Virology 528 (2019) 152–163

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Development and characterization of swine primary respiratory epithelial cells and their susceptibility to infection by four influenza virus types

Chithra C. Sreenivasan[a], Milton Thomas[a], Linto Antony[a], Tristen Wormstadt[a], Michael B. Hildreth[a], Dan Wang[a,c], Ben Hause[d], David H. Francis[b], Feng Li[a,c], Radhey S. Kaushik[a,*]

[a] Department of Biology and Microbiology, South Dakota State University, Brookings, SD 57007, USA
[b] Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, SD 57007, USA
[c] BioSNTR, Brookings, SD 57007, USA
[d] Cambridge Technologies, Oxford Street, Worthington, MN 56187, USA

ARTICLE INFO

Keywords:
Swine
Primary cells
Respiratory epithelial cells
Influenza virus
Replication kinetics

ABSTRACT

Influenza viruses are a group of respiratory pathogens that have evolved into four different types: A, B, C, and D. A common feature is that all four types are capable of replicating and transmitting among pigs. Here, we describe the development of isogenous cell culture system from the swine respiratory tract to study influenza viruses. Phenotypic characterization of swine primary nasal turbinate, trachea and lung cells revealed high expression of cytokeratin and demonstrated tissue site dependent expression of tight junction proteins. Furthermore, lectin binding assay on these cells demonstrated higher levels of Sia2–6Gal than Sia2–3Gal receptors and supported the replication of influenza A, B, C, and D viruses to appreciable levels at both 33 and 37 °C, but replication competence was dependent on virus type or temperature used. Overall, these swine primary respiratory cells showed epithelial phenotype, which is suitable for studying the comparative biology and pathobiology of influenza viruses.

1. Introduction

Influenza viruses belong to the Orthomyxoviridae family and are classified into 4 types: A, B, C, and D. All the four virus types share swine as a common host. The rapid evolutionary dynamics of the influenza viruses demand close epidemiological surveillance, which in turn depends on timely detection, isolation, and characterization of the viruses. Influenza virus isolation and propagation has been facilitated *via* embryonated chicken eggs, cell cultures, and occasionally in lab animals. Primary epithelial cells developed from chicken kidney, pig lung, human adenoids, and fetal rat brain have been employed to study the influenza pathogenesis and virulence in the past (Chan et al., 2005; Endo et al., 1996; Katz and Webster, 1992; Matrosovich et al., 2007; Seo et al., 2001; Takahashi et al., 1997; Wu et al., 2016). Nevertheless, influenza research has been extensively supported by continuous cell lines such as Madin-Darby canine kidney cells (MDCK), African green monkey kidney epithelial cells (Vero), and baby hamster kidney (BHK) cells, all of which are derived from tissues that are not commonly infected by influenza under natural conditions (Frank et al., 1979; Govorkova et al., 1996; Lau and Scholtissek, 1995). There are only very few existing cell lines or primary cell cultures that are derived from swine respiratory organs although all influenza types could cause respiratory infections in pigs (Khatri et al., 2012; Seo et al., 2001; Wu et al., 2016).

Primary cells vary in their nature of the growth, nutritional characteristics, and metabolic profile compared to transformed cells (Chang, 1961). Morphological and genetic studies in amnion cells showed that the transformed cells exhibited malignant properties, while the primary cells showed non-malignant characteristics (Nakanishi et al., 1959; Petursson and Fogh, 1963). Primary cells mimic the *in-vivo* physiological properties and are better models to study the mechanistic details of the normal or diseased conditions of the body. However, primary cells have a limited growth *in-vitro* and show considerable mitotic activity only during the first 2–4 weeks (Petursson and Fogh, 1963). Species-specific primary cell cultures have been developed and used for studying host-pathogen interactions (Connor and Marti, 1964; Easton, 1963; Greig et al., 1967; Noyes, 1965; Rehacek and Kozuch, 1964). Furthermore, primary epithelial cell cultures of swine-origin have been used for normal physiological and pathological studies of several infectious diseases (Dean et al., 2014; Huygelen and Peetermans, 1967;

* Corresponding author.
E-mail address: Radhey.Kaushik@sdstate.edu (R.S. Kaushik).

https://doi.org/10.1016/j.virol.2018.12.016
Received 16 August 2018; Received in revised form 17 December 2018; Accepted 21 December 2018
Available online 05 January 2019

Imura et al., 1983; Kasza et al., 1960; McClurkin, 1965; Semenov et al., 1961).

Epithelial surfaces of the mammalian body are equipped with highly sophisticated proteins and lipid machinery that play a crucial role in maintaining the homeostasis and cell polarity. Among these, tight junction proteins are macromolecular complexes consisting of several membrane proteins, which are important for the cell-cell interactions, cell-extracellular matrix interactions, and for transcellular and paracellular transport and permeability. Tight junctions and cell polarity play an important role in the influenza virus morphogenesis and budding (Nayak et al., 2009; Rodriguez-Boulan et al., 1983; Torres-Flores and Arias, 2015). The budding site of the influenza virus is at the apical domain of the plasma membrane of the polarized epithelial cells (Mora et al., 2002; Nayak et al., 2009). The two major spike proteins of the influenza viral envelope, hemagglutinin (HA) and neuraminidase (NA), carry apical sorting signals in their transmembrane or cytoplasmic domains, which direct these proteins to use exocytic pathways and lipid rafts for transport to the cell surface and apical sorting. Hence, an epithelial cell culture system with the inherent polarization properties of the epithelial surfaces *in-vivo* can better reflect replication, transmission, and pathogenic properties of influenza viruses in animals.

Evolution strategies and host adaptation properties of influenza virus enable it to cross species barriers from their reservoir hosts, and some of these multiple stable host switch events culminated in zoonotic infections (Garten et al., 2009; Taubenberger and Kash, 2010). With the expanding influenza viral ecology over the past years, such adaptation in humans leads to continued transmission, thereby causing the emergence of novel viruses. Further, pigs, when co-infected with various influenza A subtypes, act as mixing vessels and give rise to novel viruses with high transmissibility to humans. The swine respiratory tract possesses both Sia2–6Gal and Sia2–3Gal receptors that can bind to human and avian influenza A viruses respectively which facilitates gene reassortment between multiple influenza subtypes.

Human and swine respiratory epithelial cells have been utilized for studying the virulence, and receptor binding specificities of the influenza A viruses from different host origin, but not for other types of influenza viruses (Bateman et al., 2008, 2012, 2010; Busch et al., 2008; Kogure et al., 2006; Sreenivasan et al., 2018; Thomas et al., 2018). Recent studies have demonstrated the susceptibility of pigs to influenza B and C viruses that are primarily human pathogens (Guo et al., 1983; Kimura et al., 1997; Ran et al., 2015). Further, influenza D has been originally isolated from swine and was found to have considerable seroprevalence in the swine population across the United States (Collin et al., 2015; Hause et al., 2013). Interestingly, pigs can be infected by all four types of influenza viruses (A, B, C, and D) and are capable of transmission. Therefore, a primary cell culture system derived from the upper and lower compartments of the swine respiratory tract of the same animal would be helpful to study the influenza viral pathobiology and to dissect the specific cellular and biological factors that promote or restrict the transmission of influenza viruses originated from different hosts.

In this study, we report the development and characterization of an isogenous primary epithelial cell culture system derived from nasal turbinate, trachea and lungs of a day-old influenza-free gnotobiotic piglet, to determine its phenotype and polarization properties. We also investigated the suitability of these primary cells to support influenza viral replication. First, we analyzed the presence of Sia2–6Gal and Sia2–3Gal receptors on these cells. Then we conducted replication kinetics of eight swine and human influenza viruses belonging to all four types of influenza, at two different temperatures 33 °C and 37 °C, which represent the temperatures of upper and lower respiratory tract respectively. The differential replication kinetics of four types of influenza viruses in these three types of primary cells were compared to those in MDCK cells at both temperatures. The results of our study demonstrated that these swine primary cells of nasal turbinate, tracheal and lung origin are predominantly of epithelial phenotype and can undergo polarization. These cells expressed Sia2–6Gal more than Sia2–3Gal receptors and differentially supported the replication of all four types of influenza viruses.

2. Materials & methods

2.1. Isolation and establishment of swine primary respiratory epithelial cells

Tissues for the isolation of cells were collected from a day old gnotobiotic piglet according to the protocol approved by the South Dakota State University's Institutional Animal Care and Use Committee (IACUC). Tissues such as dorsal and ventral nasal turbinates/conchae, mid-trachea, and lung were washed with 1x PBS and cut into small pieces of 1 mm^3 and incubated with 800U of collagenase enzyme at 37 °C for 1.5 h. After collagenase digestion, the cells were strained using 70 μm cell strainers and centrifuged at 500 g for 5 min. The cell pellet was washed two times with 1X PBS and then seeded on the collagenase coated flask. Cells were incubated at 37 °C, 5% CO_2 and maintained using Dulbecco's Modified Eagle Medium-F12 (DMEM/F-12) medium (Invitrogen, Grand Island, NY) supplemented with 5% FCS (Atlanta Biologicals, GA, USA), 1% insulin-transferrin-selenium (ITS) supplement (Invitrogen, Grand Island, NY), 5 ng/ml mouse epidermal growth factor (EGF) (Invitrogen, Grand Island, NY), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Koh et al., 2008; Thomas et al., 2018). After 24 h, non-adherent cells were transferred to another flask and cultured under the same conditions. Fibroblasts were removed by treating the cells with 0.03% Trypsin every three days.

2.2. Phenotyping of primary respiratory epithelial cells by immunohistochemistry (IHC)

The primary respiratory epithelial cells were stained with antibodies against various epithelial, fibroblast and smooth muscle markers using the protocol as described previously (Kaushik et al., 2008; Thomas et al., 2018). Cytospins prepared from a cell suspension of approximately 1×10^5, were air-dried, fixed in acetone for 7 min and stored at 4 °C until staining (Cytospin 3; Thermo Shandon Inc, Cheshire, UK). For staining, slides were equilibrated at room temperature and then rehydrated in PBS. Blocking for non-specific protein binding and endogenous peroxidase activity was performed using PBS containing 1% goat serum and PBS containing 0.3% hydrogen peroxide and 0.01% sodium azide respectively. The presence of cytokeratin, vimentin, α-smooth muscle actin (ASMA) and desmin proteins was detected using monoclonal antibodies such as anti-cytokeratin (C6909), anti-vimentin (V5255), anti-ASMA (A2547) and anti-desmin (D1033). Monoclonal antibodies such as M9144 (Sigma), M9269 (Sigma) and M5170 (Sigma) were used as isotype-matched controls for IgG2a, IgG1, and IgM respectively. Cytospins were incubated with primary antibodies (Sigma) for 1 h. After PBS wash, the slides were then incubated with isotype-specific, biotinylated goat anti-mouse IgG2a, IgG1 or IgM antisera (1:2000 dilution; Caltag Laboratories) for 30 min, followed by HRP–streptavidin solution for 30 min. Ready-to-use (RTU) diaminobenzene (DAB) substrate (Vector Laboratories) was then added and cytospins were counterstained with hematoxylin and examined under the light microscope. Images were taken at 20X magnification using an Olympus AX70 microscope.

2.3. Transepithelial electric resistance (TEER) and indirect immunofluorescence assay (IFA)

Primary respiratory epithelial cells from passages 3–6 were differentiated and polarized on collagen-coated permeable supports in the cell culture media (Johnson et al., 2010). About 1×10^6 cells were seeded on a 24 mm diameter (growth area 4.7 cm^2) transwell permeable filter (24 mm x 0.4 μm pore size). About 2 ml of the medium was added to the upper and lower chamber of the inserts in a six-well plate.

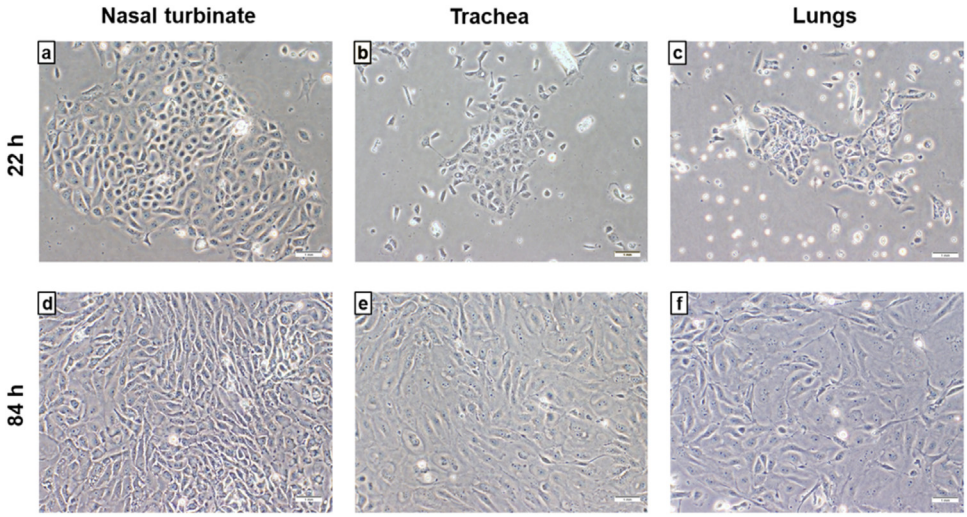

Fig. 1. Morphology of the swine primary respiratory epithelial cells. Swine nasal turbinate, trachea and lung tissues were digested with collagenase and the epithelial cell suspension was seeded on collagen-coated flasks. At P0, the primary cultures demonstrated both homogeneous and heterogeneous populations of epithelial and fibroblast-like cells. (a-c) Small clusters of epithelial-like cells with 'cobblestone morphology' characteristic of epithelial cells can be seen at 22 h for nasal turbinate, trachea, and lungs respectively. (d-f) A confluent cell monolayer was observed at 84 h for all the three types of cells with a mixed population of epithelial-like cells and fibroblasts. Scale bars, 50 μm.

The media was changed every other day and the polarization was measured by detecting the trans-epithelial electric resistance (TEER) by Evom voltmeter (World Precision Instruments, Sarasota, FL) in Ωs every 24 h. When the TEER stabilized between 2000 and 3000 Ωs, tight junction proteins were stained by IFA (Johnson et al., 2010). For staining, transwell filters were washed and fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS. The filters were blocked using 5% normal goat serum and the cells were incubated with rabbit polyclonal antibodies against claudin-1, claudin-3, ZO-1, and occludin (5 μg/ml; Zymed), followed by secondary detection with goat anti-rabbit IgG-Alexa 488 (10 μg/ml) and counterstained with propidium iodide. Normal rabbit IgG (5 μg/ml) was used as a negative control. Images were visualized using Olympus Fluoview FV1200 Laser Scanning Confocal Microscope System (Life Science Solutions, Waltham, MA, USA).

2.4. Fluorescence-activated cell sorting (FACS)

To determine the type of the lung alveolar epithelial cells, these swine primary respiratory epithelial cells were stained for pneumocyte specific markers. Approximately, 2.5×10^5 cells of swine primary respiratory epithelial cells were fixed and permeabilized using BD cytofix/cytoperm (BD Biosciences, San Jose, CA, USA). After blocking the cells with 1% goat serum in BD cytoperm solution, cells were incubated with aquaporin-5 (AQP5) antibody (D-7) (cat #sc-514022, Santa Cruz Biotechnology, TX, USA), a mouse monoclonal IgG1antibody at 5 μg/ml for staining type I pneumocytes and surfactant protein-C (SP-C) mouse monoclonal IgG2b antibody (H-8) at 2.5 μg/ml (Cat #sc-518029) for staining type II pneumocytes for 1 h, followed by goat anti-mouse Alexa Fluor 488 (IgG) antibody at 10 μg/ml for 30 min. Samples were analyzed using FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA). Cell passages from 6 to 12 were used for this experiment. A549 cells, derived from a human lung alveolar adenocarcinoma, were used as positive control.

For sialic acid specific staining, 5×10^5 cells of swine primary respiratory epithelial cells were incubated with biotinylated MAL-I, and II specific for Sia2–3Gal and SNA (Vector Laboratories, Burlingame, CA, USA) specific for Sia2–6Gal (final concentration 10 μg/ml) for 1 h. The inhibitors used for MAL II was n-acetyl neuraminic acid (NANA) at 200 μM concentration, while lactose was used at 200 μM for both SNA and MAL I (George et al., 2007; Sun et al., 2012; Thomas et al., 2018). After wash, the cells were stained with Streptavidin-FITC (1:200 dilution) for 30 min. Cells stained with only Streptavidin-FITC served as negative control cells. Samples were analyzed using FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA). Cell passages from 8 to 12 were used for this experiment.

2.5. Viral replication kinetics

Approximately 2×10^5 cells/well were seeded on collagen-coated 24 well plates and infected with 1) A/swine/Minnesota/2073/2008 (MN08) 2) A/swine/Iowa/0855/2007 (IA07) 3) A/California/04/2009 (CA04Pdm09) at 0.01 MOI; 4) B/Brisbane/60/2008 (BR08), 5) B/Florida/04/06 (FL06) at 0.1 MOI; 6) C/Johannesburg/1/1966 (C/JHB), 7) D/Swine/Oklahoma/1334/2011, 8) D/bovine/Oklahoma/660/2013 (D660) at 1 MOI. Cells from passages 5 to13 were used for the replication kinetics study. Virus growth medium for primary cells included Opti-MEM (Thermofisher Scientific, Waltham, MA), 0.001% (0.01 μg/ml) TPCK Trypsin (Pierce, Thermofisher Scientific, Waltham, MA), and 1% antibiotic-antimycotic (Thermofisher Scientific, Waltham, MA). MDCK cells were infected at the same MOI, with DMEM, 0.1% (1 μg/ml) TPCK Trypsin (Pierce, Thermofisher Scientific, Waltham, MA), and 1% antibiotic-antimycotic (Thermofisher Scientific, Waltham, MA). Samples were collected at 24 h intervals until 120 h.

Approximately, 1×10^4 MDCK cells were seeded on flat bottom 96 well plate (Greiner bio-one, NC, USA) and incubated overnight. Serial ten-fold dilutions of the samples were prepared in virus growth media and were inoculated on pre-seeded MDCK cell culture plates. The inoculated plates were incubated for 5 days at respective temperatures. The infectivity of the virus was determined by doing hemagglutination assay using 1% turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA, USA). Titers were calculated using Reed and Muench formula (Reed and Muench, 1938).

3. Results

3.1. Isolation, morphology, and growth of swine primary respiratory epithelial cells

Isolation procedure of the primary swine respiratory epithelial cells derived from nasal turbinate, trachea, and lungs is described in detail in the Materials & Methods section. Swine primary epithelial cells from nasal turbinate, trachea, and lungs formed small epithelial-like clusters by 18–24 h post-isolation on tissue culture flasks coated with type I collagen. The morphology of the cell clusters observed under the phase contrast microscope was shown (Fig. 1a-c). The adhered cells appeared heterogeneous, however, 80% of the cells attached were polygonal in shape having uniform dimensions with a cobblestone appearance. The adherent cells grown on T-25 flasks reached 80–90% confluence by 84 h (Fig. 1d-f). Swine primary respiratory cells can be cultured on the

normal tissue culture flasks but often exhibited a lag time in reaching confluence compared to the collagen I coated flask. Ciliated cells were seen in the initial cultures from nasal turbinate and trachea, which gradually disappeared during the subculture. Around 5–10% fibroblast cells that appeared as spindle-shaped cells were identified in the initial passages. The fibroblasts were removed by treating the cell monolayer with 0.03% trypsin for 3 min, every 48 h followed by PBS wash and the addition of fresh media. Primary respiratory epithelial cells were monitored daily until the cells reached confluence. These cells could be sub-cultured in normal or collagen-coated tissue culture flasks. The sub-cultured cells attached to the tissue culture surface in 24–48 h. Normally, the subcultures reached confluence in 5–7 days in a T-75 flask. At later passages, some cells appeared irregularly sized, indicative of cell differentiation.

3.2. Swine primary respiratory epithelial cells could be subcultured and are predominantly of the epithelial phenotype

To determine the phenotype, the sub-cultured cells (passage 2) from nasal turbinate, trachea and lung cells were stained with monoclonal antibodies targeting marker proteins such as cytokeratin (epithelial), vimentin (fibroblasts), desmin (smooth and striated muscles), and α-smooth muscle actin (ASMA, smooth muscle), along with their isotype controls. About 95% of the nasal turbinate cells expressed cytokeratin indicative of their epithelial phenotype (Fig. 2a). Less than 5% of the nasal turbinate cells expressed vimentin indicative of fibroblasts. Similarly, 90% of the tracheal and lung cells expressed cytokeratin (Fig. 2b, c), while only 10% of the cells expressed vimentin in both cell types. Desmin and α-smooth muscle actin were not expressed in all three types of cells from passage 2.

All three types of cells were serially passaged to determine the subculturing capacity, and immunocytochemical staining was done on alternate passages to study phenotypic stability (Fig. 3). Nasal turbinate cells were able to grow up to 18 passages, while trachea and lung cells could grow until 20–22 passages. To determine the phenotypic stability, immunocytochemical staining was performed on all three types of the cells from passage 17. Swine primary respiratory epithelial cells from nasal turbinate, trachea and lungs expressed epithelial marker cytokeratin, but the level of expression decreased during subculture. Nasal turbinate cells expressed cytokeratin and desmin in cells from passage 17, however, there was a low abundance of cytokeratin and high abundance of desmin, indicating that cell differentiation has occurred over time (Fig. 3a, d, g, j). Tracheal cells expressed mainly cytokeratin, while few cells expressed ASMA (Fig. 3b, e, h, k). Cytokeratin was the major protein expressed by the lung cells; however, a low expression of desmin and ASMA could be seen (Fig. 3c, f, i, l). Isotype controls were also included to determine the specificity of the antibodies used in the staining and were stained negative.

3.3. Swine primary respiratory epithelial cells could polarize, express tight junction proteins and produce TEER

Nasal turbinate, trachea and lung primary epithelial cells from passages 4 to 6 were grown on transwell inserts, expressed the tight junction proteins and polarized to form the apical and basolateral surfaces. The morphology of the cells under phase contrast microscope at day 15, is shown (Fig. 4a-c). Primary tracheal epithelial cells polarized faster to produce the maximum trans-epithelial electric resistance (TEER). Tracheal epithelial cells reached a maximum TEER of 2600 Ωs by 12 days, and then decreased to 2500 Ωs on day13. Lung cells showed a TEER with a maximum of 2240 Ωs in 16 days and nasal turbinate cells polarized to reach the maximum TEER of 2030 Ωs by day 18 (Fig. 4d). Swine intestinal epithelial cells (IPEC-1), another established cell line derived from the small intestine of a neonatal piglet was used as a positive control (Nossol et al., 2011), along with a negative control (no cells on the transwell insert).

We tested the presence of the tight junction proteins by staining the polarized transwell filters by indirect immunofluorescence assay (IFA). All three types of cells were stained for tight junction proteins such as claudin-1, claudin-3, occludin and zona occludens-1, along with the isotype antibody controls. Primary nasal turbinate epithelial cells expressed tight junction proteins such as claudin 1, claudin-3, and occludin distributed mainly on the cell membrane/cell-cell junctions except for claudin-3 which showed some nuclear localization (Fig. 5a, d, g, j). Swine primary tracheal epithelial cells expressed claudin-1, −3, occludin, and zona occludens-1 (ZO-1), where claudin-3 and ZO-1 showed some nuclear localization along with the cell-cell junctions (Fig. 5b, e, h, k). Primary lung alveolar epithelial cells expressed only claudin 1 and claudin 3 (Fig. 5c, f). Interestingly, claudin 3 was localized in the nuclei and cell membrane/junctions in the nasal turbinate and tracheal cells but only seen in the cell membrane/junctions in the case of lung cells (Fig. 5d-f). Isotype controls were also included to check the specificity of the antibodies used for staining and were stained negative.

To further determine the phenotype, undifferentiated swine primary lung alveolar epithelial cells were stained for type I and II pneumocyte specific proteins such as aquaporin-5 and surfactant protein-C (SPC) respectively (Fig. 6). A549 cells, a human lung adenocarcinoma cell line was also used in the experiment as a positive control. Similar to A549 cells, swine primary lung alveolar epithelial cells were predominantly type II pneumocytes. SP-C was expressed in 32.02% and 33.65% in swine primary lung alveolar epithelial cells and A549 cells respectively (Fig. 6g). Aquaporin-5 was expressed in 15.42% and 23.22% of the swine primary lung alveolar epithelial cells and A549 cells respectively (Fig. 6g). Histograms showing the stained negative control cell populations (Cells + Alexa Fluor 488) in swine lung alveolar epithelial cells and A549 were given in Fig. (6a-b); Stained type I and II pneumocyte cell populations for both cells were demonstrated in Fig. 6c, d, e, f. Isotype control was also included to confirm the specificity of the antibody used for the staining.

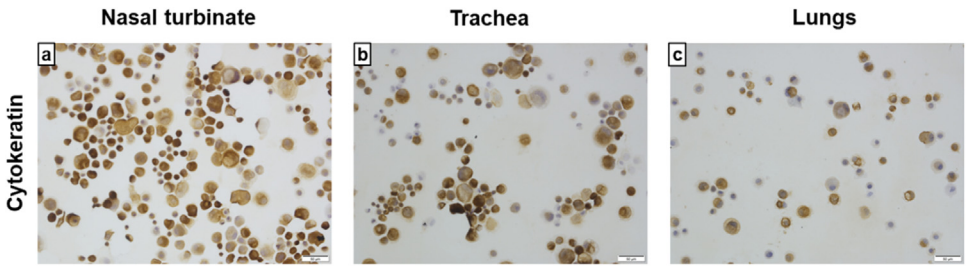

Fig. 2. Immunocytochemical staining of the swine primary respiratory epithelial cells for cytokeratin. To determine the phenotype of the three types of swine primary respiratory epithelial cells, cytospins were prepared from early passage (P2) and stained for cytokeratin specific monoclonal antibody. Brown color cytokeratin positive cells can be seen in all the three types of the cells, indicative of their epithelial phenotype. Swine primary (a) nasal turbinate (b), trachea and (c) lung alveolar epithelial cells expressed cytokeratin, an important cell marker for the epithelial phenotype. The images are representative of three independent experiments. Scale bars, 50 μm.

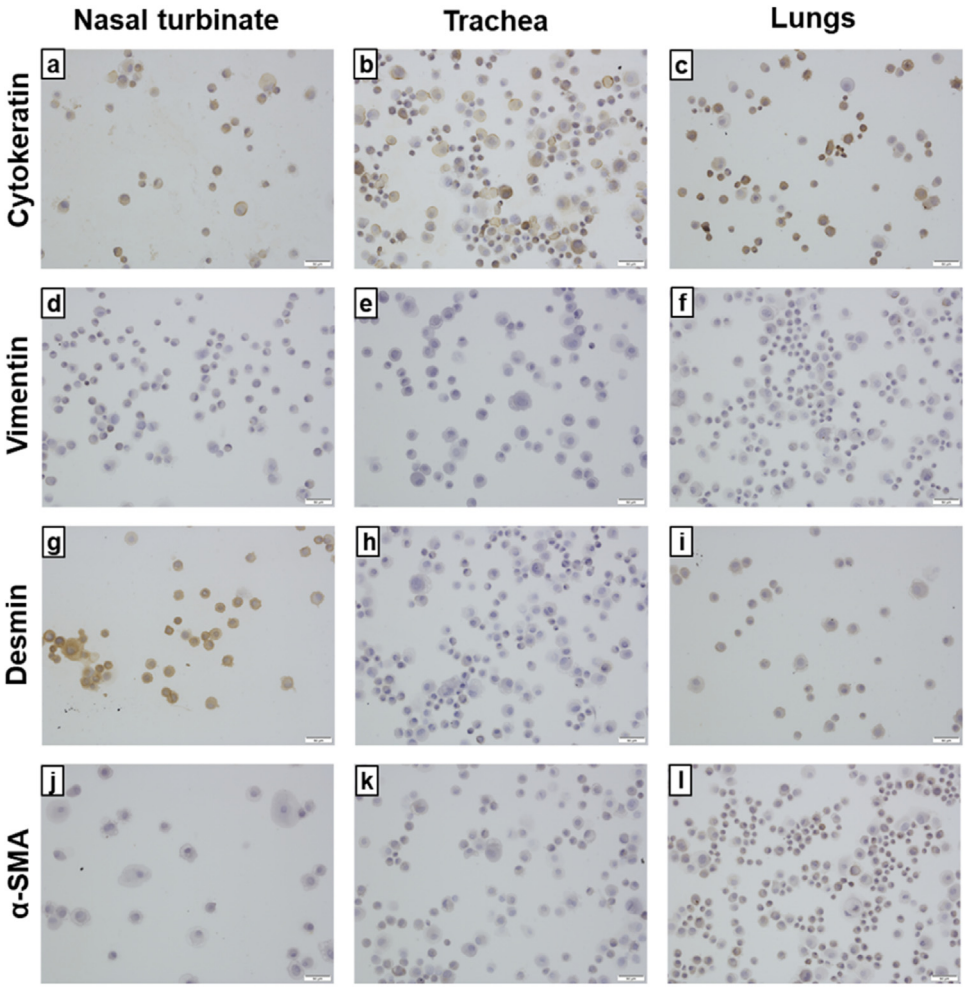

Fig. 3. Immunocytochemical staining of swine primary respiratory epithelial cells for the phenotype stability. Cytospins of swine primary nasal turbinate, trachea and lung alveolar epithelial cells were prepared and stained with phenotypic markers such as cytokeratin, vimentin, α-smooth muscle actin (ASMA), and desmin, along with isotype controls for antibodies to confirm the specificity of the antibodies. Expression of cell markers: (a-c) cytokeratin, (d-f) vimentin, (g-i) desmin, and (j-l) ASMA for the nasal turbinate, trachea and lung cells were shown. Brown color stained cells indicate positive cells. The images are representative of three independent experiments. Scale bars, 50 μm.

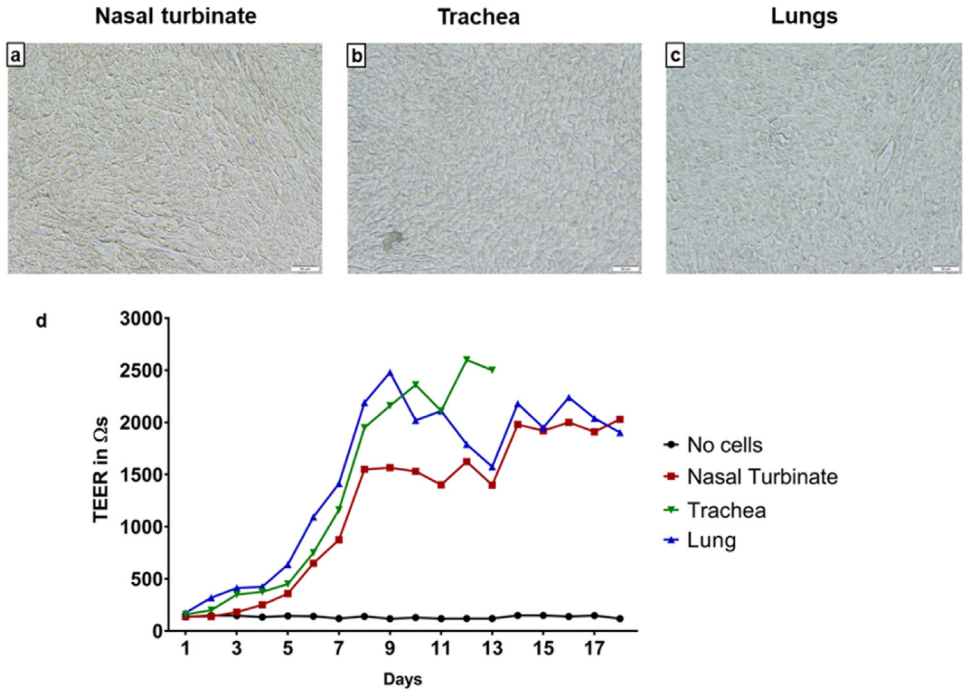

Fig. 4. Swine primary respiratory epithelial cells could undergo polarization. Swine primary nasal turbinate, trachea and lung alveolar epithelial cells were grown on transwell filter inserts and TEER was measured with a volt-ohmmeter (World Precision Instruments) over time. Cellular morphology of the polarized (a) nasal turbinate, (b) trachea and (c) lungs under phase contrast microscope was shown. Scale bars, 50 μm. (d) Primary swine respiratory epithelial cells polarized to develop transepithelial electric resistance (TEER) measured in ohms were plotted against the function of time.

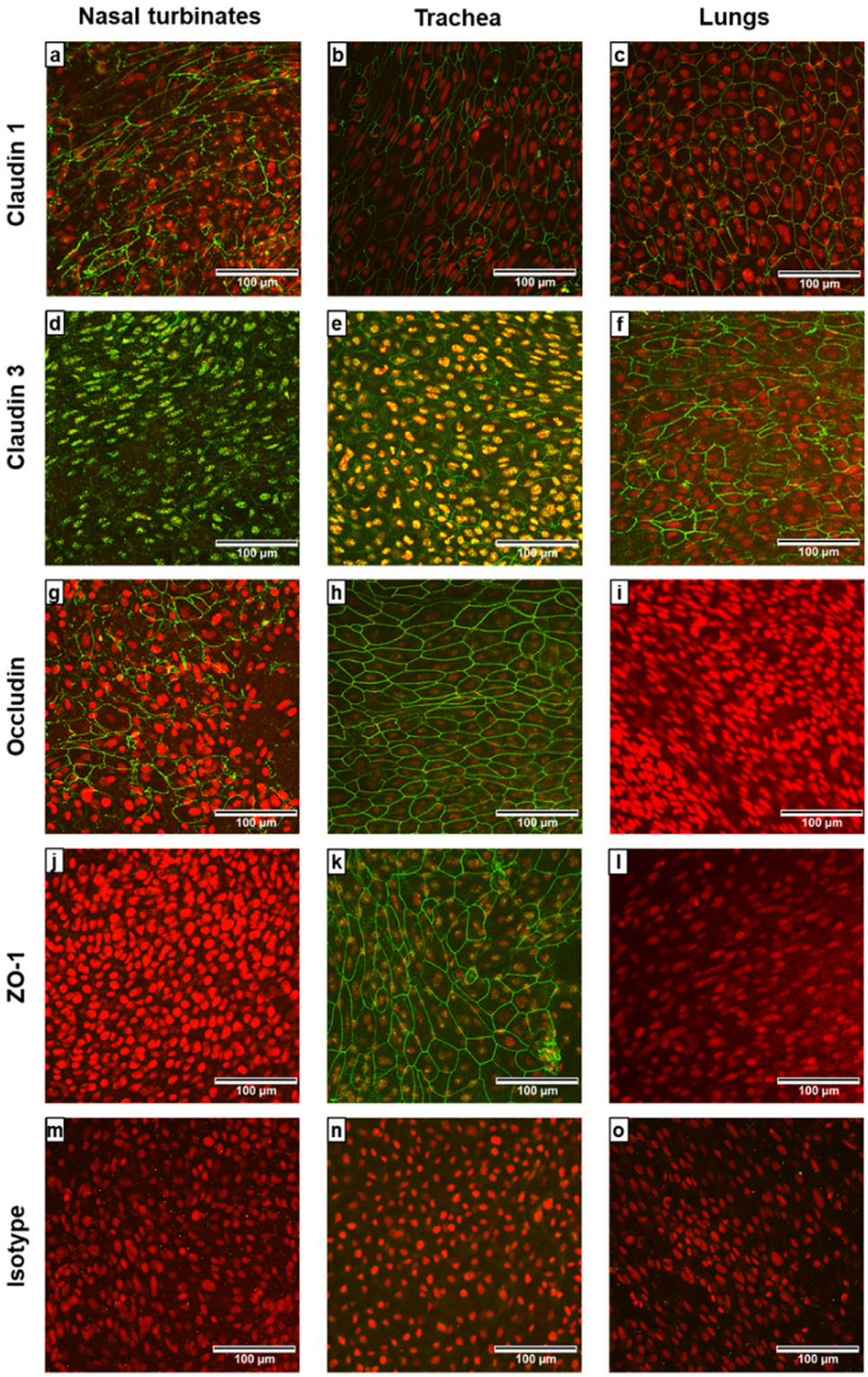

Fig. 5. Tight junction proteins expressed by the swine primary respiratory epithelial cells visualized by confocal microscopy. Swine primary nasal turbinate, trachea and lung alveolar epithelial cells were grown on transwell filter inserts and TEER was measured with a volt-ohmmeter (World Precision Instruments) over time. When the TEER reached a point of no increase, the polarized cells on the filters were stained for tight junction proteins with Alexa Flour-488 tagged antibodies and nucleus counterstained by Propidium iodide (PI). The different tight junction proteins expressed by these cells (a-c) claudin-1; (d-f) claudin-3; (g-i) occludin; (j-l) ZO-1 and (m-o) isotype controls. Representative images were merged Z stack confocal images, scale bars 100 µm.

3.4. Swine primary respiratory epithelial cells expressed more Sia2–6 galactose receptors than Sia2–3 galactose receptors

Biotinylated Sambucus nigra agglutinin (SNA) specific for Sia2–6Gal and Maackia amurensis lectins such as Maackia amurensis leucoagglutinin (MAL-I) and Maackia amurensis hemagglutinin (MAL-II) specific for Sia2–3Gal were used for staining the cells and the receptor specificity was measured by flow cytometry. MAL I and MAL II lectins differentially bind to Sia α-2,3 Galβ1–4 GlcNAc (Nicholls et al., 2007) and Sial α-2,3 Galβ1–3 GalNAc (Brinkman-Van der Linden et al., 2002) glycans respectively. Swine primary respiratory epithelial cells derived from nasal turbinate, trachea, and lungs showed more expression for Sia2–6Gal receptors than for Sia2–3Gal receptors. Sia2–6Gal expression by nasal turbinate, trachea, and lungs was 92.01%, 81.72%, and 86.23% respectively (Fig. 7). MAL I receptors were more expressed than MAL II receptors in tracheal and lung alveolar epithelial cells, while MAL II was more in nasal turbinate cells. Sia2–3Gal receptors specific for MAL I were 31.03%, 58.69%, and 45.66%, in the nasal

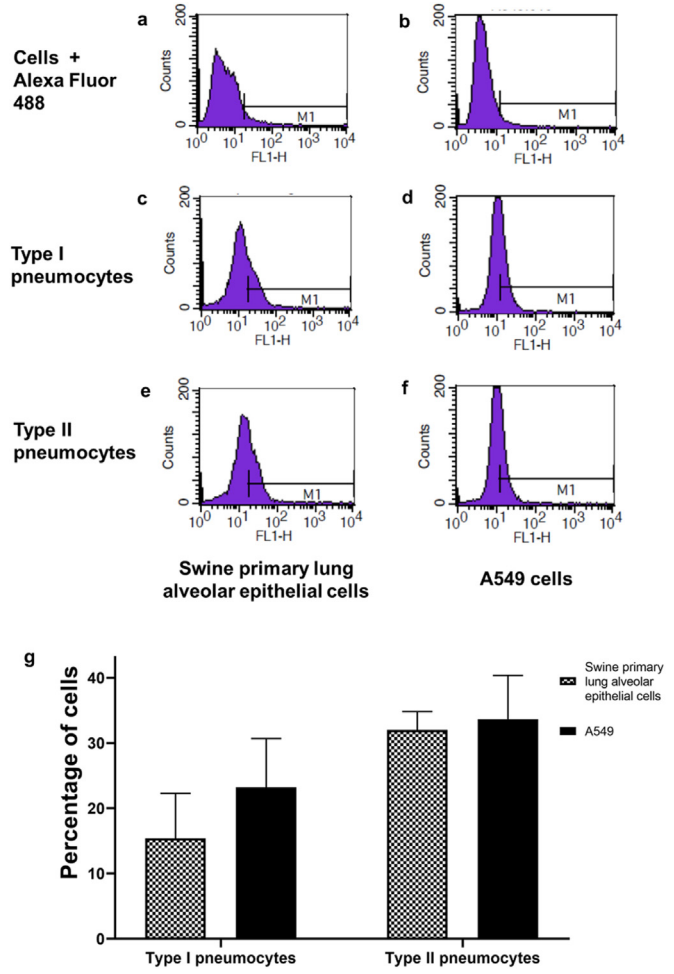

Fig. 6. Swine primary lung alveolar epithelial cells comprise type I and II pneumocytes. Swine primary lung alveolar epithelial cells were stained for type-specific pneumocyte markers, aquaporin-5 (type I) and SP-C (type II) to determine their phenotype. A549 cells, human lung alveolar adenocarcinoma cell line widely used in the influenza studies were also used in the experiment. Histogram plots showing the stained cell populations for (a, b) Alexa Fluor 488 (c, d) type I and (e, f) type II stained for Aquaporin-5 and SP-C respectively were shown. (g) The percentage of the cell populations of type I and type II pneumocytes in swine primary lung alveolar epithelial cells and A549 cells. Data represent two independent experiments ± SE.

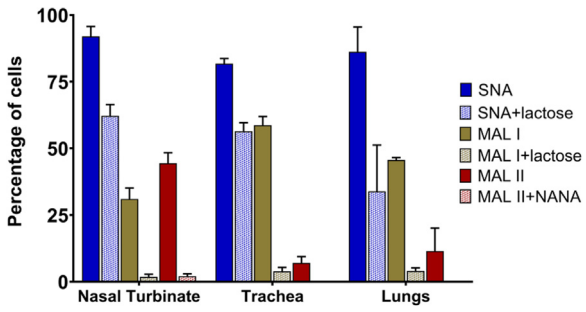

Fig. 7. Expression of Sia2–6Gal and Sia2–3Gal in swine primary respiratory epithelial cells. To determine the expression of sialic acid receptors, swine primary nasal turbinate, trachea, and lung alveolar epithelial cells were stained with biotinylated lectins binding to Sial2–6Gal (SNA) and Sial2–3Gal (MAL I and MAL II) and were stained using streptavidin-FITC and analyzed using a FACS Calibur flow cytometer. Lactose and NANA, the inhibitors for the lectins were used to show the binding specificity. The percentage of cells expressing SNA, MAL I, and MAL II are shown. Data represent three independent experiments ± SE.

turbinate, trachea, and lungs respectively (Fig. 7). The percentages of cells expressing Sia2–3Gal receptors specific for MAL II were 44.48%, 7.10%, and 11.50% respectively. The expression of the Sia2–6Gal and Sia2–3Gal decreased when the cells were treated with lactose (inhibitor for SNA, and MAL I) and n-acetylneuraminic acid (inhibitor for MAL-II), indicating the assay specificity (Fig. 7).

3.5. Swine primary respiratory epithelial cells supported the replication of four types of influenza

Swine primary respiratory epithelial cells derived from nasal turbinate, trachea, and lungs were infected by all four types of influenza viruses affecting animal or human populations at 33 °C and 37 °C. We used swine IAV/MN08 (H1N1), swine IAV/IA07 (H3N2), human IAV/CA04 Pdm09 (H1N1), human IBV/FL06 and IBV/BR08, human ICV/JHB/1966, swine IDV/OK/2011 (swine-IDV) and bovine IDV/660/2013 (bovine-IDV). All the viruses used for the replication kinetics studies were propagated in MDCK cells and Tissue Culture Infectious Dose50 (TCID50) were determined. The $TCID_{50}$ titers of the stock viruses were 7.6, 7.16, 6.84 logs for IAV/MN08/H1N1, IAV/IA07/H3N2, and human IAV/CA04 H1N1Pdm09 viruses. Both the B viruses had 5.4 $\log_{10}$ $TCID_{50}$/ml. The $TCID_{50}$ titer of the stock ICV/JHB/1966 is 5.74 logs while swine IDV/OK/2011 (swine-IDV) and bovine IDV/660/2013 (bovine-IDV) titers were 6.16 and 6.5 logs respectively.

A titration experiment on these respiratory epithelial cells to determine the infective dose demonstrated that MOI of 0.01 caused appreciable titers for IAV, whereas such an infection with non-influenza A viruses such as IBV, ICV, and IDV failed to yield appreciable viral titers and hence prevented us from using 0.01 MOI. So, the MOI used for the replication kinetics study was 0.01 (IAV); 0.1 (IBV); and 1.0 for ICV and IDV. MDCK cells were used as a positive control for each experiment and same MOI of 0.01 (IAV); 0.1 (IBV); and 1.0 for ICV and IDV was used for infection. A concentration of 0.01 μg/ml TPCK trypsin (0.001%) which caused minimum cell toxicity was used on the primary epithelial cells, while 1 μg/ml (0.1%) was used for infecting MDCK cells.

Virus replication kinetics studies demonstrated that all the three influenza A viruses replicated productively in these primary cells at both 33 °C and 37 °C (Fig. 8). Swine IAV/MN08 (H1N1) replicated more efficiently in tracheal and lung primary cells than the nasal turbinate cells. However, there was no significant difference in the titers for swine IAV/MN08 (H1N1) grown on these three different cells. Our data also showed that swine IAV/MN08 (H1N1) replicated more efficiently at 37 °C compared to 33 °C. MDCK cells supported swine IAV/MN08 (H1N1) almost the same, with a slightly higher virus titer at 33 °C (Fig. 8a).

On the contrary, swine IAV/IA07 H3N2 replicated in the nasal turbinate and lung cells better than tracheal cells. Interestingly, the temperature did not substantially affect the virus kinetics. MDCK cells also supported swine IAV/IA07 H3N2 growth at both temperatures (Fig. 8b). Human IAV/CA04 Pdm09 (H1N1) virus replicated to a higher peak at 33 °C than 37 °C, in all the three types of the primary cells and in MDCK cells. Lung primary cells supported human IAV/CA04 Pdm09 (H1N1) virus more than the nasal turbinate and tracheal cells (Fig. 8c).

Human influenza B virus kinetics on the three types of primary cells were also compared to MDCK cells (Fig. 8d,e). We used human IBV/BR08 and IBV/FL06 viruses that belonged to the Victoria and Yamagata lineages respectively. All the three primary cells supported human IBV/FL06 virus at both 33 °C and 37 °C, with a slightly higher peak at 33 °C in both nasal turbinate and tracheal cells than at 37 °C, while lung cells, like MDCK cells, supported equally well at both temperatures. However, tracheal cells at 33 °C showed the highest peak (5.345 logs) that is greater than the peak viral titer in MDCK cells (4.995 logs). Human IBV/BR08 on MDCK cells demonstrated the highest peak at 48 h, with a titer of 4.33 and 4.4 logs at 33 °C and 37 °C respectively while lung cells showed a peak titer of 4.96 logs at 72 h at 33 °C. The peak titers of

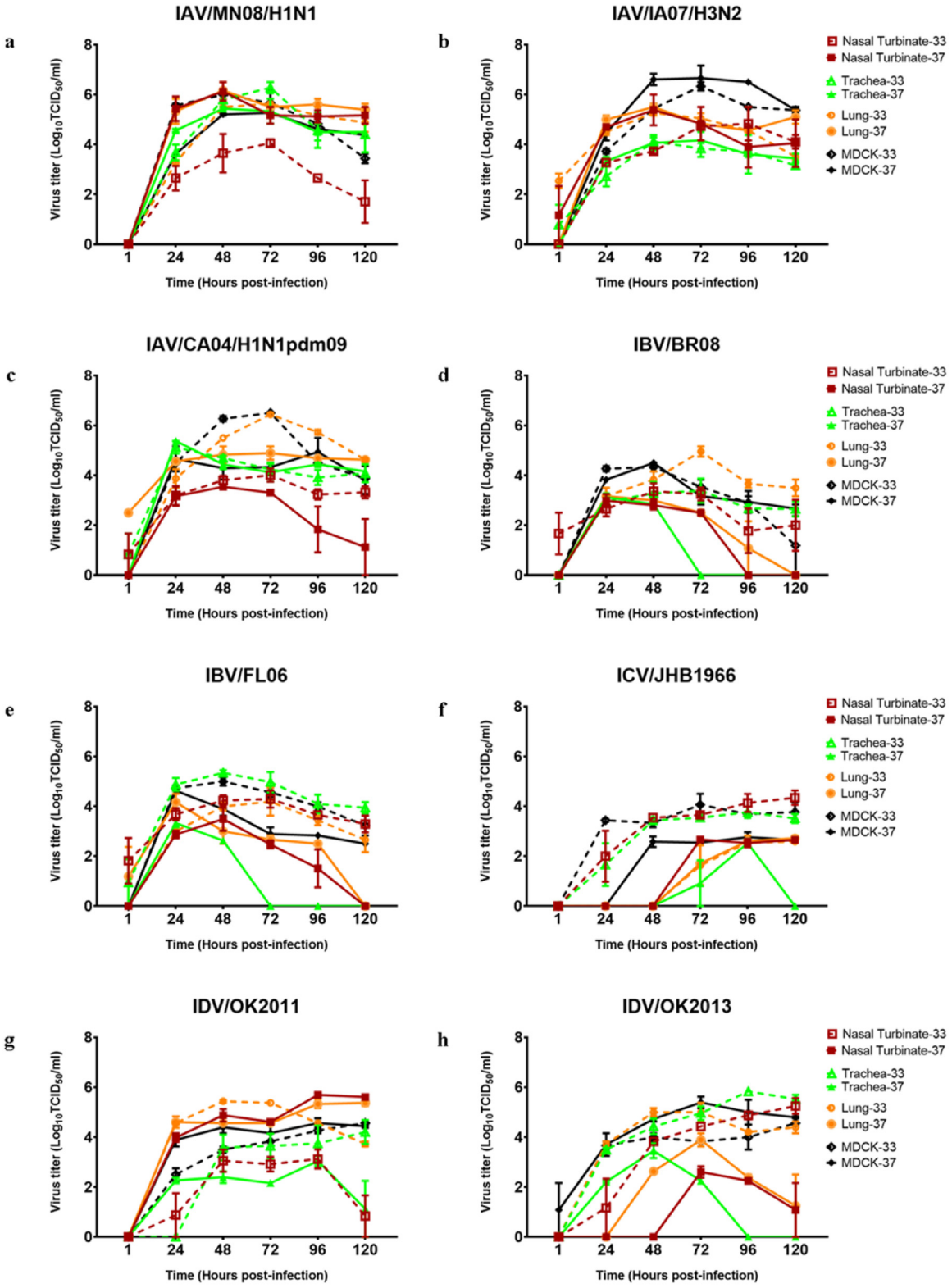

Fig. 8. Comparison of virus growth kinetics of swine primary respiratory epithelial cells to MDCK cells upon infection with IAV, IBV, ICV, and IDV at 33 °C and 37 °C. Approximately 2×10^5 cells of swine primary nasal turbinate, trachea and lung alveolar epithelial cells were infected with all four types of influenza viruses and incubated at 33 °C and 37 °C. Replication kinetics of eight influenza viruses were compared to MDCK cells at both temperatures. Samples were taken at 24 h intervals for 5 days and titrated on MDCK cells. Virus-specific replication kinetics for the swine primary respiratory epithelial cells compared to MDCK cells were demonstrated in (a) IAV/MN08/H1N1 (0.01) (b) IAV/IA07/H3N2 at 0.01 MOI (c) IAV/CA04Pdm09/H1N1 (0.01 MOI); (d) IBV/BR08 and (e) IBV/FL06 at 0.1 MOI; (f) ICV/JHB1966 (g) IDV/OK2011 and (h) IDV/OK2013 at 1 MOI. Virus titers were expressed in $\log_{10}TCID_{50}$/ml. Dotted line represents the replication kinetics at 33 °C, while the bold line represents the kinetics at 37 °C. Data shown here are representative of three independent experiments performed in duplicate ± SE.

human IBV/BR08 in nasal turbinate and tracheal cells at both temperatures were 3.25 and 3.38 logs respectively (Fig. 8d).

Human ICV/JHB1966 demonstrated better replication kinetics in nasal turbinate and tracheal cells at 33 °C compared to 37 °C, same as MDCK cells. ICV has a restricted replication, with less than 3 logs at all time points in lung primary cells at both temperatures. Among the influenza D viruses, swine IDV/OK2011 strain replicated at both temperatures, with a slightly higher peak at 37 °C in all the three primary cells. In the case of bovine IDV/OK2013, 33 °C promoted a high virus peak in all the three different types of primary cells, while MDCK cells demonstrated slightly higher peak at 37 °C (Fig. 8f-h).

4. Discussion

Historically, Madin-Darby Canine Kidney (MDCK) cells are widely used for influenza research for the isolation and propagation of the influenza viruses irrespective of the host origin. A possible reason attributed to the high permissibility of the influenza viruses in MDCK cells is the lack of anti-influenza virus activity of canine Mx proteins (interferon-induced large GTPases with antiviral properties), compared to that in human, mice, cotton rats, and pigs (Dittmann et al., 2008; Seitz et al., 2010). Therefore, it is pertinent to question the suitability of MDCK cells for influenza comparative studies and host-pathogen interactions. The broad resemblance between the porcine and human lungs in the morphological structure and airway distribution coupled with the closely shared histological features make pigs and swine-derived primary cell cultures good models for human influenza research (Judge et al., 2014; Lee et al., 2018; Meyerholz et al., 2016; Monticello and Haschek, 2016). The goal of this study was to establish a non-transformed, non-tumorigenic, pathogen-free swine primary respiratory cell culture derived from the upper and lower respiratory tract of the same animal that can support influenza growth. These isogenous cell cultures will minimize donor-to-donor genetic variations and would be an excellent platform to study the host resistance, susceptibility, and disease outcomes. However, little is known about such isogenous primary cell cultures derived from swine. The main questions we wanted to address were 1) whether we can establish primary cell cultures with epithelial phenotypic characteristics from different organs of the swine respiratory system and what would be the stability of the epithelial phenotype and the longevity of the cells, 2) whether these cells possess sialic acid receptors that are necessary for the influenza binding, 3) could these swine-derived primary respiratory epithelial cells support all four types of influenza viruses and whether the virus growth is comparable to that in MDCK cells, and 4) whether these cells need exogenous proteases for influenza virus infectivity or propagation.

The immunocytochemical staining demonstrated that the swine primary respiratory epithelial cells derived from nasal turbinate, trachea, and lungs were predominantly of the epithelial phenotype and can be sub-cultured while retaining the epithelial phenotype up to 17 passages. Additionally, these cells were sub-cultured till 18–22 passages before the cells entered senescence. Further characterization revealed that these primary cells polarized to form functionally specialized apical and basolateral domains due to the specific distribution of the tight junction proteins. Swine primary respiratory cells polarized and produced TEER comparable to IPEC-1 cells, which is an established swine intestinal epithelial cell line (Nossol et al., 2011). Influenza infection leads to the loss of integrity of the tight junction proteins, especially claudin-4, and thus disrupts the epithelial barrier (Short et al., 2016). In this study, we found tissue site dependent variation in the expression of claudin −1, −3, occludin, and ZO-1. The mammalian bronchi and bronchioles express claudin-1 and −3 while alveolar epithelial cells predominantly express claudin-3 (Gunzel and Yu, 2013). Although the nuclear translocation of claudin-3 has been reported earlier (Tokuhara et al., 2018), the biological significance of this occurrence has yet to be elucidated. It is hypothesized that translocated claudins could have non-canonical functions and influence the cell proliferation and apoptosis. Occludin and ZO-1 were not expressed by lung cells, while tracheal cells had both the proteins in the nucleus and cell junction. This was in contrast to the finding that ZO-1 was expressed in pig type II alveolar epithelial cells when grown on transwell membranes (Li et al., 2012). Additionally, ZO-1 was expressed in laryngeal epithelium such as mouse, rat, guinea pig, rabbit and pigs which justifies the expression of ZO-1 in tracheal cells in our results (Gill et al., 2005). In a previous characterization study, human nasal epithelial cells that were grown *in-vitro* expressed occludin, and various claudins, while the human nasal mucosa *in-vivo* expressed ZO-1, occludin, and claudins (Kojima et al., 2013). The findings of our phenotypic characterization answered our first question towards establishing an isogenous swine primary respiratory epithelial cells.

Based on cellular staining for pneumocyte markers, we found that swine lung alveolar epithelial cells demonstrated a mixed type I and type II alveolar epithelial cell phenotype. Swine primary lung alveolar epithelial cells comprise type I and II pneumocytes but predominantly express type II specific marker SP-C, similar to A549, which is one of the widely used human alveolar epithelial cells for influenza studies (Kumar et al., 2017; Zhou et al., 2017). It was also demonstrated that human-adapted H1N1, H3N2 viruses and classic swine and avian viruses were detected in type II pneumocytes of fresh human lung explants (Weinheimer et al., 2012). It should be noted that anti-human monoclonal antibodies against the marker proteins were used for staining in this experiment. Although cross-reactivity to the swine antigens was observed, it is possible that antibody affinity towards the marker proteins could have affected the end results.

The lectins SNA and MAL bind to Sia α-2,6Gal and Sia α-2,3Gal receptors of the host cells respectively. Maackia amurensis lectins extracted from *M. amurensis* seed extracts contain a mixture of MAL I and Mal II lectins (Varki and Varki, 2007). Even though both are structurally similar, MAL I and MAL II lectins differentially bind to Sia α-2,3Galβ1–4 GlcNAc (Nicholls et al., 2007) and Sial α-2,3 Galβ1–3 GalNAc (Brinkman-Van der Linden et al., 2002) glycans respectively. Avian and equine IAV bind to Sia α-2,3Gal receptor and human viruses bind to Sia α-2,6Gal receptor while swine viruses can bind to both the receptors (Kogure et al., 2006). Glycan array studies conducted to characterize the receptor binding specificities of human influenza B viruses revealed that the Yamagata-like strains predominantly bound to α-2,6-linkage glycans while Victoria-like strains bound to both α-2,3- and α-2,6-linkage glycans (Velkov, 2013; Wang et al., 2012). The Sia α-2,6Gal was expressed by 80–100% of the cells derived from all three sites indicating the uniform distribution of human A and B influenza receptors throughout the swine respiratory tract. These findings were also similar to that observed in the epithelial cell lining of the porcine respiratory tract *in vivo* and explants derived from swine upper and lower respiratory tract (Trebbien et al., 2011; Van Poucke et al., 2010). In this study, similar to previous findings, MAL I receptors were more expressed than MAL II in the epithelial lining of lung alveoli (Nelli et al., 2010), however, our finding that MAL I receptors were more expressed in tracheal epithelial cells was incongruent to the findings in the same study. Trebbien et al. demonstrated that SA-alpha-2,6 was expressed in 80–100% of the lining epithelial cells of the swine respiratory tract while SA-alpha-2,3 was expressed in 20–40% of bronchiolar and alveolar epithelial cells and not expressed (0%) in the epithelial lining of upper respiratory tract such as epithelial cells of nose, trachea, and most bronchi (Nelli et al., 2010; Trebbien et al., 2011). Our findings with Sia α-2,3Gal receptor expression were not consistent with these studies as sialic acid distribution varies with age and breed within species. Trebbien et al. used two months old piglets for the comparison of sialic acid distribution between humans and pigs, however, the tissues we used were derived from the gnotobiotic piglets. It was demonstrated that age-dependent variation in the sialic acid distribution can occur, as an increased expression of α 2,6 SA in the large intestine was observed in ducks and geese as they aged. Similarly, the α 2,6 SA was also increased in the trachea of turkeys as they aged

(Kimble et al., 2010). Human influenza C and bovine/swine D viruses bind to the 9-O-Ac-Neu5Ac-carrying oligosaccharide chains, irrespective of glycosidic linkages (Song et al., 2016; Suzuki et al., 1992). The distribution of these receptors in the primary cells was not investigated in this study.

The swine primary respiratory epithelial cells, especially from the trachea and lung, have been used in influenza virus pathogenesis studies previously (Bateman et al., 2012, 2010; Busch et al., 2008; Hauser et al., 2013; Seo et al., 2001). Previously, swine nasal epithelial cultures have been used to study influenza A, but not for any other types of influenza (Bravo-Vasquez et al., 2017). In this study, swine IAV replicated in all the three primary cells to titers comparable to MDCK cells at both temperatures. This was similar to previous findings where efficient replication of swine IAV was demonstrated in tissue explants from the porcine upper and lower respiratory tract (Van Poucke et al., 2010). A higher viral load in nasal turbinates compared to the trachea and lungs was observed in isogenic guinea pigs, after intranasal and intratracheal inoculation of 2009 pandemic human H1N1 originated from swine, and histopathological changes were largely confined to the nasal epithelium (Wiersma et al., 2015). The higher viral replication competence demonstrated by human CA04/Pdm09 H1N1 at 33 °C in all the three primary cells and MDCK cells is in consensus with this observation. In general, the results of the virus kinetics study reflected the influenza infection profile in swine under natural conditions. It is very evident that IAV is superior to IBV, ICV, and IDV in replicating in these swine primary respiratory epithelial cells at a lower MOI (0.01), which agrees with large part of the previous influenza research that IAV is widespread in swine, while other non-influenza A viruses have very sporadic occurrence (Guo et al., 1983; Hause et al., 2014; Mitra et al., 2016; Tsai and Tsai, 2018; Vincent et al., 2014). Interestingly, human IBV of both lineages were able to replicate in these swine primary epithelial cells. This observation seems to be in good agreement with our previous study where we showed that 7.3% (41/560) of tested swine serum samples were positive for IBV antibodies and IBV genomes were detected in nasal swabs from diseased pigs, despite that the virus isolation was unsuccessful due to the inefficient growth of IBV compared to IAV (Ran et al., 2015).

Both human ICV and bovine/swine IDV replicated in all the three cell types, in a similar fashion at a higher MOI compared to IAV and IBV, which justifies the fact that both these virus types have been isolated infrequently from pigs and pigs are not thought as primary hosts for ICV and IDV (Guo et al., 1983). Strikingly, a temperature dependent differential replication of human ICV was evident in all the three primary cells and MDCK cells. ICV is considered as a ubiquitous pathogen of the upper respiratory tract and has a restricted replication at higher temperatures owing to the intrinsic temperature sensitivity of viral RNA polymerase and hemagglutinin-esterase fusion (HEF) protein (Wang and Veit, 2016). The two IDV strains used for the study, replicated efficiently in all the three primary cells at both temperatures, with titers comparable to MDCK cells. These results agreed with our previous research in that IDV-HEF has high temperature and acid stability compared to other influenza types and can replicate in upper and lower respiratory tract *in-vivo* (Sreenivasan et al., 2015; Yu et al., 2017). Additionally, the ability of these swine primary respiratory epithelial cells to yield viral titers comparable to MDCK cells, despite using 100 times less TPCK trypsin, suggests that these cells may possess endogenous proteases needed for influenza replication.

In summary, we characterized the swine primary respiratory epithelial cells derived from nasal turbinate, trachea, and lungs of the same animal and tested the utility of these cells to study all four types of influenza viruses. There are only very few cell lines of swine respiratory origin according to the published data. To our knowledge, this is the first study that described the development and characterization of isogenous swine primary respiratory cell cultures from both upper and lower respiratory tract tissues and demonstrated the susceptibility to swine and human influenza viruses, to grow into higher titers will largely benefit influenza research. The uniqueness and the utility of these cells are due to the origin of these cells from the same animal, which excludes the genetic variability factor of the host that could influence the susceptibility to the pathogens. These cells were derived from one day old gnotobiotic piglet, which provides a clean platform for pathogenesis studies. The utility of this primary cell culture system could be extended to study the cellular and immune responses at different sites of the respiratory tract when exposed to respiratory pathogens.

Acknowledgments

Radhey S. Kaushik, Chithra C. Sreenivasan, Milton Thomas, Tristen Wormstadt and this research project were funded and supported by USDA NIFA SDSU Agricultural Experiment Station Hatch grant SD00H547-15. Tristen Wormstadt is an undergraduate student. We acknowledge use of the SDSU-FGCF supported in part by NSF/EPSCoR Grant No. 0091948 and by the State of South Dakota. This study was also partially funded by NIH R15 grant number 1R15AI089690-01A1. This study was also in part supported by SDSU-AES 4AH-477, USDA/NIFA 2016-67016-24949, NIH R21AI107379, National Science Foundation/EPSCoR (http://www.nsf.gov/od/iia/programs/epscor/index.jsp) award IIA-1335423, and the State of South Dakota's Governor's Office of Economic Development as a South Dakota Research Innovation Center.

Conflicts of interest

The authors declare no conflict of interest.

References

Bateman, A.C., Busch, M.G., Karasin, A.I., Bovin, N., Olsen, C.W., 2008. Amino acid 226 in the hemagglutinin of H4N6 influenza virus determines binding affinity for alpha2,6-linked sialic acid and infectivity levels in primary swine and human respiratory epithelial cells. J. Virol. 82, 8204–8209.

Bateman, A.C., Busch, M.G., Karasin, A.I., Olsen, C.W., 2012. Infectivity phenotypes of H3N2 influenza A viruses in primary swine respiratory epithelial cells are controlled by sialic acid binding. Influenza Other Respir. Virus. 6, 424–433.

Bateman, A.C., Karamanska, R., Busch, M.G., Dell, A., Olsen, C.W., Haslam, S.M., 2010. Glycan analysis and influenza A virus infection of primary swine respiratory epithelial cells: the importance of NeuAc{alpha}2-6 glycans. J. Biol. Chem. 285, 34016–34026.

Bravo-Vasquez, N., Karlsson, E.A., Jimenez-Bluhm, P., Meliopoulos, V., Kaplan, B., Marvin, S., Cortez, V., Freiden, P., Beck, M.A., Hamilton-West, C., Schultz-Cherry, S., 2017. Swine influenza virus (H1N2) characterization and transmission in ferrets, Chile. Emerg. Infect. Dis. 23, 241–251.

Brinkman-Van der Linden, E.C., Sonnenburg, J.L., Varki, A., 2002. Effects of sialic acid substitutions on recognition by Sambucus nigra agglutinin and Maackia amurensis hemagglutinin. Anal. Biochem. 303, 98–104.

Busch, M.G., Bateman, A.C., Landolt, G.A., Karasin, A.I., Brockman-Schneider, R.A., Gern, J.E., Suresh, M., Olsen, C.W., 2008. Identification of amino acids in the HA of H3 influenza viruses that determine infectivity levels in primary swine respiratory epithelial cells. Virus Res. 133, 269–279.

Chan, M.C., Cheung, C.Y., Chui, W.H., Tsao, S.W., Nicholls, J.M., Chan, Y.O., Chan, R.W., Long, H.T., Poon, L.L., Guan, Y., Peiris, J.S., 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir. Res. 6, 135.

Chang, R.S., 1961. A comparative study of the growth, nutrition, and metabolism of the primary and the transformed human cells in vitro. J. Exp. Med. 113, 405–417.

Collin, E.A., Sheng, Z., Lang, Y., Ma, W., Hause, B.M., Li, F., 2015. Cocirculation of two distinct genetic and antigenic lineages of proposed influenza D virus in cattle. J. Virol. 89, 1036–1042.

Connor, J.D., Marti, A., 1964. Use of primary human cell cultures in titering certain human adenoviruses for infectivity. In: Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N. Y.), vol. 117, pp. 38–45.

Dean, N., Ranganath, N.K., Jones, B., Zhang, S., Skinner, D., Rowe, S.M., Sorscher, E.J., Woodworth, B.A., 2014. Porcine nasal epithelial cultures for studies of cystic fibrosis sinusitis. Int. Forum Allergy Rhinol. 4, 565–570.

Dittmann, J., Stertz, S., Grimm, D., Steel, J., Garcia-Sastre, A., Haller, O., Kochs, G., 2008. Influenza A virus strains differ in sensitivity to the antiviral action of Mx-GTPase. J. Virol. 82, 3624–3631.

Easton, J.M., 1963. Electron microscopic observations of simian virus-40 in primary rhesus kidney tissue cultures. In: Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.), vol.

114, pp. 663–665.
Endo, Y., Carroll, K.N., Ikizler, M.R., Wright, P.F., 1996. Growth of influenza A virus in primary, differentiated epithelial cells derived from adenoids. J. Virol. 70, 2055–2058.
Frank, A.L., Couch, R.B., Griffis, C.A., Baxter, B.D., 1979. Comparison of different tissue cultures for isolation and quantitation of influenza and parainfluenza viruses. J. Clin. Microbiol. 10, 32–36.
Garten, R.J., Davis, C.T., Russell, C.A., Shu, B., Lindstrom, S., Balish, A., Sessions, W.M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C.B., Emery, S.L., Hillman, M.J., Rivailler, P., Smagala, J., de Graaf, M., Burke, D.F., Fouchier, R.A.M., Pappas, C., Alpuche-Aranda, C.M., López-Gatell, H., Olivera, H., López, I., Myers, C.A., Faix, D., Blair, P.J., Yu, C., Keene, K.M., Dotson, P.D., Boxrud, D., Sambol, A.R., Abid, S.H., George, K.S., Bannerman, T., Moore, A.L., Stringer, D.J., Blevins, P., Demmler-Harrison, G.J., Ginsberg, M., Kriner, P., SteveWaterman, Smole, S., Guevara, H.F., Belongia, E.A., Clark, P.A., Beatrice, S.T., Donis, R., Katz, J., Finelli, L., Bridges, C.B., Shaw, M., Jernigan, D.B., Uyeki, T.M., Smith, D.J., Klimov, A.I., Cox, N.J., 2009. Antigenic and genetic characteristics of the early isolates of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325, 197–201.
George, S., Oh, Y., Lindblom, S., Vilain, S., Rosa, A.J.M., Francis, D.H., Brözel, V.S., Kaushik, R.S., 2007. Lectin binding profile of the small intestine of five-week-old pigs in response to the use of chlortetracycline as a growth promotant and under gnotobiotic conditions1,2. J. Anim. Sci. 85, 1640–1650.
Gill, G.A., Buda, A., Moorghen, M., Dettmar, P.W., Pignatelli, M., 2005. Characterisation of adherens and tight junctional molecules in normal animal larynx; determining a suitable model for studying molecular abnormalities in human laryngopharyngeal reflux. J. Clin. Pathol. 58, 1265–1270.
Govorkova, E.A., Murti, G., Meignier, B., de Taisne, C., Webster, R.G., 1996. African green monkey kidney (Vero) cells provide an alternative host cell system for influenza A and B viruses. J. Virol. 70, 5519–5524.
Greig, A.S., Boulanger, P., Bannister, G.L., 1967. African swine fever. V. cultivation of the virus in primary pig kidney cells. Can. J. Comp. Med. Vet. Sci. 31, 24–31.
Gunzel, D., Yu, A.S., 2013. Claudins and the modulation of tight junction permeability. Physiol. Rev. 93, 525–569.
Guo, Y.J., Jin, F.G., Wang, P., Wang, M., Zhu, J.M., 1983. Isolation of influenza C virus from pigs and experimental infection of pigs with influenza C virus. J. Gen. Virol. 64 (Pt 1), 177–182.
Hause, B.M., Collin, E.A., Liu, R., Huang, B., Sheng, Z., Lu, W., Wang, D., Nelson, E.A., Li, F., 2014. Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family. mBio 5 (e00031-00014).
Hause, B.M., Ducatez, M., Collin, E.A., Ran, Z., Liu, R., Sheng, Z., Armien, A., Kaplan, B., Chakravarty, S., Hoppe, A.D., Webby, R.J., Simonson, R.R., Li, F., 2013. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. PLoS Pathog. 9, e1003176.
Hauser, M.J., Dlugolenski, D., Culhane, M.R., Wentworth, D.E., Tompkins, S.M., Tripp, R.A., 2013. Antiviral responses by Swine primary bronchoepithelial cells are limited compared to human bronchoepithelial cells following influenza virus infection. PLoS One 8, e70251.
Huygelen, C., Peetermans, J., 1967. Isolation of a hemagglutinating picornavirus from a primary swine kidney cell culture. Arch. Gesamt. Virusforsch. 20, 260–262.
Imura, S., Hirohata, K., McDuffie, F.C., 1983. An immunoelectron microscopic study of Mycoplasma hyosynoviae in primary swine kidney cell culture. Kobe J. Med. Sci. 29, 1–15.
Johnson, A.M., Kaushik, R.S., Hardwidge, P.R., 2010. Disruption of transepithelial resistance by enterotoxigenic Escherichia coli. Vet. Microbiol. 141, 115–119.
Judge, E.P., Hughes, J.M., Egan, J.J., Maguire, M., Molloy, E.L., O'Dea, S., 2014. Anatomy and bronchoscopy of the porcine lung. A model for translational respiratory medicine. Am. J. Respir. Cell Mol. Biol. 51, 334–343.
Kasza, L., Bohl, E.H., Jones, D.O., 1960. Isolation and cultivation of swine pox virus in primary cell cultures of swine origin. Am. J. Vet. Res. 21, 269–273.
Katz, J.M., Webster, R.G., 1992. Amino acid sequence identity between the HA1 of influenza A (H3N2) viruses grown in mammalian and primary chick kidney cells. J. Gen. Virol. 73 (Pt 5), 1159–1165.
Kaushik, R.S., Begg, A.A., Wilson, H.L., Aich, P., Abrahamsen, M.S., Potter, A., Babiuk, L.A., Griebel, P., 2008. Establishment of fetal bovine intestinal epithelial cell cultures susceptible to bovine rotavirus infection. J. Virol. Methods 148, 182–196.
Khatri, M., Goyal, S.M., Saif, Y.M., 2012. Oct4+ stem/progenitor swine lung epithelial cells are targets for influenza virus replication. J. Virol. 86, 6427–6433.
Kimble, B., Nieto, G.R., Perez, D.R., 2010. Characterization of influenza virus sialic acid receptors in minor poultry species. Virol. J. 7, 365.
Kimura, H., Abiko, C., Peng, G., Muraki, Y., Sugawara, K., Hongo, S., Kitame, F., Mizuta, K., Numazaki, Y., Suzuki, H., Nakamura, K., 1997. Interspecies transmission of influenza C virus between humans and pigs. Virus Res. 48, 71–79.
Kogure, T., Suzuki, T., Takahashi, T., Miyamoto, D., Hidari, K.I., Guo, C.T., Ito, T., Kawaoka, Y., Suzuki, Y., 2006. Human trachea primary epithelial cells express both sialyl(alpha2-3)Gal receptor for human parainfluenza virus type 1 and avian influenza viruses, and sialyl(alpha2-6)Gal receptor for human influenza viruses. Glycoconj. J. 23, 101–106.
Koh, S.Y., George, S., Brözel, V., Moxley, R., Francis, D., Kaushik, R.S., 2008. Porcine intestinal epithelial cell lines as a new in vitro model for studying adherence and pathogenesis of enterotoxigenic Escherichia coli. Vet. Microbiol. 130, 191–197.
Kojima, T., Go, M., Takano, K.-i., Kurose, M., Ohkuni, T., Koizumi, J.-i., Kamekura, R., Ogasawara, N., Masaki, T., Fuchimoto, J., Obata, K., Hirakawa, S., Nomura, K., Keira, T., Miyata, R., Fujii, N., Tsutsumi, H., Himi, T., Sawada, N., 2013. Regulation of tight junctions in upper airway epithelium. BioMed. Res. Int. 2013, 11.
Kumar, D., Tiwari, K., Rajala, M.S., 2017. Analysis of A549 cell proteome alteration in response to recombinant influenza A virus nucleoprotein and its interaction with cellular proteins, a preliminary study. Acta Virol. 61, 56–65.
Lau, S.C., Scholtissek, C., 1995. Abortive infection of Vero cells by an influenza A virus (FPV). Virology 212, 225–231.
Lee, S.L., Dempsey-Hibbert, N.C., Vimalachandran, D., Wardle, T.D., Sutton, P.A., Williams, J.H.H., 2018. Primary colorectal cells culture as a translational research model. Methods Mol. Biol. 1709, 97–105.
Li, X., Comellas, A.P., Karp, P.H., Ernst, S.E., Moninger, T.O., Gansemer, N.D., Taft, P.J., Pezzulo, A.A., Rector, M.V., Rossen, N., Stoltz, D.A., McCray, P.B., Welsh, M.J., Zabner, J., 2012. CFTR is required for maximal transepithelial liquid transport in pig alveolar epithelia. Am. J. Physiol. - Lung Cell. Mol. Physiol. 303, L152–L160.
Matrosovich, M., Matrosovich, T., Uhlendorff, J., Garten, W., Klenk, H.D., 2007. Avian-virus-like receptor specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium. Virology 361, 384–390.
McClurkin, A.W., 1965. Studies on transmissible gastroenteritis of swine. I. The isolation and identification of a cytopathogenic virus of transmissible gastroenteritis in primary swine kidney cell cultures. Can. J. Comp. Med. Vet. Sci. 29, 46–53.
Meyerholz, D.K., Lambertz, A.M., Reznikov, L.R., Ofori-Amanfo, G.K., Karp, P.H., McCray, P.B., Welsh, M.J., Stoltz, D.A., 2016. Immunohistochemical detection of markers for translational studies of lung disease in pigs and humans. Toxicol. Pathol. 44, 434–441.
Mitra, N., Cernicchiaro, N., Torres, S., Li, F., Hause, B.M., 2016. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. J. Gen. Virol. 97, 1771–1784.
Monticello, T.M., Haschek, W.M., 2016. Swine in translational research and drug development. Toxicol. Pathol. 44, 297–298.
Mora, R., Rodriguez-Boulan, E., Palese, P., García-Sastre, A., 2002. Apical budding of a recombinant influenza A virus expressing a hemagglutinin protein with a basolateral ocalization signal. J. Virol. 76, 3544–3553.
Nakanishi, Y.H., Fernandes, M.V., Mizutani, M., Pomerat, C.M., 1959. On the chromosome numbers of human amnion cells in primary and strain cultures. Tex. Rep. Biol. Med. 17, 345–353.
Nayak, D.P., Balogun, R.A., Yamada, H., Zhou, Z.H., Barman, S., 2009. Influenza virus morphogenesis and budding. Virus Res. 143, 147–161.
Nelli, R.K., Kuchipudi, S.V., White, G.A., Perez, B.B., Dunham, S.P., Chang, K.C., 2010. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. BMC Vet. Res. 6, 4.
Nicholls, J.M., Bourne, A.J., Chen, H., Guan, Y., Peiris, J.S., 2007. Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Respir. Res. 8, 73.
Nossol, C., Diesing, A.K., Walk, N., Faber-Zuschratter, H., Hartig, R., Post, A., Kluess, J., Rothkotter, H.J., Kahlert, S., 2011. Air-liquid interface cultures enhance the oxygen supply and trigger the structural and functional differentiation of intestinal porcine epithelial cells (IPEC). Histochem. Cell Biol. 136, 103–115.
Noyes, W.F., 1965. Studies on the human wart virus. II. Changes in primary human cell cultures. Virology 25, 358–363.
Petursson, G., Fogh, J., 1963. The chromosomes of primary human amnion cells and Fl cells. Cancer Res. 23, 1021–1024.
Ran, Z., Shen, H., Lang, Y., Kolb, E.A., Turan, N., Zhu, L., Ma, J., Bawa, B., Liu, Q., Liu, H., Quast, M., Sexton, G., Krammer, F., Hause, B.M., Christopher-Hennings, J., Nelson, E.A., Richt, J., Li, F., Ma, W., 2015. Domestic pigs are susceptible to infection with influenza B viruses. J. Virol. 89, 4818–4826.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.
Rehacek, J., Kozuch, O., 1964. Comparison of the susceptibility of primary tick and chick embryo cell cultures to small amounts of tickborne encephalitis virus. Acta Virol. 8, 470–471.
Rodriguez-Boulan, E., Paskiet, K.T., Sabatini, D.D., 1983. Assembly of enveloped viruses in Madin-Darby canine kidney cells: polarized budding from single attached cells and from clusters of cells in suspension. J. Cell Biol. 96, 866–874.
Seitz, C., Frensing, T., Hoper, D., Kochs, G., Reichl, U., 2010. High yields of influenza A virus in Madin-Darby canine kidney cells are promoted by an insufficient interferon-induced antiviral state. J. Gen. Virol. 91, 1754–1763.
Semenov, B.F., Bogomolova, N.N., Akopova, I.I., 1961. Multiplication of tick-borne encephalitis virus in primary swine embryo kidney epithelial cell cultures. Probl. Virol. 6, 155–159.
Seo, S.H., Goloubeva, O., Webby, R., Webster, R.G., 2001. Characterization of a porcine lung epithelial cell line suitable for influenza virus studies. J. Virol. 75, 9517–9525.
Short, K.R., Kasper, J., van der Aa, S., Andeweg, A.C., Zaaraoui-Boutahar, F., Goeijenbier, M., Richard, M., Herold, S., Becker, C., Scott, D.P., Limpens, R.W., Koster, A.J., Barcena, M., Fouchier, R.A., Kirkpatrick, C.J., Kuiken, T., 2016. Influenza virus damages the alveolar barrier by disrupting epithelial cell tight junctions. Eur. Respir. J. 47, 954–966.
Song, H., Qi, J., Khedri, Z., Diaz, S., Yu, H., Chen, X., Varki, A., Shi, Y., Gao, G.F., 2016. An open receptor-binding cavity of hemagglutinin-esterase-fusion glycoprotein from newly-identified influenza D virus: basis for its broad cell tropism. PLoS Pathog. 12, e1005411.
Sreenivasan, C., Thomas, M., Sheng, Z., Hause, B.M., Collin, E.A., Knudsen, D.E., Pillatzki, A., Nelson, E., Wang, D., Kaushik, R.S., Li, F., 2015. Replication and transmission of the novel bovine influenza D virus in a guinea pig model. J. Virol. 89, 11990–12001.
Sreenivasan, C.C., Jandhyala, S.S., Luo, S., Hause, B.M., Thomas, M., Knudsen, D.E.B., Leslie-Steen, P., Clement, T., Reedy, S.E., Chambers, T.M., Christopher-Hennings, J., Nelson, E., Wang, D., Kaushik, R.S., Li, F., 2018. Phylogenetic analysis and characterization of a sporadic isolate of quine influenza A H3N8 from an unvaccinated horse in 2015. Viruses 10.
Sun, Z., Huber, V.C., McCormick, K., Kaushik, R.S., Boon, A.C., Zhu, L., Hause, B., Webby,

R.J., Fang, Y., 2012. Characterization of a porcine intestinal epithelial cell line for influenza virus production. J. Gen. Virol. 93, 2008–2016.
Suzuki, Y., Nakao, T., Ito, T., Watanabe, N., Toda, Y., Xu, G., Suzuki, T., Kobayashi, T., Kimura, Y., Yamada, A., et al., 1992. Structural determination of gangliosides that bind to influenza A, B, and C viruses by an improved binding assay: strain-specific receptor epitopes in sialo-sugar chains. Virology 189, 121–131.
Takahashi, M., Yamada, T., Nakanishi, K., Fujita, K., Nakajima, K., Nobusawa, E., Yamamoto, T., Kato, T., Okada, H., 1997. Influenza a virus infection of primary cultured cells from rat fetal brain. Park. Relat. Disord. 3, 97–102.
Taubenberger, J.K., Kash, J.C., 2010. Influenza virus evolution, host adaptation and pandemic formation. Cell Host Microbe 7, 440–451.
Thomas, M., Pierson, M., Uprety, T., Zhu, L., Ran, Z., Sreenivasan, C.C., Wang, D., Hause, B., Francis, D.H., Li, F., Kaushik, R.S., 2018. Comparison of porcine airway and intestinal epithelial cell lines for the susceptibility and expression of pattern recognition receptors upon influenza virus infection. Viruses 10.
Tokuhara, Y., Morinishi, T., Matsunaga, T., Sakai, M., Sakai, T., Ohsaki, H., Kadota, K., Kushida, Y., Haba, R., Hirakawa, E., 2018. Nuclear expression of claudin-3 in human colorectal adenocarcinoma cell lines and tissues. Oncol. Lett. 15, 99–108.
Torres-Flores, J.M., Arias, C.F., 2015. Tight junctions go viral!. Viruses 7, 5145–5154.
Trebbien, R., Larsen, L.E., Viuff, B.M., 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virol. J. 8, 434.
Tsai, C.P., Tsai, H.J., 2018. Influenza B viruses in pigs, Taiwan. Influenza Other Respir. Virus.
Van Poucke, S.G., Nicholls, J.M., Nauwynck, H.J., Van Reeth, K., 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol. J. 7, 38.
Varki, N.M., Varki, A., 2007. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab. Investig. 87, 851–857.
Velkov, T., 2013. The specificity of the influenza B virus hemagglutinin receptor binding pocket: what does it bind to? J. Mol. Recognit. 26, 439–449.
Vincent, A., Awada, L., Brown, I., Chen, H., Claes, F., Dauphin, G., Donis, R., Culhane, M., Hamilton, K., Lewis, N., Mumford, E., Nguyen, T., Parchariyanon, S., Pasick, J., Pavade, G., Pereda, A., Peiris, M., Saito, T., Swenson, S., Van Reeth, K., Webby, R., Wong, F., Ciacci-Zanella, J., 2014. Review of influenza A virus in swine worldwide: a call for increased surveillance and research. Zoonoses Public Health 61, 4–17.
Wang, M., Veit, M., 2016. Hemagglutinin-esterase-fusion (HEF) protein of influenza C virus. Protein Cell 7, 28–45.
Wang, Y.F., Chang, C.F., Chi, C.Y., Wang, H.C., Wang, J.R., Su, I.J., 2012. Characterization of glycan binding specificities of influenza B viruses with correlation with hemagglutinin genotypes and clinical features. J. Med. Virol. 84, 679–685.
Weinheimer, V.K., Becher, A., Tonnies, M., Holland, G., Knepper, J., Bauer, T.T., Schneider, P., Neudecker, J., Ruckert, J.C., Szymanski, K., Temmesfeld-Wollbrueck, B., Gruber, A.D., Bannert, N., Suttorp, N., Hippenstiel, S., Wolff, T., Hocke, A.C., 2012. Influenza A viruses target type II pneumocytes in the human lung. J. Infect. Dis. 206, 1685–1694.
Wiersma, L.C., Vogelzang-van Trierum, S.E., van Amerongen, G., van Run, P., Nieuwkoop, N.J., Ladwig, M., Banneke, S., Schaefer, H., Kuiken, T., Fouchier, R.A., Osterhaus, A.D., Rimmelzwaan, G.F., 2015. Pathogenesis of infection with 2009 pandemic H1N1 influenza virus in isogenic guinea pigs after intranasal or intratracheal inoculation. Am. J. Pathol. 185, 643–650.
Wu, N.H., Yang, W., Beineke, A., Dijkman, R., Matrosovich, M., Baumgartner, W., Thiel, V., Valentin-Weigand, P., Meng, F., Herrler, G., 2016. The differentiated airway epithelium infected by influenza viruses maintains the barrier function despite a dramatic loss of ciliated cells. Sci. Rep. 6, 39668.
Yu, J., Hika, B., Liu, R., Sheng, Z., Hause, B.M., Li, F., Wang, D., 2017. The hemagglutinin-esterase fusion glycoprotein Is a primary determinant of the exceptional thermal and acid stability of influenza D virus. mSphere 2.
Zhou, B., Li, J., Liang, X., Yang, Z., Jiang, Z., 2017. Transcriptome profiling of influenza A virus-infected lung epithelial (A549) cells with lariciresinol-4-beta-D-glucopyranoside treatment. PLoS One 12, e0173058.