3N2 infections.⁸⁴

In conclusion, our 3D-cultured ALI-exposed HSAEpCs closely resembled the *in vivo* airway epithelium. The 3D-cultured HSAEpCs showed higher cell viability and marker protein expression, and exhibited *in vivo*-like cell morphology and distribution. The 3D-cultured HSAEpCs also closely recapitulated *in vivo* immune responses to two major IAV strains. Thus, the 3D-cultured HSAEpCs are a close approximation of the lower airway, and is the first step in development of a 3D-HTLM, which may have wide applicability in studying pulmonary pathophysiology and therapeutics development.

Acknowledgments

The authors are grateful to Dr. Gillian M. Air, in the Department of Biochemistry and Molecular Biology, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK for donating the A/Hong Kong/

01/68 H3N2 strain. This work was supported (in part) by the National Institute of General Medical Sciences under Award Number P20GM103648.

Disclosure Statement

No competing financial interests exist.

References

- Cheng, P.Y., Palekar, R., Azziz-Baumgartner, E., *et al.* Burden of influenza-associated deaths in the Americas, 2002–2008. *Influenza Other Respir Viruses* **9**(Suppl 1), 13, 2015.
- Health in the Americas: Pan American Health Organization; 2012. Available from: https://www.paho.org/salud-en-las-americas-2012/index.php?option=com_content&view=article&id=68:united-states&Itemid=174&lang=en (last accessed September 15, 2017).
- Wu, W., Booth, J.L., Duggan, E.S., *et al.* Innate immune response to H3N2 and H1N1 influenza virus infection in a human lung organ culture model. *Virology* **396**, 178, 2010.
- CDC Estimates of 2009 H1N1 Influenza Cases, Hospitalizations and Deaths in the United States: Centers for Disease Control, 2014. Available from: http://cdc.gov/h1n1flu/estimates_2009_h1n1.htm/ (last accessed September 15, 2017).
- Scalera, N.M., and Mossad, S.B. The first pandemic of the 21st century: a review of the 2009 pandemic variant influenza A (H1N1) virus. *Postgrad Med* **121**, 43, 2009.
- Osterlund, P., Pirhonen, J., Ikonen, N., *et al.* Pandemic H1N1 2009 influenza A virus induces weak cytokine responses in human macrophages and dendritic cells and is highly sensitive to the antiviral actions of interferons. *J Virol* **84**, 1414, 2010.
- Woo, P.C., Tung, E.T., Chan, K.H., Lau, C.C., Lau, S.K., and Yuen, K.Y. Cytokine profiles induced by the novel

- swine-origin influenza A/H1N1 virus: implications for treatment strategies. *J Infect Dis* **201**, 346, 2010.
8. Liu, Q., Zhou, Y.H., and Yang, Z.Q. The cytokine storm of severe influenza and development of immunomodulatory therapy. *Cell Mol Immunol* **13**, 3, 2016.
 9. Ronni, T., Matikainen, S., Sareneva, T., *et al.* Regulation of IFN- α /beta, MxA, 2',5'-oligoadenylate synthetase, and HLA gene expression in influenza A-infected human lung epithelial cells. *J Immunol* **158**, 2363, 1997.
 10. Ito, Y., Correll, K., Zemans, R.L., Leslie, C.C., Murphy, R.C., and Mason, R.J. Influenza induces IL-8 and GM-CSF secretion by human alveolar epithelial cells through HGF/c-Met and TGF- α /EGFR signaling. *Am J Physiol Lung Cell Mol Physiol* **308**, L1178, 2015.
 11. Tumpey, T.M., Garcia-Sastre, A., Taubenberger, J.K., *et al.* Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *J Virol* **79**, 14933, 2005.
 12. Bouvier, N.M., and Lowen, A.C. Animal models for influenza virus pathogenesis and transmission. *Viruses* **2**, 1530, 2010.
 13. Kirchenbaum, G.A., and Ross, T.M. Generation of monoclonal antibodies against immunoglobulin proteins of the domestic ferret (*Mustela putorius furo*). *J Immunol Res* **2017**, 5874572, 2017.
 14. Mestas, J., and Hughes, C.C. Of mice and not men: differences between mouse and human immunology. *J Immunol* **172**, 2731, 2004.
 15. Shieh, W.J., Blau, D.M., Denison, A.M., *et al.* 2009 Pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. *Am J Pathol* **177**, 166, 2010.
 16. Subbarao, K., Klimov, A., Katz, J., *et al.* Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **279**, 393, 1998.
 17. Bhowmick, R., and Gappa-Fahlenkamp, H. Cells and culture systems used to model the small airway epithelium. *Lung* **194**, 419, 2016.
 18. Arndt, U., Wennemuth, G., Barth, P., *et al.* Release of macrophage migration inhibitory factor and CXCL8/interleukin-8 from lung epithelial cells rendered necrotic by influenza A virus infection. *J Virol* **76**, 9298, 2002.
 19. Bussfeld, D., Kaufmann, A., Meyer, R.G., Gemsa, D., and Sprenger, H. Differential mononuclear leukocyte attracting chemokine production after stimulation with active and inactivated influenza A virus. *Cell Immunol* **186**, 1, 1998.
 20. Fesq, H., Bacher, M., Nain, M., and Gemsa, D. Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. *Immunobiology* **190**, 175, 1994.
 21. Seo, S.H., Goloubeva, O., Webby, R., and Webster, R.G. Characterization of a porcine lung epithelial cell line suitable for influenza virus studies. *J Virol* **75**, 9517, 2001.
 22. Knight, B., Laukaitis, C., Akhtar, N., Hotchin, N.A., Edlund, M., and Horwitz, A.R. Visualizing muscle cell migration in situ. *Curr Biol* **10**, 576, 2000.
 23. Roskelley, C.D., Desprez, P.Y., and Bissell, M.J. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proc Natl Acad Sci U S A* **91**, 12378, 1994.
 24. Booth, J.L., Coggeshall, K.M., Gordon, B.E., and Metcalf, J.P. Adenovirus type 7 induces interleukin-8 in a lung slice model and requires activation of Erk. *J Virol* **78**, 4156, 2004.
 25. Leemasawatdigul, K., and Gappa-Fahlenkamp, H. Development of a mathematical model to describe the transport of monocyte chemoattractant protein-1 through a three-dimensional collagen matrix. *Cardiovasc Pathol* **21**, 219, 2012.
 26. Subramanian, R., Bhowmick, R., and Gappa-Fahlenkamp, H. Design of a novel method for the spatial distribution of cells within a porous scaffold for tissue engineering applications. *J Tissue Sci Eng* **8**, 201, 2017.
 27. Kroeker, A.L., Ezzati, P., Halayko, A.J., and Coombs, K.M. Response of primary human airway epithelial cells to influenza infection: a quantitative proteomic study. *J Proteome Res* **11**, 4132, 2012.
 28. Bhowmick, R., Ghosal, A., Das, B., *et al.* Intestinal adherence of *Vibrio cholerae* involves a coordinated interaction between colonization factor GbpA and mucin. *Infect Immun* **76**, 4968, 2008.
 29. Blank, N., Gabler, C., Schiller, M., Kriegel, M., Kalden, J.R., and Lorenz, H.M. A fast, simple and sensitive method for the detection and quantification of detergent-resistant membranes. *J Immunol Methods* **271**, 25, 2002.
 30. Livak, K.J., and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402, 2001.
 31. Ekert, J.E., Johnson, K., Strake, B., *et al.* Three-dimensional lung tumor microenvironment modulates therapeutic compound responsiveness in vitro—implication for drug development. *PLoS One* **9**, e92248, 2014.
 32. Kreft, M.E., Jerman, U.D., Lasic, E., *et al.* The characterization of the human cell line Calu-3 under different culture conditions and its use as an optimized in vitro model to investigate bronchial epithelial function. *Eur J Pharm Sci* **69**, 1, 2015.
 33. Giard, D.J., Aaronson, S.A., Todaro, G.J., *et al.* In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst* **51**, 1417, 1973.
 34. Godfrey, R.W. Human airway epithelial tight junctions. *Microsc Res Tech* **38**, 488, 1997.
 35. Swain, R.J., Kemp, S.J., Goldstraw, P., Tetley, T.D., and Stevens, M.M. Assessment of cell line models of primary human cells by Raman spectral phenotyping. *Biophys J* **98**, 1703, 2010.
 36. Stueckle, T.A., Davidson, D.C., Derk, R., *et al.* Effect of surface functionalizations of multi-walled carbon nanotubes on neoplastic transformation potential in primary human lung epithelial cells. *Nanotoxicology* **11**, 613, 2017.
 37. Auger, F., Gendron, M.C., Chamot, C., Marano, F., and Dazy, A.C. Responses of well-differentiated nasal epithelial cells exposed to particles: role of the epithelium in airway inflammation. *Toxicol Appl Pharmacol* **215**, 285, 2006.
 38. Million, K., Tournier, F., Houcine, O., Ancian, P., Reichert, U., and Marano, F. Effects of retinoic acid receptor-selective agonists on human nasal epithelial cell differentiation. *Am J Respir Cell Mol Biol* **25**, 744, 2001.
 39. Frohlich, E., Bonstingl, G., Hofer, A., *et al.* Comparison of two in vitro systems to assess cellular effects of nanoparticles-containing aerosols. *Toxicol In Vitro* **27**, 409, 2013.
 40. Yang, C., Su, L., Wang, Y., and Liu, L. UTP regulation of ion transport in alveolar epithelial cells involves distinct mechanisms. *Am J Physiol Lung Cell Mol Physiol* **297**, L439, 2009.

41. Zhao, L., Yee, M., and O'Reilly, M.A. Transdifferentiation of alveolar epithelial type II to type I cells is controlled by opposing TGF-beta and BMP signaling. *Am J Physiol Lung Cell Mol Physiol* **305**, L409, 2013.
42. Shannon, J.M., Mason, R.J., and Jennings, S.D. Functional differentiation of alveolar type II epithelial cells in vitro: effects of cell shape, cell-matrix interactions and cell-cell interactions. *Biochim Biophys Acta* **931**, 143, 1987.
43. Rubin, B.K. Physiology of airway mucus clearance. *Respir Care* **47**, 761, 2002.
44. Larstad, M., Almstrand, A.C., Larsson, P., *et al.* Surfactant protein A in exhaled endogenous particles is decreased in chronic obstructive pulmonary disease (COPD) patients: a pilot study. *PLoS One* **10**, e0144463, 2015.
45. Mackay, R.M., Grainge, C.L., Lau, L.C., Barber, C., Clark, H.W., and Howarth, P.H. Airway surfactant protein D deficiency in adults with severe asthma. *Chest* **149**, 1165, 2016.
46. Franzdottir, S.R., Axelsson, I.T., Arason, A.J., Baldursson, O., Gudjonsson, T., and Magnusson, M.K. Airway branching morphogenesis in three dimensional culture. *Respir Res* **11**, 162, 2010.
47. Schamberger, A.C., Staab-Weijnitz, C.A., Mise-Racek, N., and Eickelberg, O. Cigarette smoke alters primary human bronchial epithelial cell differentiation at the air-liquid interface. *Sci Rep* **5**, 8163, 2015.
48. Smirnova, N.F., Schamberger, A.C., Nayakanti, S., Hatz, R., Behr, J., and Eickelberg, O. Detection and quantification of epithelial progenitor cell populations in human healthy and IPF lungs. *Respir Res* **17**, 83, 2016.
49. Stone, K.C., Mercer, R.R., Gehr, P., Stockstill, B., and Crapo, J.D. Allometric relationships of cell numbers and size in the mammalian lung. *Am J Respir Cell Mol Biol* **6**, 235, 1992.
50. Funaki, H., Yamamoto, T., Koyama, Y., *et al.* Localization and expression of AQP5 in cornea, serous salivary glands, and pulmonary epithelial cells. *Am J Physiol* **275**, C1151, 1998.
51. Kondo, H., Miyoshi, K., Sakiyama, S., Tangoku, A., and Noma, T. Differential regulation of gene expression of alveolar epithelial cell markers in human lung adenocarcinoma-derived A549 clones. *Stem Cells Int* **2015**, 165867, 2015.
52. Speer, C.P., Sweet, D.G., and Halliday, H.L. Surfactant therapy: past, present and future. *Early Hum Dev* **89**(Suppl 1), S22, 2013.
53. Lopez, I.P., Pineiro-Hermida, S., Pais, R.S., Torrens, R., Hoefflich, A., and Pichel, J.G. Involvement of Igflr in bronchiolar epithelial regeneration: role during repair kinetics after selective club cell ablation. *PLoS One* **11**, e0166388, 2016.
54. Nikolic, M.Z., and Rawlins, E.L. Lung organoids and their use to study cell-cell interaction. *Curr Pathobiol Rep* **5**, 223, 2017.
55. O'Boyle, N., Sutherland, E., Berry, C.C., and Davies, R.L. Temporal dynamics of ovine airway epithelial cell differentiation at an air-liquid interface. *PLoS One* **12**, e0181583, 2017.
56. Hong, K.U., Reynolds, S.D., Watkins, S., Fuchs, E., and Stripp, B.R. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am J Pathol* **164**, 577, 2004.
57. Tam, A., Wadsworth, S., Dorscheid, D., Man, S.F., and Sin, D.D. The airway epithelium: more than just a structural barrier. *Ther Adv Respir Dis* **5**, 255, 2011.
58. Wu, W., Zhang, W., Duggan, E.S., Booth, J.L., Zou, M.H., and Metcalf, J.P. RIG-I and TLR3 are both required for maximum interferon induction by influenza virus in human lung alveolar epithelial cells. *Virology* **482**, 181, 2015.
59. Hamilton, S.B., Wyatt, D.E., Wahlgren, B.T., *et al.* Higher titers of some H5N1 and recent human H1N1 and H3N2 influenza viruses in Mv1 Lu vs. MDCK cells. *Virol J* **8**, 66, 2011.
60. Waugh, T., Ching, J.C., Zhou, Y., and Loewen, M.E. Influenza A virus (H1N1) increases airway epithelial cell secretion by up-regulation of potassium channel KCNN4. *Biochem Biophys Res Commun* **438**, 581, 2013.
61. Minor, D.M., and Proud, D. Role of human rhinovirus in triggering human airway epithelial-mesenchymal transition. *Respir Res* **18**, 110, 2017.
62. Carterson, A.J., Honer zu Bentrup, K., Ott, C.M., *et al.* A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for *Pseudomonas aeruginosa* pathogenesis. *Infect Immun* **73**, 1129, 2005.
63. Influenza virus infections in humans: World Health Organization; 2014. Available from: http://who.int/influenza/human_animal_interface/virology_laboratories_and_vaccines/influenza_virus_infections_humans_feb14.pdf?ua=1 (last accessed September 15, 2015).
64. van Riel, D., Munster, V.J., de Wit, E., *et al.* Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. *Am J Pathol* **171**, 1215, 2007.
65. Wang, J., Oberley-Deegan, R., Wang, S., *et al.* Differentiated human alveolar type II cells secrete antiviral IL-29 (IFN-lambda 1) in response to influenza A infection. *J Immunol* **182**, 1296, 2009.
66. David, J., Sayer, N.M., and Sarkar-Tyson, M. The use of a three-dimensional cell culture model to investigate host-pathogen interactions of *Francisella tularensis* in human lung epithelial cells. *Microbes Infect* **16**, 735, 2014.
67. Nakajima, N., Sato, Y., Katano, H., *et al.* Histopathological and immunohistochemical findings of 20 autopsy cases with 2009 H1N1 virus infection. *Mod Pathol* **25**, 1, 2012.
68. Hofer, C.C., Woods, P.S., and Davis, I.C. Infection of mice with influenza A/WSN/33 (H1N1) virus alters alveolar type II cell phenotype. *Am J Physiol Lung Cell Mol Physiol* **308**, L628, 2015.
69. Liu, N., Song, W., Wang, P., *et al.* Proteomics analysis of differential expression of cellular proteins in response to avian H9N2 virus infection in human cells. *Proteomics* **8**, 1851, 2008.
70. Moll, R., Divo, M., and Langbein, L. The human keratins: biology and pathology. *Histochem Cell Biol* **129**, 705, 2008.
71. Vester, D., Rapp, E., Gade, D., Genzel, Y., and Reichl, U. Quantitative analysis of cellular proteome alterations in human influenza A virus-infected mammalian cell lines. *Proteomics* **9**, 3316, 2009.
72. Yu, G., Liang, W., Liu, J., *et al.* Proteomic analysis of differential expression of cellular proteins in response to avian H9N2 virus infection of A549 cells. *Front Microbiol* **7**, 1962, 2016.
73. Greenbaum, D., Colangelo, C., Williams, K., and Gerstein, M. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol* **4**, 117, 2003.
74. Amsen, D., de Visser, K.E., and Town, T. Approaches to determine expression of inflammatory cytokines. *Methods Mol Biol* **511**, 107, 2009.
75. Le, T., Leung, L., Carroll, W.L., and Schibler, K.R. Regulation of interleukin-10 gene expression: possible mechanisms accounting for its upregulation and for maturational

- differences in its expression by blood mononuclear cells. *Blood* **89**, 4112, 1997.
76. Petrilli, V., Papin, S., and Tschopp, J. The inflammasome. *Curr Biol* **15**, R581, 2005.
 77. Vogel, C., and Marcotte, E.M. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **13**, 227, 2012.
 78. Towne, J.E., Harrod, K.S., Krane, C.M., and Menon, A.G. Decreased expression of aquaporin (AQP)1 and AQP5 in mouse lung after acute viral infection. *Am J Respir Cell Mol Biol* **22**, 34, 2000.
 79. Lewis, C.C., Yang, J.Y., Huang, X., *et al.* Disease-specific gene expression profiling in multiple models of lung disease. *Am J Respir Crit Care Med* **177**, 376, 2008.
 80. Leeming, G.H., Kipar, A., Hughes, D.J., *et al.* Gamma-herpesvirus infection modulates the temporal and spatial expression of SCGB1A1 (CCSP) and BPIFA1 (SPLUNC1) in the respiratory tract. *Lab Invest* **95**, 610, 2015.
 81. Ranaware, P.B., Mishra, A., Vijayakumar, P., *et al.* Genome wide host gene expression analysis in chicken lungs infected with avian influenza viruses. *PLoS One* **11**, e0153671, 2016.
 82. Preusse, M., Tantawy, M.A., Klawonn, F., Schughart, K., and Pessler, F. Infection- and procedure-dependent effects on pulmonary gene expression in the early phase of influenza A virus infection in mice. *BMC Microbiol* **13**, 293, 2013.
 83. Sanpui, P., Zheng, X., Loeb, J.C., *et al.* Single-walled carbon nanotubes increase pandemic influenza A H1N1 virus infectivity of lung epithelial cells. *Part Fibre Toxicol* **11**, 66, 2014.
 84. Yap, J., Tan, C.H., Cook, A.R., *et al.* Differing clinical characteristics between influenza strains among young healthy adults in the tropics. *BMC Infect Dis* **12**, 12, 2012.

Address correspondence to:
Heather Gappa-Fahlenkamp, PhD
School of Chemical Engineering
Oklahoma State University
420 Engineering North
Stillwater, OK 74078

E-mail: heather.fahlenkamp@okstate.edu

Received: October 24, 2017

Accepted: April 19, 2018

Online Publication Date: June 26, 2018