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Original Article

Differentiated swine airway epithelial cell cultures for the investigation of influenza A virus infection and replication

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Background Differentiated human airway epithelial cell cultures have been utilized to investigate cystic fibrosis, wound healing, and characteristics of viral infections. These cultures, grown at an air–liquid interface (ALI) in media with defined hormones and growth factors, recapitulate many aspects of the *in vivo* respiratory tract and allow for experimental studies at the cellular level.

Objectives To optimize growth conditions for differentiated swine airway epithelial cultures and to use these cultures to examine influenza virus infection and replication.

Methods Primary swine respiratory epithelial cells were grown at an air–liquid interface with varying amounts of retinoic acid and epidermal growth factor. Cells grown with optimized concentrations of these factors for 4 weeks differentiated into multilayer epithelial cell cultures resembling the lining of the swine respiratory tract. Influenza virus infection and replication were examined in these cultures.

Results/Conclusions Retinoic acid promoted ciliogenesis, whereas epidermal growth factor controlled the thickness of the pseudoepithelium. The optimal concentrations for differentiated swine cell cultures were 1·5 ng/ml epidermal growth factor and 100 nm retinoic acid. Influenza A viruses infected and productively replicated in these cultures in the absence of exogenous trypsin, suggesting that the cultures express a protease capable of activating influenza virus hemagglutinin. Differences in virus infection and replication characteristics found previously in pigs *in vivo* were recapitulated in the swine cultures. This system could be a useful tool for a range of applications, including investigating influenza virus species specificity, defining cell tropism of influenza viruses in the swine respiratory epithelium, and studying other swine respiratory diseases.

Keywords Cultures, epithelial, influenza virus, replication, swine.

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Introduction

In the late 1990s 'triple reassortant' H3N2 (rH3N2) viruses containing genes from swine-, avian-, and human-lineage influenza viruses emerged and spread widely throughout the US swine population.[1–3] Further reassortment led to the appearance of rH1N1, rH1N2, rH3N1, and rH2N3 viruses in North American pigs.[4–7] The emergence of these reassortant swine viruses has had major health implications for both pigs and humans, as many of these viruses have caused substantial disease in pigs and contributed six nucleic acid segments to the 2009 H1N1 human pandemic virus.[8,9]

To determine what allowed the initial rH3N2 viruses to successfully emerge, spread, and be maintained in the swine population, we previously compared a representative rH3N2 virus [A/Swine/Minnesota/593/99 (Sw/MN)] with a wholly human-lineage virus isolated from a single piglet during the same time period [A/Swine/Ontario/00130/97 (Sw/ONT)]. Sw/ONT has not been subsequently isolated from pigs, while the rH3N2 viruses spread and have established a stable lineage in the North American swine population. To determine whether the differences in epidemiological outcomes between Sw/MN and Sw/ONT could be due to inherent differences between the viruses, infection experiments were performed in pigs *in vivo*. Sw/MN was infectious at lower doses, exhibited more rapid and more extensive nasal shedding and caused more severe lung lesions than Sw/ONT.[10] Using reverse genetics(rg)-created viruses in which we exchanged the hemagglutinin (HA) and neuraminidase (NA) genes between Sw/MN and Sw/ONT, we previously found that the infectivity and

replication phenotypes of rgMN and rgONT *in vivo* are dependent on the HA and/or NA genes.[11] We subsequently examined the infection of these viruses in relatively undifferentiated monolayers of primary swine respiratory epithelial cells (SRECs) grown submerged in media *in vitro*. RgMN infected a significantly higher proportion of SRECs than rgONT, and these phenotypes could be reversed by exchanging the HA and NA genes, as seen *in vivo*.[12] In addition, by exchanging the HA or NA individually, we showed that the HA alone (and specific amino acids within the HA) controlled infectivity in SRECs.

The SREC model system allows the examination of fine details of the infection process in primary cells,[13,14] but these submerged monolayer cells do not fully recapitulate the cellular architecture of the swine respiratory epithelium. As such, we sought to develop a cell culture model that mimics the three-dimensional architecture of the swine respiratory tract. Differentiated human airway epithelial cultures have been widely used to study influenza virus infection characteristics.[15–21] These multi-layered cultures are grown at an air–liquid interface (ALI), contain ciliated and goblet cells, and faithfully mimic the architecture of *in vivo* respiratory epithelium.

We describe here the development of differentiated ALI swine airway epithelial (SAE) cell cultures and the use of these cultures to investigate infection and replication characteristics of influenza viruses, with a particular focus on the previously well-characterized rgMN and rgONT viruses. By modulating growth media conditions used for human respiratory epithelial cell cultures,[22] we found that retinoic acid (RA) promotes ciliogenesis, and epidermal growth factor (EGF) controls the thickness (number of layers of cells) of the differentiated SAE pseudoepithelium. Influenza viruses replicated successfully in these cultures, even in the absence of exogenous trypsin. Furthermore, infection and replication characteristics of the rgMN and rgONT viruses in these cultures were similar to results seen previously *in vivo* and in SRECs cultured submerged in media.

Materials and methods

Isolation of human and swine respiratory epithelial cells (HRECs and SRECs)

The HRECs were isolated as described previously,[23] and the HREC protocol originally designed to grow these cells submerged in media was adapted to isolate and grow submerged SRECs.[12] Briefly, distal tracheal specimens from healthy, untreated pigs [raised for research and free (based on serologic testing) from infection with influenza virus, porcine reproductive and respiratory syndrome virus, and *Mycoplasma hyopneumoniae*] were placed in a pronase/DNAse solution for 72 hours at 4°C. The cells were then collected by centrifugation, resuspended, and fibroblasts were removed by differential adherence to plastic. Non-adherent (epithelial) cells were collected and seeded into type VI collagen (Sigma Chemical Co., St. Louis, MO, USA)-coated tissue culture flasks. The SRECs were grown in serum-free, hormone-supplemented bronchial epithelial growth media (BEGM, Lonza, Walkersville, MD, USA) at 37°C in a 5% CO_2 atmosphere. The collection and use of HRECs and SRECs were approved by the Institutional Review Board of the University of Wisconsin-Madison School of Medicine and Public Health and the Animal Care and Use Committee of the School of Veterinary Medicine.

Growth of differentiated airway epithelial cultures

At passage two, HRECs and SRECs grown submerged in media were plated (approximately 125 000 cells per well) onto type VI collagen-coated Transwell permeable supports (12 mm insert, 0·4 μm polyester membrane, Costar, Corning Incorporated, Corning, NY, USA) in 12-well plates with BEGM in the apical and basal compartments, and incubated at 37°C in a 5% CO_2 atmosphere. On the second day after plating, the apical media were removed, inducing an ALI, and the basal media were changed to 'modified Gray's' media (i.e., modified from media described in [22,24]). This media consists of a 1:1 mixture of DMEM (Mediatech, Manassas, VA, USA):BEBM with 50 μg/ml insulin, 1·4 μm hydrocortisone, 2·7 μm epinephrine, 9·7 nm triiodothyronine, 10 ng/ml transferrin, 1% fatty acid-free bovine serum albumin (all from Lonza BEGM SingleQuots), 1% bovine pituitary extract (0·4% from Lonza SingleQuots + 0·6% from Sigma), 1% penicillin/streptomycin/amphotericin (Invitrogen, Carlsbad, CA, USA), and the indicated concentrations of epidermal growth factor (EGF, Sigma) and retinoic acid (RA, Sigma). Basal media were changed the day after inducing an ALI and every other day thereafter. (Henceforth, the term SRECs will be used for submerged swine cells, while SAE cultures will refer to differentiated swine cell cultures grown at an ALI.)

Quantification of cell proliferation and ciliogenesis

After cultures were grown for 4 weeks at an ALI with various concentrations of EGF and RA, the membrane was removed from the Transwell, cultures were fixed with 10% formalin, and samples were embedded in wax, cut in transverse sections, and stained with hematoxylin and eosin (H&E). The number of cell layers constituting the pseudoepithelium was determined by counting the nuclei per vertical section of the culture. For each of three independent cultures, three microscope fields (40× magnification) were randomly chosen, and the thickness of three randomly chosen areas of each microscope field was determined ($n = 27$ for each concentration of EGF). Ciliogenesis was assessed by counting the number of ciliated cells (cells with cilia attached to the apical surface) per randomly chosen

microscope field (40× magnification) for each of three independent cultures (n = 9 for each concentration of RA).

Measurement of trans-epithelial electrical resistance (TEER)

Each day that the media were changed, the TEER across the membrane was measured using an EVOM Epithelial Voltohmmeter with an STX2 chopstick electrode (World Precision Instruments, Sarasota, FL, USA). A blank resistance reading (membrane only) was subtracted from the resistance reading of the SAE cultures, and unit area resistance (Ω cm^2) was calculated by multiplying the meter reading by the surface area of the filter membrane, as per the manufacturer's instructions.

Lectin staining of differentiated airway epithelial cultures

Four weeks after induction of an ALI, the Transwell membranes were removed, cultures were fixed with 10% formalin, and samples were embedded in wax and cut in transverse sections. To detect sialic acids on ALI cultures, sections were stained with lectins using the Roche DIG glycan differentiation kit (Roche Diagnostics, Mannheim, Germany). The lectins used were digoxigenin (DIG)-conjugated *Sambucus nigra* (SNA, which predominantly labels α2-6-linked sialic acids [25]) (Roche Diagnostics) and biotinylated *Maackia amurensis* I and II (MAA I and MAA II, which predominantly label α2-3-linked sialic acids [26]) (Vector Laboratories, Burlingame, CA, USA). Sections of swine lower trachea were stained for comparison. Following deparaffinization, sections were treated with the DIG glycan differentiation kit (Roche) blocking reagent for 30 minutes at room temperature (RT). Sections were then washed three times in TBS, incubated in buffer 1 for 1 hour at RT with either SNA (1:1000), MAA I (1:4000), or MAA II (1:2000), and washed three times with TBS. Sections stained with SNA were incubated for 1 hour at RT with anti-DIG alkaline phosphatase (1:1000) in TBS, while sections stained with MAA I and MAA II were incubated for 1 hour at RT with streptavidin–alkaline phosphatase (1:200, Vector) in TBS. Following three washes in TBS, sections were developed for 5 minutes at RT with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) diluted 1:50 in buffer 2 to reveal dark blue staining.

Immunostaining of differentiated airway epithelial cultures

Sections of SAE cultures and swine lower trachea were fixed, sectioned, blocked as described earlier, and incubated with mouse anti-pancytokeratin AE1/AE3 antibody (Zymed, San Francisco, CA, USA) diluted 1:100 in phosphate-buffered saline (PBS) for 1 hour at RT. Following three washes in PBS, sections were incubated with Vectastain Elite ABC Kit (Vector) biotinylated anti-mouse secondary antibody for 30 minutes at RT, washed three times in PBS, incubated with streptavidin-containing Vectastain Elite ABC Reagent, washed three times in PBS, and incubated in AEC (Invitrogen) for 5 minutes at RT to reveal red staining.

Viruses

Stocks of rgMN, rgONT, and reassortant viruses in which the HA and/or NA genes were exchanged between rgMN and rgONT were generated as described previously.[10–12,27] These reassortant viruses, as well as the field isolates A/Brazil/1137/99 (human H1N1), A/Brazil/02/99 (human H3N2), A/Sw/NC/44173/00 (swine H1N1), and A/Sw/IN/9K035/99 (swine H1N2), were grown and titered in Madin Darby canine kidney (MDCK) cells as described previously.[12]

Virus infection of differentiated SAE cultures

Four weeks after induction of an ALI, SAE cultures were either left unwashed or washed 10 times with 500 µl sterile PBS. Cultures were then overlaid with 3×10^6 50% tissue culture infectious dose ($TCID_{50}$) of virus in 200 µl of inoculation media [Minimal Essential Medium (Invitrogen) + 0·2% bovine serum albumin + 1% penicillin/streptomycin/amphotericin (Invitrogen) + 0·01% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA)] for 1 hour at 37°C. The number of cells per Transwell was approximately 1×10^6 (data not shown), meaning that this inoculation approximated a multiplicity of infection (MOI) of 3. However, not all cells in SAE cultures are apical, so the virus inoculum did not have access to all cells. If only the cells that the virus inoculum can access are included, the MOI would clearly be higher than 3. Following incubation, the virus inoculum was removed from the Transwell, cells were washed twice with 500 µl sterile PBS, and cultures were incubated for 11 hours at 37°C. Cells were then fixed with 10% formalin, and the infected cells were identified by immunocytochemical (ICC) staining with a mouse anti-influenza A nucleoprotein monoclonal antibody as previously published.[10,12]

Virus replication in differentiated SAE cultures

Differentiated cultures within the Transwell compartments were washed 10 times with sterile PBS and overlaid with 3×10^5 $TCID_{50}$ of virus in 200 µl inoculation media for 1 hour at 37°C. Following incubation, cells were washed twice with 500 µl sterile PBS with or without 1 µg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin and incubated at an ALI at 37°C. At indicated times, 200 µl of PBS with or without TPCK-treated trypsin was added to cells for 10 minutes, removed, and frozen at −80°C for future virus titration using a $TCID_{50}$

assay. Samples of media in the basal compartment (below the culture) were also collected at various times and titered to determine whether virus was present in the basal media compartments.

Titer determination

A $TCID_{50}$ assay was used to measure viral titers following virus replication in differentiated SAE cultures. MDCK cells in 96-well plates were inoculated with 1:7 serial dilutions of sample and incubated for 48 h in the presence of 1 μg/ml TPCK-treated trypsin. We have previously determined that serial 1:7 dilutions result in a more precise measure of infectious virus titer than standard 10-log dilutions (data not shown). Cells were then fixed with formalin, permeabilized by methanol, and infected cells were visualized by ICC staining. Titers were determined using the method of Reed and Muench.[28]

Statistical analysis

Comparison of pseudoepithelium thickness with regard to EGF levels was analyzed by a Student's *t*-test using the R statistical software (http://www.R-project.org). Comparison of viral replication titers was analyzed by anova-protected *t*-tests with the Bonferroni correction using SAS 9.2 (SAS Institute Ind., Cary, NC, USA).

Results

Effect of epidermal growth factor on cell proliferation

Multiple protocols exist to produce differentiated human epithelial cell cultures, but a commonly utilized protocol is that of Gray *et al.*[15,22,24,29,30] In an attempt to produce differentiated swine respiratory epithelial cultures, we initially examined human and swine cell cultures grown using the Gray *et al.* methods and media (which contains 0·5 ng/ml EGF and 50 nm RA). Similar to previously published reports,[15,22] the human cells differentiated after approximately 4 weeks into multilayered cultures that contained ciliated and mucin-producing cells (data not shown). However, swine cells grown using this method and media resulted in a thin, poorly differentiated epithelial layer (only 1–2 cells thick) that contained very few ciliated cells (data not shown).

We hypothesized that additional EGF could increase cell proliferation and the thickness of the swine pseudoepithelium. To examine the effect of EGF, swine cell cultures were grown in modified Gray's media containing a constant amount of RA (50 nm), but various amounts of EGF. A clear dose-response was seen, where increasing amounts of EGF led to an increase in cell proliferation (Figure 1). Cultures grown in the absence of EGF were essentially monolayers only one cell thick, whereas cultures grown with

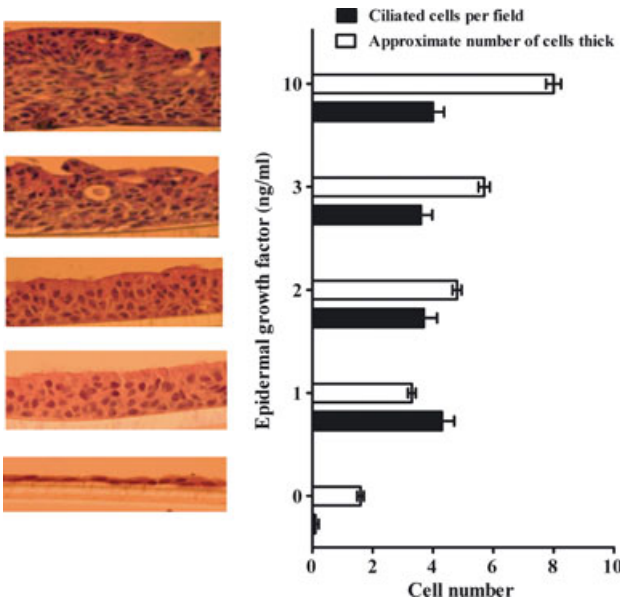

Figure 1. Effect of epidermal growth factor on cell proliferation. SAE cultures were grown at an ALI for 4 weeks with a constant amount of RA (50 nm) and various concentrations of EGF, followed by histological sectioning and H&E staining. Representative histological sections (100× magnification) are shown. The number of cell layers constituting the pseudoepithelium was determined by counting the nuclei per vertical section of the culture. The results shown are the mean ± SEM of three areas counted per microscope field (40× magnification), three fields per culture, and three independent cultures (n = 27 total). Ciliogenesis was assessed by counting the number of ciliated cells per microscope field (40× magnification). Results shown are the mean ± SEM of three microscope fields per culture and three independent cultures (n = 9 total).

1 ng/ml or more EGF were thicker and contained significantly more layers of cells ($P < 0{\cdot}001$, Student's *t*-test). The absence of EGF also led to an absence of cilia, but cultures grown with all other EGF concentrations contained approximately the same number of ciliated cells (as measured by counting the number of ciliated cells per microscope field, Figure 1). Thus, EGF appears to induce cell proliferation of SAE cultures in a dose-dependent manner, and EGF is required at some minimal level to support ciliogenesis.

Effect of retinoic acid on ciliogenesis

Increasing the concentration of EGF led to increased pseudoepithelium thickness, but the cultures did not express as many ciliated cells as the natural swine respiratory tract *in vivo*. (Wallace *et al.*[31] estimate that approximately 70% of swine tracheal epithelial cells are ciliated.) Previous studies reported that retinoic acid (RA) is important for ciliogenesis and in supporting epithelial cell differentiation. Specifically, when RA is not present, cells exhibit a squamous phenotype, while RA inhibits squamous morphology and induces ciliated epithelial cell

differentiation.[22,32–34] To determine whether RA could induce growth of ciliated cells, the cultures were grown in media containing a constant amount of EGF (1 ng/ml), but various concentrations of RA. Consistent with previous reports, lack of RA led to a squamous cell phenotype, whereas increasing amounts of RA resulted in a pseudoepithelium with ciliated cells (Figure 2), yet varying the RA concentration had little to no effect on the number of cells in the cell layer. Taken together, the data indicate that RA is a major determinant of ciliogenesis in SAE cultures, but does not greatly affect cell proliferation.

Characterization of differentiated SAE cultures

To confirm cell confluence and the presence of functionally intact tight junctions, TEER readings of SAE cultures were taken. Initial readings were approximately 100 Ω cm^2, and after 12 days of growth at ALI, TEER increased to approximately 800 Ω cm^2, indicating a confluent cell layer (Figure 3). The TEER subsequently decreased so that 18 days after ALI induction, TEER decreased to 250–300 Ω cm^2 and remained at this level. The virus infection experiments (described below), which involved inoculating SAE cultures from the apical side after 4 weeks of growth at ALI, did not substantially affect TEER readings (data not shown). In addition, results indicated that at 250–300 Ω cm^2 the culture maintained distinct apical and basal compartments, as virus was not detected in the basal media by $TCID_{50}$ assay (data not shown).

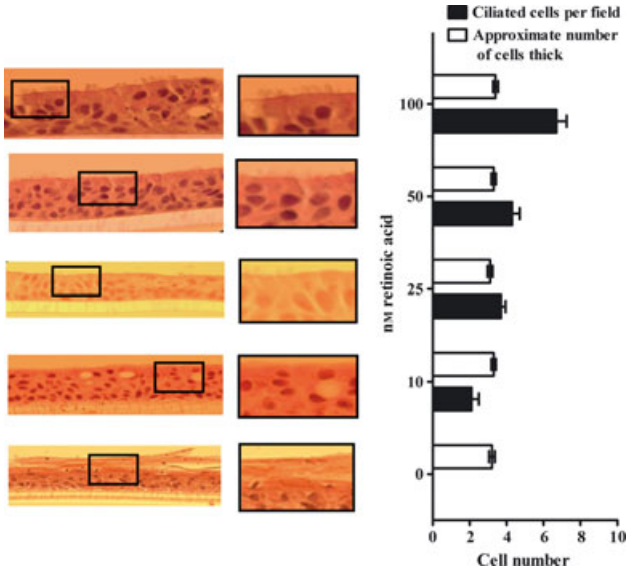

Figure 2. Effect of retinoic acid on ciliogenesis. SAE cultures were grown at an ALI for 4 weeks with a constant amount of EGF (1 ng/ml) and various concentrations of RA, followed by histological sectioning and H&E staining. Representative histological sections (100× magnification) are shown, with inserts to show ciliated cell surface. The thickness of the pseudoepithelium and the number of ciliated cells per microscope field were determined as in Figure 1.

To determine how well the differentiated SAE cell cultures mimic the swine respiratory tract as it exists *in vivo*, histological sections of SAE cultures were compared to sections of respiratory tract tissues excised from pigs. Well-differentiated SAE cultures were defined as multilayered cultures that expressed many ciliated cells and contained mucin-producing cells. By this definition, the cultures grown at ALI for 2 weeks with optimal levels of EGF and RA (1·5 ng/ml and 100 nm, respectively) were partially differentiated, similar to the 'poorly differentiated' human cells (as defined by Pickles *et al.*[30]). These partially differentiated swine cultures contained thin cell layers (the cultures were approximately two cells thick) and expressed very few cilia (data not shown). However, SAE cultures grown for 4 weeks with optimal levels of EGF and RA became well differentiated, with a culture thickness and number of ciliated cells comparable to that of the swine lower trachea (where primary cells were harvested; Figure 3B). This amount of time at ALI is similar to the time necessary for human cells to become well differentiated ([24,35] and data not shown). One difference noted, however, was that while well-differentiated SAE cultures exhibited similar thickness and number of ciliated cells as the swine lower trachea, SAE cultures contained a somewhat more disordered architecture without clearly columnar surface cells, while sections of excised pig trachea showed more ordered columnar cells (Figure 3).

Influenza viruses initiate infection by binding to cell surface sialic acids that are linked either α2-3 or α2-6 to underlying carbohydrates.[36] We sought to use SAE cultures to study influenza virus infection and replication, and lectin staining was used to define sialic acid expression. Swine lower trachea and SAE cultures were stained with SNA, which predominantly labels α2-6-linked sialic acids,[25] or MAAI and MAA II, two isoforms of a lectin that predominantly label α2-3-linked sialic acids.[26] The SNA lectin strongly stained the apical surface of both SAE cultures and swine lower trachea, while MAA I did not stain either samples (Figure 3B). The MAA II lectin did not stain the apical surface, but did react with sub-apical cells of both the SAE culture and swine lower trachea (Figure 3B).

To ensure that the SAE cultures were of epithelial origin, cultures were stained with a pan-cytokeratin antibody. Swine lower trachea was used as a comparison. The apical cells of SAE cultures and swine lower trachea stained cytokeratin-positive, indicating that SAE cultures are indeed composed of epithelial cells (Figure 3B).

Influenza virus infection of differentiated SAE cultures

To determine whether influenza viruses could infect SAE cultures, and to determine whether viruses retain previously documented infectivity characteristics in SAE

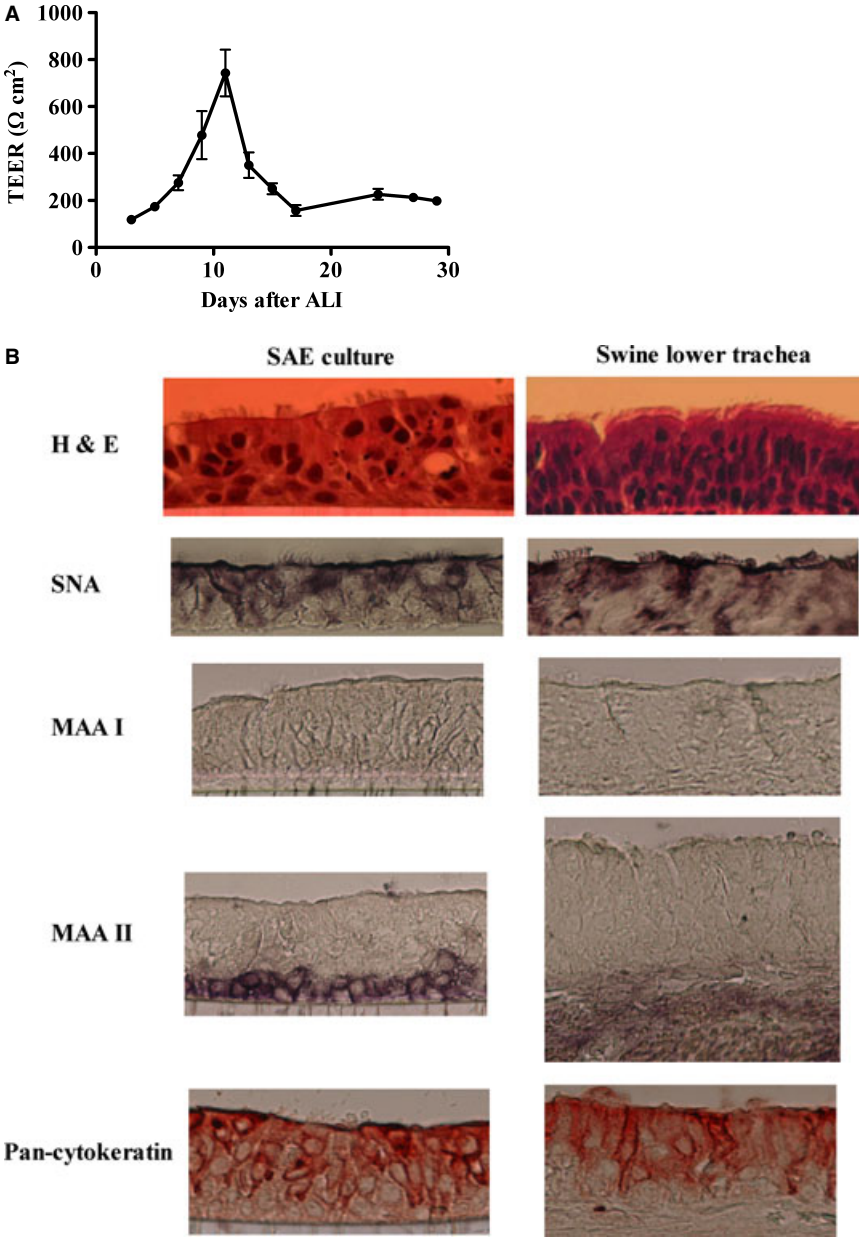

Figure 3. Characterization of SAE cultures. (A) TEER of SAE cultures. Results are mean ± SEM of twelve independent cultures. (B) Four-week-old SAE cultures and swine lower trachea were sectioned and stained with either H&E, SNA lectin, MAA I lectin, MAA II lectin, or pan-cytokeratin antibody. Dark blue staining indicates positive lectin reactivity, and red staining indicates positive cytokeratin reactivity. A larger area of swine lower trachea stained with MAA II is shown to depict positive staining of the sub-apical layer, similar to MAA II reactivity with SAE cultures. 100× magnification.

cultures, we infected differentiated (grown for 4 weeks at an ALI) SAE cultures with rgMN and rgONT. We have previously shown that in pigs, rgMN was infectious at lower doses than rgONT and that in submerged SRECs rgMN infected a significantly higher proportion of cells than rgONT.[10,12] To determine whether these differences could be recapitulated in SAE cultures, the cultures were infected with 3×10^6 $TCID_{50}$ of each virus. In addition, to determine whether a secreted substance inhibits influenza virus infection of SAE cultures, cells were either washed extensively (10 times) before inoculating or inoculated directly without washing. RgMN infected many more cells than rgONT (Figure 4), demonstrating that the previously described virus infectivity phenotypes are recapitulated in SAE cultures. Further, both rgMN and rgONT infected fewer cells in unwashed SAE cultures, indicating that viruses are inhibited by a substance on the SAE culture surface, possibly mucins (high molecular weight glycosylated

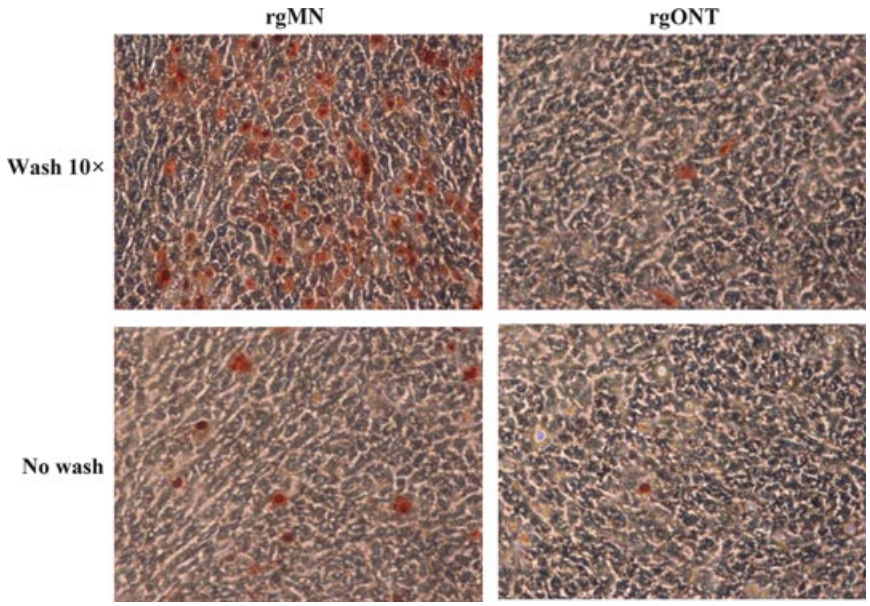

Figure 4. Representative photomicrographs of SAE cultures infected with rgMN and rgONT with or without prior washing of the cultures. Cultures were stained 12 h.p.i. Red staining indicates influenza virus nucleoprotein positive cells, indicative of productive infection. Magnification, 30×.

molecules that are believed to inhibit influenza virus infection of differentiated human respiratory cell cultures[29]). The inhibition is more visibly apparent with rgMN, because this virus has a higher infectivity phenotype than rgONT. Taken together, these data indicate that SAE cultures are susceptible to influenza virus infection and that the cultures secrete compounds, possibly mucin glycoproteins, which can inhibit influenza virus infection.

Influenza virus replication in SAE cultures

To determine whether influenza viruses can replicate in SAE cultures (beyond the initially infected cells), and to examine whether rgMN and rgONT replication phenotypes in pigs are recapitulated in SAE cultures, we examined the multiple-round replication of rgMN and rgONT viruses. In pigs, rgMN exhibited more rapid and more extensive nasal shedding than rgONT, and this phenotype could be largely reversed by exchanging the HA and NA genes between the viruses.[11] In SAE cultures, all examined viruses replicated and were shed into the apical compartment, and as expected, differences in titers were seen (Figure 5). RgMN replicated to high titers and replacing the HA and NA genes with those of rgONT decreased the peak viral titer approximately 100-fold (Figure 5A). Replacing the rgMN HA alone with the HA of rgONT had little effect, while replacing NA decreased viral replication to the level of rgMN + ONT HA/NA, demonstrating that the NA gene has a dominant effect on viral replication and/or virus release from cells in this model system. Similarly, rgONT replicated in SAE cultures and replacing the HA and NA genes with those of rgMN increased the peak viral titer approximately 100-fold (Figure 5B). Replacing rgONT HA and/or NA with that of rgMN led to increased viral titers, but replacement with the NA alone increased viral production more than replacing the HA alone, again indicating the importance of the NA gene in viral replication/release into the apical compartment. Taken together the results indicate that, consistent with results from *in vivo* infections, exchanging the HA and NA genes between rgMN and rgONT has substantial effects on virus replication. Furthermore, the NA gene appears to play a more dominant role in this phenotype than the HA.

Virus replication in the absence of exogenous protease

The influenza virus HA protein must be cleaved from the precursor molecule HA0 into HA1 and HA2 in order for the virus to be infectious.[37] If a cell line does not express an HA-activating protease, exogenous protease is added to facilitate multiple-round viral replication. To determine whether SAE cultures express such a protease, rgMN and rgONT titers in the apical compartment were examined in the presence and in the absence of TPCK-treated trypsin. Viral titers were similar in both the presence and absence of added trypsin (Figure 6), demonstrating that influenza viruses can replicate in SAE cultures in the absence of exogenous protease and indicating that SAE cultures express a protease capable of activating the virus HA.

In contrast to SAE cultures, submerged SRECs do not appear to express an HA-activating protease, as titers of

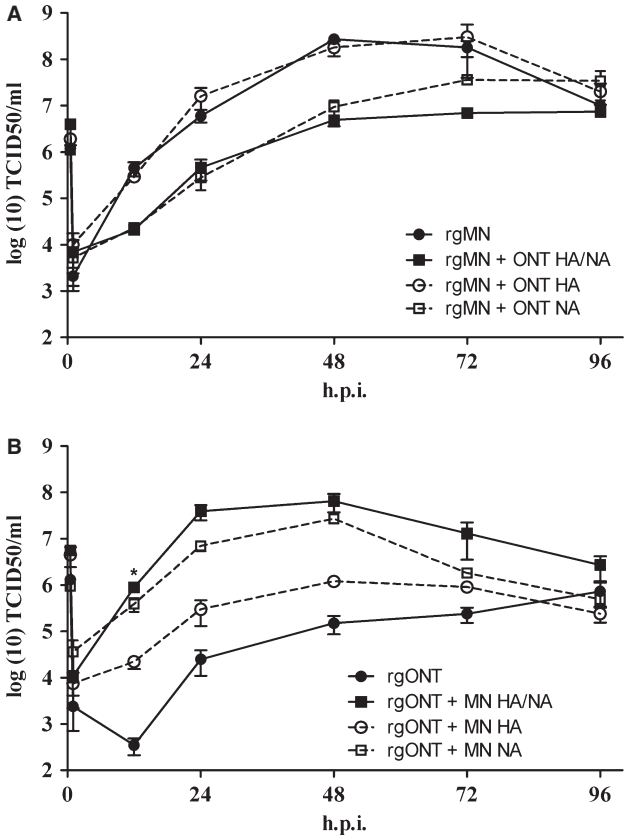

Figure 5. Influenza virus replication (virus titers in the apical compartment) in SAE cultures. (A) Replication of viruses with rgMN backbone and selected genes from rgONT, in the presence of 1 μg/ml TPCK-treated trypsin. (B) Replication of viruses with rgONT backbone and selected genes from rgMN, in the presence of 1 μg/ml TPCK-treated trypsin. Shown are the mean ± SEM of three independent experiments. *, compared to rgONT, $P < 0.0028$ (i.e., $P < 0.05$ after the Bonferroni correction for multiple comparisons).

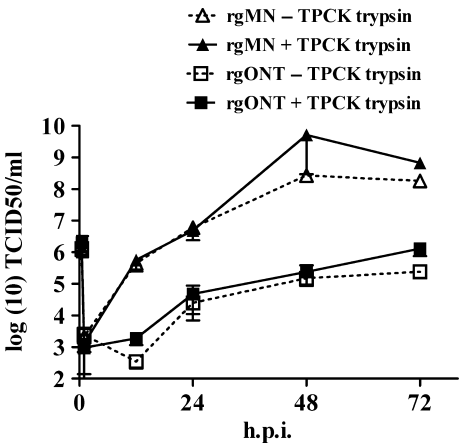

Figure 6. Replication (virus titers in the apical compartment) of rgMN and rgONT in SAE cultures, with and without TPCK-treated trypsin. Shown are the mean ± SEM of three independent experiments. Using *t*-tests with the Bonferroni correction for multiple comparisons, no statistically significant differences at any timepoint were seen between rgMN + TPCK and rgMN - TPCK, or between rgONT + TPCK and rgONT - TPCK.

rgMN and rgONT in the presence of added trypsin were much higher than in the absence (Figure 7A,B). In the absence of trypsin, the increase in titer from 1 to 24 h.p.i. was most likely due to virus produced by initially infected cells, but in the absence of an HA-activating protease, the newly produced virus could not infect other cells to sustain replication. As such, virus titers in the absence of trypsin decreased slightly after 24 h.p.i., whereas titers in the presence of trypsin continued to increase. Immunocytochemical staining of these cells demonstrated intact monolayers and few infected cells in the absence of trypsin, but extensive cytopathic effect and the vast majority of cells were infected in the presence of trypsin (Figure 7C).

To determine whether human- versus swine-origin of influenza viruses impacts virus replication in SAE cultures, we examined the replication of human- and swine-origin viruses, with and without exogenous TPCK-treated trypsin. The four human- and swine-origin viruses examined replicated to high titers in the apical compartment in the absence of exogenous trypsin, and addition of trypsin did not impact virus titers (Figure 8), again demonstrating that the SAE cultures likely express an HA-activating protease.

Discussion

Differentiated SAE cultures constitute a more authentic model system for the study of influenza viruses and other respiratory pathogens of pigs, compared to primary cells submerged in cell culture media and lacking ALI morphology. Herein we describe the optimization of EGF and RA concentrations for the growth of differentiated SAE cell cultures. Influenza viruses productively infected and replicated in differentiated SAE cultures, and differences in virus infection characteristics seen *in vivo* and in media-submerged SRECs were recapitulated in SAE cultures. In addition, our results show that while the HA gene of rgMN and rgONT is most important for virus infectivity levels in submerged SRECs,[12] the NA gene appears to be the dominant factor that controls virus replication and/or release of virus into the apical compartment of differentiated SAE cultures.

The finding that EGF controls the proliferation of cells in differentiated SAE cultures is consistent with previous studies of cultures of human airway epithelial cells, wherein either keratinocyte growth factor or EGF induced cellular proliferation.[38,39] These studies, coupled with results described here, demonstrate that these growth factors induce both swine and human airway epithelial cells to proliferate, but that species differences exist with respect to

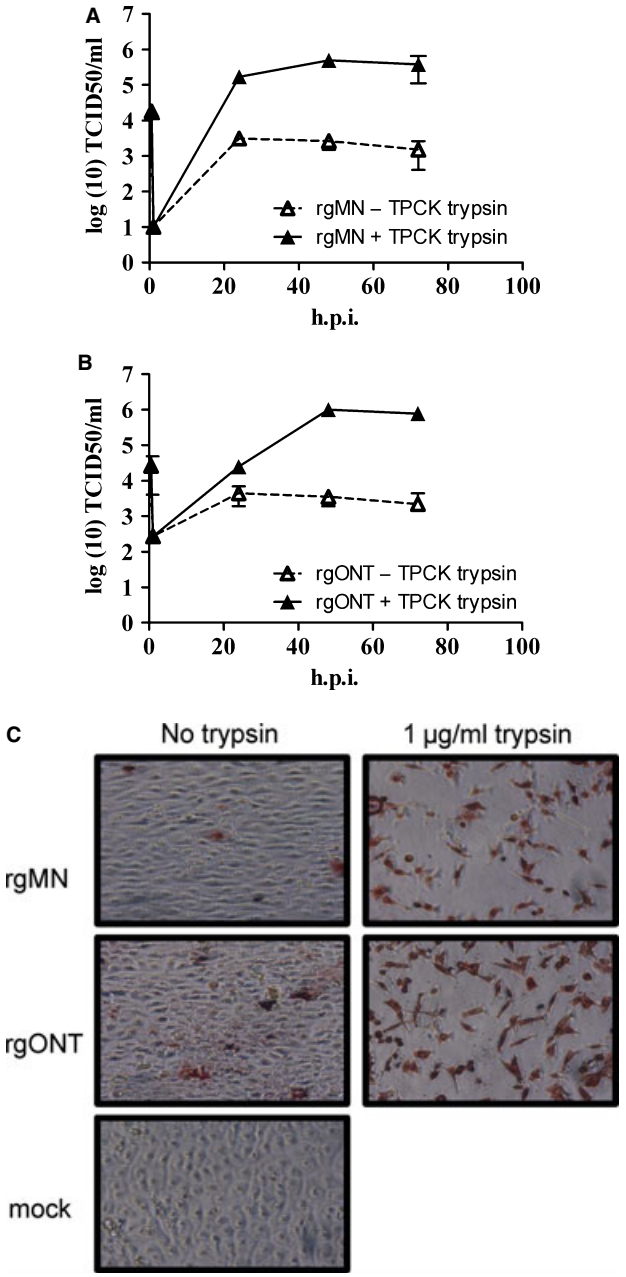

Figure 7. Replication (virus titers in cell culture media) of rgMN and rgONT in submerged monolayers of SRECs in the presence and in the absence of TPCK-treated trypsin. Confluent monolayers were overlayed with virus (0.01 $TCID_{50}$/cell) for 1 hour, after which the virus inoculum was washed and replaced with new BEGM media in the presence or in the absence of 1 µg/ml TPCK-treated trypsin. At various timepoints, the media were removed and frozen at −80°C for future virus titration. Virus was titrated on MDCK cells using 1:7 serial dilutions, as in Figures 5 and 6. (A–B) Virus titers at each timepoint. Shown are the mean ± SEM of two independent experiments. (C) Immunocytochemical staining of SRECs. At 3 d.p.i. cells were fixed and stained as described earlier. Representative micrographs are pictured. Magnification, 30×.

the exact concentrations necessary for optimal cell culture proliferation and differentiation. Retinoic acid has also been reported previously to affect both ciliogenesis and mucin secretion in human airway epithelial cultures.[22,40,41] Taken together, the identification of EGF and RA as important factors in optimizing SAE culture growth and differentiation may also inform cell culture studies of other species.

Ciliated cells are an integral part of the 'mucociliary escalator' that sweeps unwanted particles out of the respiratory system and helps to keep the lower respiratory tract virtually sterile.[42,43] The SAE cultures described here contain many ciliated cells, consistent with the swine respiratory epithelium *in vivo* (Figure 3B). In addition, the expression of α2-3- and α2-6- linked sialic acids on SAE cultures is similar to that in the swine lower trachea (Figure 3B). These patterns also parallel lectin binding studies reported in swine sections and swine explant cultures,[44–46] indicating that α2-3- and α2-6- linked sialic acid expression in SAE cultures faithfully mimics that in swine trachea. Thus, the comparable degree of ciliation and the similarity of sialic acid expression between SAE cultures and swine lower trachea make SAE cultures valuable as an *in vitro* surrogate to study swine respiratory epithelial cells.

We found that washing the apical surface of SAE cultures greatly increased the number of cells infected by influenza viruses. This effect has been recognized by others with respect to differentiated human respiratory cell cultures, and infection inhibition is believed to be due to mucins, a heterogeneous group of high molecular weight glycosylated molecules that are either apically secreted or cell-associated on respiratory epithelial cells.[29,47,48] Human influenza virus preference for use of α2-6- over α2-3-linked sialic acids as receptors for cell infection may be an evolutionary adaptation to avoid mucin inactivation; mucins contain mainly α2-3-linked sialic acids, whereas human upper airway cells predominantly express α2-6-linked sialic acids.[49–51] Swine cells express both α2-3- and α2-6-linked sialic acids,[12,52] but swine influenza viruses also preferentially bind [53] and utilize [13,14] α2-6-linked sialic acids for infection, suggesting that a similar mechanism of inhibitory α2-3-linked sialic acids and functional α2-6-linked sialic acids may occur in the swine respiratory tract.

In the absence of TPCK-treated trypsin, the viruses examined here replicated in SAE cultures (Figure 6), but not in submerged SREC monolayers (Figure 7). Thus, the process of differentiation from submerged SRECs to differentiated SAE cultures likely leads to the expression of an HA-activating protease. The expression of such a protease in SAE cultures is not unexpected, as differentiated cultures of human airway epithelia have also been shown to express such proteases.[54] In fact, in concordance with the virus replication results presented here, Chan *et al.*[55] demonstrated

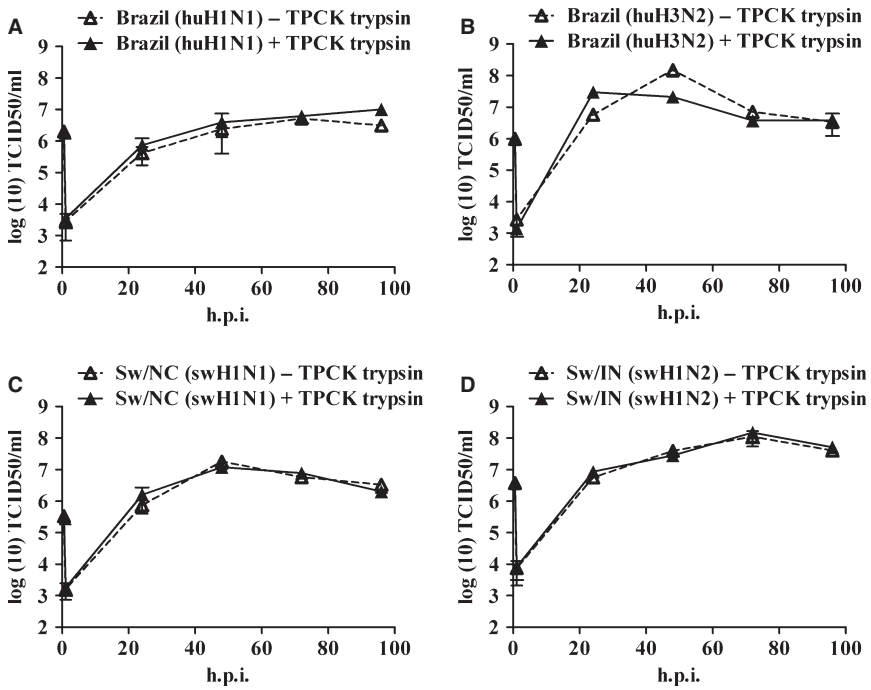

Figure 8. Replication (virus titers in the apical compartment) of various human- and swine-lineage influenza viruses in SAE cultures in the presence and in the absence of TPCK-treated trypsin. Differentiated cultures were infected and virus titers were determined as in Figures 5 and 6. Shown are the mean ± SEM of three independent experiments.

that differentiated human respiratory cells express higher levels of an HA-activating protease than non-differentiated cells.

Others have described differentiated swine airway cell cultures grown at ALI.[56–59] The present data complement these reports by detailing the effects of EGF and RA, as well as defining the expression of sialic acids known to be important in influenza virus infection. Lastly, unpassaged cells were used for many of the previous reports.[56–58] In contrast, the culture systems described here and by Lam *et al.*[59] allow for cell expansion before differentiation, which improves the reproducibility of data because of the ability to conduct more experiments from an individual donor pig.

The differentiated nature of SAE cultures will allow for the comparison of swine cell tropism of influenza viruses isolated from various animal hosts. Previous studies have examined influenza virus tropism in differentiated human respiratory cell cultures.[15,17–19,26] As pigs can be infected with human-, swine-, and avian-lineage influenza A viruses,[60–62] it is important to accurately determine the cell tropism of these viruses in swine cells. The SAE cultures may also prove useful in the study of other swine respiratory diseases, such as those caused by porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*, and may also be of use outside the infectious disease field. For instance, differentiated human cultures have long been utilized in cystic fibrosis research,[63] and with the advent of transgenic pigs carrying a mutated cystic fibrosis transmembrane conductance regulator gene,[64] pigs are poised to be a potentially crucial model animal in this field in the future. To this end, SAE cultures may be important for *in vitro* studies to determine gene transfer efficiency by viral vectors.[30,65,66]

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