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Characterization of a porcine intestinal epithelial cell line for influenza virus production

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We have developed a porcine intestine epithelial cell line, designated SD-PJEC for the propagation of influenza viruses. The SD-PJEC cell line is a subclone of the IPEC-J2 cell line, which was originally derived from newborn piglet jejunum. Our results demonstrate that SD-PJEC is a cell line of epithelial origin that preferentially expresses receptors of oligosaccharides with Sia2-6Gal modification. This cell line is permissive to infection with human and swine influenza A viruses and some avian influenza viruses, but poorly support the growth of human-origin influenza B viruses. Propagation of swine-origin influenza viruses in these cells results in a rapid growth rate within the first 24 h post-infection and the titres ranged from 4 to 8 $\log_{10}$ $TCID_{50}$ ml^{-1}. The SD-PJEC cell line was further tested as a potential alternative cell line to Madin–Darby canine kidney (MDCK) cells in conjunction with 293T cells for rescue of swine-origin influenza viruses using the reverse genetics system. The recombinant viruses A/swine/North Carolina/18161/02 (H1N1) and A/swine/Texas/4199-2/98 (H3N2) were rescued with virus titres of 7 and 8.25 $\log_{10}$ $TCID_{50}$ ml^{-1}, respectively. The availability of this swine-specific cell line represents a more relevant substrate for studies and growth of swine-origin influenza viruses.

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INTRODUCTION

The family *Orthomyxoviridae* contains three genera of influenza virus, which are identified by antigenic differences in their nucleoprotein (NP) and matrix protein. Influenza A virus infects humans, other mammals and birds, and causes all flu pandemics (Webster *et al.*, 1992). Influenza B virus infects humans and seals (Osterhaus *et al.*, 2000). Influenza C virus infects humans and pigs (Buonagurio *et al.*, 1986). Viruses of the family *Orthomyxoviridae* contain six to eight segments of linear negative-sense ssRNA genome, influenza A and B viruses consists of eight RNA segments. The viral haemagglutinin protein is a major envelope glycoprotein encoded by one of these RNA segments. During the infection, viruses bind to a cell through interactions between the HA glycoprotein and sialic acid sugars on the surfaces of epithelial cells.

An important aspect of the influenza virus pathogenesis is the mechanism of cross-species transmission. Pigs are considered to be a mixing vessel, from which novel virus reassortants could emerge to cause pandemics (Scholtissek *et al.*, 1983). Previous studies have demonstrated that HA receptor specificity can determine the infection of species (Maines *et al.*, 2011; Shinya *et al.*, 2006). Human influenza viruses preferentially attach to host cells expressing α2,6-linked sialic acids (Hatakeyama *et al.*, 2005; Matrosovich *et al.*, 2000; Webby & Webster, 2003), while avian influenza viruses prefer to attach to α2,3-linked sialic acids (Suzuki *et al.*, 2000). It has been well documented that both α2,6- and α2,3-linked sialic acid receptors are present at the pig

mucosal surfaces (Bateman *et al.*, 2008; Chutinimitkul *et al.*, 2010; Nicholls *et al.*, 2008; Rogers *et al.*, 1985; Takemae *et al.*, 2010), allowing for the simultaneous infection of pigs with both avian- and human-origin influenza viruses (Trebbien *et al.*, 2011).

The most common cellular model for influenza virus studies is the Madin–Darby canine kidney (MDCK) cells, and this cell line represents the most applicable alternative to egg-based virus isolation and propagation (Hussain *et al.*, 2010; Roth *et al.*, 2012). Other cell lines, including Vero, baby hamster kidney (BHK) and A549 also support the growth of influenza viruses (Govorkova *et al.*, 1999a, 1999b; Rimmelzwaan *et al.*, 2004). However, these cells may not represent the most relevant cell line for evaluation of influenza virus infection within pigs. Therefore, we developed the hypothesis that a more relevant swine-origin cell line would be very useful to study the host cell contributions to cross-species transmission and viral pathogenesis. To test this hypothesis, we developed a porcine epithelial cell line, SD-PJEC. The SD-PJEC is a subclone of the IPEC-J2 cell line, which is a non-transformed, non-tumorigenic small intestinal epithelial cell line originally derived from jejunal epithelia of a neonatal, unsuckled piglet (Berschneider, 1989; Rhoads *et al.*, 1994). The capability of this cell line to support influenza virus replication was determined by using a panel of influenza A viruses of human, swine and avian origin, and influenza B viruses of human origin. We further explored the potential application of SD-PJEC cells in a reverse genetics system.

RESULTS

SD-PJEC cells determined to be epithelial phenotype

The SD-PJEC cell line was generated from a subclone of IPEC-J2 cells (Berschneider, 1989; Rhoads *et al.*, 1994). The cell line appears to be a more homogeneous cell population than that of the IPEC-J2 cell line. To determine the phenotype of the SD-PJEC cells, cells were stained with antibodies that recognized the marker proteins for different cell phenotypes, including cytokeratin (epithelial), vimentin (fibroblast), alpha smooth muscle actin (ASMA, smooth muscle) and desmin (smooth and striated muscles). As shown in Fig. 1, all SD-PJEC cells were positively stained for cytokeratin and few cells faintly stained for vimentin. This result indicates that the SD-PJEC cell line has an epithelial phenotype.

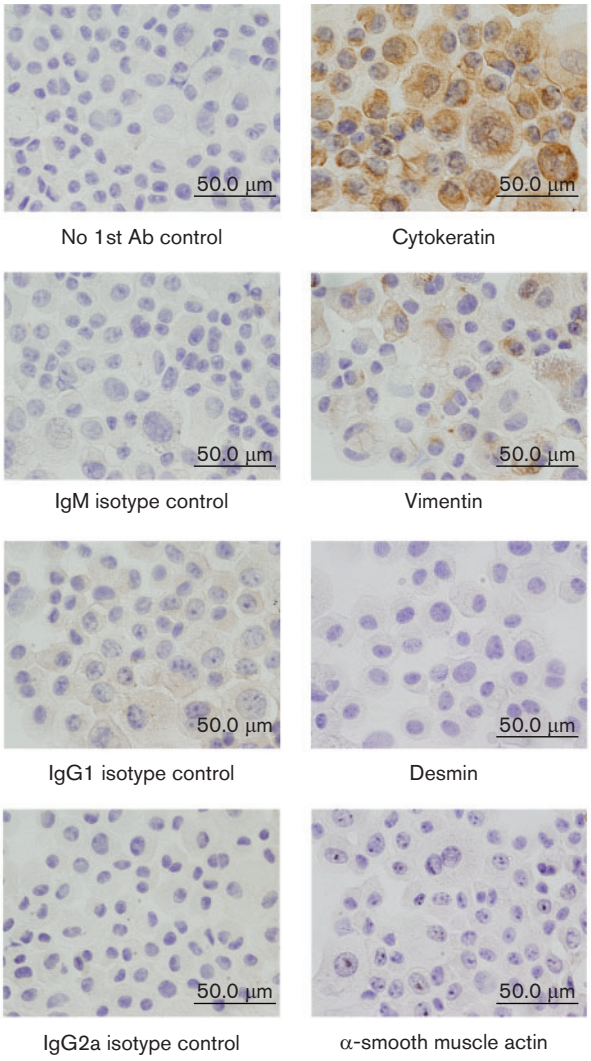

Fig. 1. Staining of SD-PJEC cells for various epithelial, fibroblast and smooth muscle markers. Cytospins (1×10^5 SD-PJEC cells) were fixed in acetone. The presence of cytokeratin, vimentin, alpha smooth muscle actin (ASMA) and desmin proteins was detected by immunohistochemical (IHC) staining using mAbs specific for these proteins. Staining without primary antibody but only secondary antibody of mouse IgM, IgG1 and IgG2a were used as negative and isotype controls. After washing, cells were incubated with biotinylated goat anti-mouse isotype-specific Abs. This was followed by incubation of cells with the ABC solution. Then DAB substrate was added and cells were counterstained with haematoxylin. Note, all SD-PJEC cells were positively stained for cytokeratin and few cells faintly stained for vimentin, indicating their epithelial phenotype.

SD-PJEC cells express Sia2-6 galactose oligosaccharides receptor

Previous studies determined that both influenza virus receptors, Sia2-6Gal and Sia2-3Gal, are present at the pig mucosal surfaces (Ito *et al.*, 1998). We further determined whether these receptors were present on the SD-PJEC cells. As a comparison, MDCK cells were included in the analysis. Biotinylated Maackia amurensis lectin-2 (MAL-II) specific for Sia2-3Gal and Sambucus nigra agglutinin (SNA) specific for Sia2-6Gal were used to stain both cell lines as described previously (Meroz *et al.*, 2011). Receptor specificity was evaluated by flow cytometric analysis. As

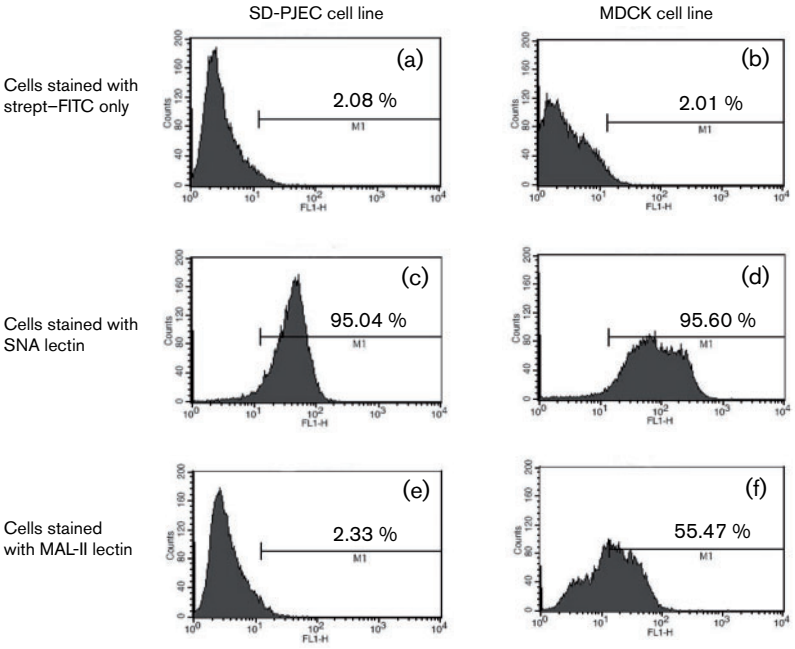

Fig. 2. Influenza virus receptor expression in SD-PJEC and MDCK cell lines. Both SD-PJEC and MDCK cell lines were incubated with biotinylated MAL-II and SNA lectins followed by staining with streptavidin–FITC. The negative control cells for both cell lines were stained with streptavidin–FITC only. Stained samples were subjected to flow cytometric analysis. A representative experiment out of four experiments is shown here.

shown in Fig. 2(a) and (b), only background fluorescent signals were detected in the negative control cells that were only stained with FITC-conjugated streptavidin. Both SD-PJEC and MDCK cells expressed Sia2-6Gal receptor showing positive SNA staining (Fig. 2c and d). However, the Sia2-3Gal receptors were not expressed on the surface of SD-PJEC cells, and only background fluorescent signal was detected for MAL-II lectin staining (Fig. 2e). In contrast, MDCK cells stained positive for MAL-II lectin (Fig. 2f). These results clearly indicate that the SD-PJEC cells mainly express Sia2-6Gal receptor, while the Sia2-3Gal receptor is expressed at minimal levels. The experiment was repeated four different times using SD-PJEC cells from passages 18 to 22. There were no significant changes in their staining pattern or percentages of cells stained for MAL-II (0.25–0.65 %) and SNA (84.16–92.96 %) across the passage 18–22 cells. This suggests that SD-PJEC cells were phenotypically stable over many cell passages tested in this study.

Replication efficiencies of human, avian and swine influenza viruses in SD-PJEC cells

To determine whether the SD-PJEC cells are permissive to influenza virus infection, we inoculated the cells with an influenza A virus, A/swine/Texas/4199-2/98 (H3N2). At 24 h post-infection (p.i.), cells were fixed and the expression of viral nucleocapsid protein (NP) was detected by immunofluorescence assay (IFA) using NP-specific antibody. As shown in Fig. 3, a specific fluorescent staining pattern was observed in nuclear and cytoplasm compartments of infected cells, while no fluorescent signal was detected in uninfected cells, suggesting that the cells were permissive for influenza A virus infection.

We further compared the replication efficiency of influenza viruses in SD-PJEC cells with that in MDCK cells. A panel of human, swine and avian-origin influenza virus isolates were titrated on both MDCK and SD-PJEC cells in order to quantify replication efficiency in the newly developed SD-PJEC cells. Some of the influenza A viruses of swine origin grew to higher titres in SD-PJEC cells (Table 1). The titres ranged from 4 to 8 $\log_{10}$ $TCID_{50}$ ml^{-1}, with most of the viruses having titres of 6–7 $\log_{10}$ $TCID_{50}$ ml^{-1}. The

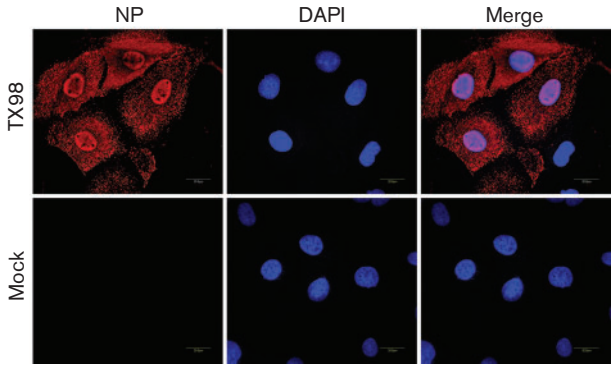

Fig. 3. Immunofluorescence microscopy detection of NP expression in SD-PJEC-infected cells by A/swine/Texas/4199-2/98 (TX98). Confluent cells were infected with the influenza virus at an m.o.i. of 0.01. At 24 h p.i., cells were fixed and stained with a primary mAb to NP and Alexa Fluor 546-labelled goat anti-mouse antibody was used as secondary antibody. The nucleus was stained with DAPI. Mock-infected cells were used as a control. Specimens were visualized on a Zeiss LSM510 confocal microscope. A 0.8 mm slice through the nucleus is shown in each image.

replication of two H7 subtype avian influenza viruses was also assessed. Contemporary North American lineage influenza H7 viruses were reported to possess human receptor specificity (Belser *et al.*, 2008) and we therefore predicted that these viruses would replicate well in the SD-PJEC cells. The results showed that both A/mallard/Alberta/177/04 (H7N9) and A/shorebird/Delaware/22/06 (H7N3) avian influenza A viruses replicated well in the SD-PJEC cells with less than 10-fold difference in virus titre between MDCK and SD-PJEC cells. Influenza B viruses have been reported to only naturally infect humans and seals (Osterhaus *et al.*, 2000), and within humans, two distinct genetic lineages are co-circulating (McCullers *et al.*, 2004). Using influenza B viruses that represent the genotypes of two human lineages, B/Memphis/13/03 (Victoria87 lineage) and B/Yamanashi/166/98 (Yamagata88 lineage), no CPE was observed in SD-PJEC cells. Influenza B viruses appeared to grow poorly in these cells, lack of cell-to-cell spreading, and very low virus titres were detected in the cell culture supernatant at 72 h p.i. (Table 1). In contrast, these two viruses replicated well in MDCK cells, with the virus titres reaching 6.5 and 7.4 $\log_{10}$ $TCID_{50}$ ml^{-1}, respectively.

To determine the kinetics of influenza virus replication in SD-PJEC cells, we compared the growth curves of a representative virus (A/swine/Texas/4199-2/98) in SD-PJEC cells and MDCK cells. Supernatants were harvested at different time points p.i., and virus titre was determined by titration in MDCK cells. The results showed that both viruses reach their peak titre at 36 h p.i. However, there was an approximately 100-fold higher peak viral titre in SD-PJEC cells, with a peak titre of 9.3 $\log_{10}$ $TCID_{50}$ ml^{-1} in SD-PJEC cells in comparison to the peak titre of 7.3 $\log_{10}$ $TCID_{50}$ ml^{-1} in MDCK cells (Fig. 4). Stability of the viruses in SD-PJEC cells was further investigated by serial passage of A/swine/Texas/4199-2/98 virus 10 times in these cells. We observed no mutations in HA gene of passage 10 virus, and the virus titre remains at a similar level to that of passage 1 virus (P1=5.5 $\log_{10}$ $TCID_{50}$ ml^{-1}; P10=6.5 $\log_{10}$ $TCID_{50}$ ml^{-1}).

Table 1. Comparison of propagation of influenza A and B viruses within MDCK and SD-PJEC cells

Virus isolate	Species	MDCK*	SD-PJEC
A/New Jersey/11/76-H1N1	Human	6.00	6.40
A/Brisbane/59/07-H1N1	Human	7.00	6.60
A/California/4/09-H1N1	Human	5.00	4.00
A/swine/South Dakota/01/09-H1N1	Swine	7.40	6.40
A/swine/South Dakota/02/09-H1N1	Swine	7.50	6.40
A/swine/Minnesota/4390/11-H1N1	Swine	3.50	4.50
A/swine/North Carolina/31/12-H1N1	Swine	5.00	8.00
A/swine/Texas/042995-27/07-H1N2	Swine	9.00	8.40
A/swine/Missouri/4460/11-H1N2	Swine	6.50	6.50
A/swine/North Carolina/4478/11-H1N2	Swine	4.00	6.00
A/swine/Minnesota/4579/11-H1N2	Swine	4.50	6.50
A/swine/Minnesota/21/12-H1N2	Swine	2.50	3.50
A/swine/North Carolina/28/12-H1N2	Swine	6.50	8.00
A/swine/North Carolina/29/12-H1N2	Swine	8.33	6.75
A/swine/Oklahoma/52/12-H1N2	Swine	7.50	7.00
A/swine/Minnesota/4393/11-H3N2	Swine	7.00	6.00
A/swine/Minnesota/4395/11-H3N2	Swine	3.50	5.50
A/swine/Texas/2/98-H3N2	Swine	5.00	5.50
A/swine/Illinois/26/12-H3N2	Swine	3.50	4.50
A/swine/Minnesota/50/12-H3N2	Swine	4.00	5.00
A/swine/Ohio/51/12-H3N2	Swine	7.50	5.50
A/mallard/Alberta/177/04 (H7N9)	Avian	7.70	6.80
A/shorebird/Delaware/22/06 (H7N3)	Avian	8.50	6.70
B/Yamanashi/166/98-Yam88	Human	7.40	4.00
B/Memphis/13/03-Vic87	Human	6.50	4.00

*Values reported as $\log_{10}$ $TCID_{50}$ ml^{-1}.

Application of the SD-PJEC cell line in reverse genetics

Reverse genetics systems allow for the production of influenza viruses from cloned viral cDNA (Hoffmann *et al.*, 2000, 2002; Hoffmann & Webster, 2000). The commonly used cell lines to obtain influenza viruses from cDNA are 293T and MDCK cells. These two types of cells are co-cultured for initial transfection and virus rescue in order to achieve the maximal viral yield. However, MDCK cells may not be the optimum cell line for rescue of viruses that contain the genome segment(s) of swine origin. In this study, we explored the possibility of using the SD-PJEC cell line as an alternative to MDCK cells in the co-culture system. Two sets of eight-plasmid cDNA reverse genetics system for swine-origin influenza viruses, A/swine/North Carolina/18161/02 (H1N1) and A/swine/Texas/4199-2/98 (H3N2), were tested (Meroz *et al.*, 2011; Solórzano *et al.*, 2005). The virus rescue efficiency was compared between 293T/MDCK and 293T/SD-PJEC co-culture systems. As a control, we also rescued the virus on 293T cell alone. At 12, 24, 36, 48 and 60 h after addition of trypsin, the titres of virus in the cell culture supernatant were determined. For A/swine/North Carolina/18161/02, there was no significant difference ($P>0.05$) in virus titres in the supernatant from both co-culture systems, whereas virus titres in 293T cells alone were reduced about 3–4 $\log_{10}$ $TCID_{50}$ ml^{-1} (Fig. 5a). In contrast, a significantly higher amount of A/swine/Texas/4199-2/98 viruses ($P<0.05$) was rescued from 293T/SD-PJEC co-culture system at 60 h p.i. The peak titre reached 7 $\log_{10}$ $TCID_{50}$ ml^{-1} (60 h p.i.), which is 2.5 log higher than that rescued from the 293T/MDCK co-culture system. Again, the 293T cells alone recovered the lowest amount of recombinant viruses in comparison to the other two co-culture systems (Fig. 5b).

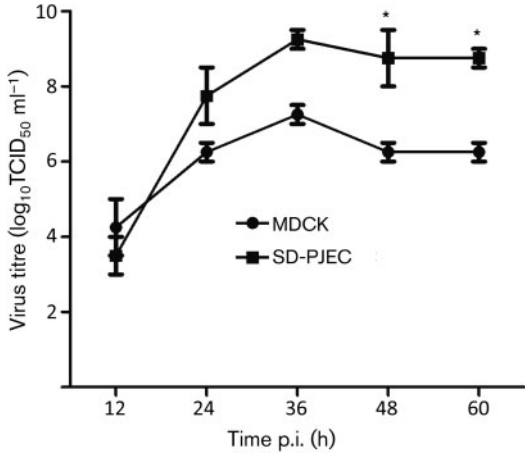

Fig. 4. Comparison of growth kinetics of A/swine/Texas/4199-2/98 (H3N2) in MDCK and SD-PJEC cells. Confluent MDCK and SD-PJEC cells were infected with the influenza virus at an m.o.i. of 0.01. Virus titres were determined at 12 h intervals. The results shown are mean values from three replicates. Error bars show SEM. Asterisks indicate that mean virus titres from different cell types differ significantly ($P<0.05$).

DISCUSSION

In this study, we characterized a porcine small intestinal epithelial cell line (SD-PJEC) that supports the productive replication of certain influenza viruses. The SD-PJEC cell line represents an *in vitro* model system for evaluation of influenza virus infection within porcine cells. The recent emergence and pandemic classification of a triple reassortant virus containing swine, human and avian genetic components have raised greater concerns over the swine-origin viruses (Smith *et al.*, 2009). There are significant concerns that these novel viruses would undergo further reassortment events within either the human or swine populations to yield viruses with increased virulence (Smith *et al.*, 2009). Based on our current knowledge of influenza virus reassortment within the swine population (Khiabanian *et al.*, 2010), the pandemic potential for a given influenza virus reassortant depends on the fitness of these reassortants within the swine host.

While studies aim to evaluate influenza virus virulence and pathogenesis require animal hosts (Ozawa *et al.*, 2011), *in vitro* studies to determine the interaction between the virus and host that yield optimal virus replication and fitness can be performed using cell lines. To date, the majority of *in vitro* influenza virus experiments have been performed using MDCK cells (Sidorenko & Reichl, 2004; Heynisch *et al.*, 2010), which may differ significantly from either human (Chakrabarti *et al.*, 2010) or swine cells. Most studies for influenza pathogenesis in swine have been performed in animal infection models. Elucidation of the *in vitro* infection mechanisms depends on a reliable, continuous porcine cell line, and the SD-PJEC cell line represents such a model.

Since the SD-PJEC cells were generated from small intestine, this cell line is particularly suitable to study the pathogenic mechanism of influenza virus in digestive systems. Influenza infection normally causes symptoms, including coughing, sore throat, headache, fever, weakness, muscle aches, diarrhoea and vomiting. The seasonal influenza usually attacks the respiratory system, and is rarely associated with gastrointestinal symptoms such as vomiting or diarrhoea. However, when 2009 pandemic virus emerged, patients showed disease not only in the respiratory tract, but also within the digestive system, with vomiting and/or diarrhoea reported by nearly 30 % of

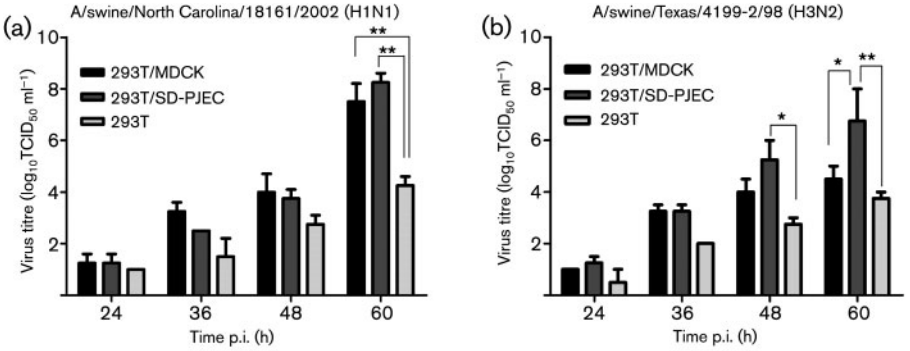

Fig. 5. Rescue of recombinant influenza viruses from 293T/SD-PJEC co-culture system. Eight plasmids containing individual gene segments of A/swine/North Carolina/18161/2002 (a) or A/swine/Texas/4199-2/98 (b) were used to transfect 293T/SD-PJEC, 293T/MDCK or 293T cells alone. The amount of virus produced in culture supernatants was determined at 12 h intervals. The results shown at each time point are mean values from three independent experiments conducted with each virus. Error bars show SEM. Asterisks indicate that mean virus titres from different culture methods differ significantly (*$P<0.05$; **$P<0.01$).

people with laboratory-confirmed influenza infection (Shinde *et al.*, 2009). This suggests that the influenza virus might be able to replicate in intestine cells, but to date, the knowledge concerning the pathogenic mechanism of influenza virus within the digestive system is limited due to a lack of a good *in vitro* cellular system. We believe that the intestinal epithelial cell line we present here would make an appropriate cellular model for evaluation of pathogenic mechanism of influenza viruses, especially for those viruses containing genome segment(s) of swine-origin influenza virus.

SD-PJEC cells were determined to have an epithelial phenotype with strong staining for epithelial marker cytokeratin. However, they were also weakly positive for vimentin staining. In a recent study, it was clearly shown that cultured bovine intestinal epithelial cells co-expressed the epithelial marker cytokeratin and the mesenchymal marker vimentin. It is known that vimentin protein is not expressed in intestinal epithelial cells *in vivo* although these cells can carry vimentin mRNA. In contrast, a post-transcriptional inhibition of vimentin synthesis observed *in vivo* is suppressed *in vitro* (Rusu *et al.*, 2005). Thus, epithelial cells could express lower levels of vimentin along with cytokeratin *in vitro*. Another study on pig intestinal cells also reported similar findings (Kaeffer *et al.*, 1993). Our results were consistent with these studies, and SD-PJEC cells strongly stained for cytokeratin but also weakly stained for vimentin.

It is intriguing that the SD-PJEC cells predominantly express the α2,6-galactose receptor. It is well-known that both α2,6- and α2,3-galactose receptors are expressed on the cell surface of the pig respiratory tract (Ito *et al.*, 1998). Receptor expression levels differ in respiratory and intestine cells; therefore it would be useful to study the difference of influenza pathogenic mechanisms between the two different compartments (respiratory tract versus intestine). Since SD-PJEC cells lack the α2,3-galactose receptor, these cells could also be useful in viral receptor-based analyses, including those associated with the development of novel therapies based on sialic acid binding properties (Malakhov *et al.*, 2006).

Our results further demonstrate that SD-PJEC could be used as an alternative cell line in a reverse genetics system. Application of 293T/SD-PJEC co-cultures would be useful for situations where 293T/MDCK co-cultures are unsuccessful for creation of swine-origin influenza viruses (Wanitchang *et al.*, 2010). In addition, targeted mutations could be created to elucidate the molecular basis of the subsequent growth adaptation of a specific influenza virus in host cells using the 293T/SD-PJEC co-cultures and the reverse genetics system.

Besides its application in influenza pathogenesis studies, the SD-PJEC cell line could be a potential candidate to use in vaccine production. There is a great demand for the development of improved cell culture systems for human vaccine production (Lee & Hu, 2012), since the traditional embryonic egg-based method for virus propagation can result in antigenic changes (Fedson, 2008). Several influenza virus permissive cell lines have been explored previously (Hussain *et al.*, 2010). For example, baby hamster kidney (BHK) cells were capable of supporting influenza virus propagation, but like eggs, receptor-binding variants of human influenza viruses were generated when growing in this cell line (Govorkova *et al.*, 1999b). Also, while African green monkey kidney (Vero) cells were reported to fully support the replication of influenza A and B viruses, the viruses have to be adapted before they can be grown in these cells (Govorkova *et al.*, 1999a). MDCK cells are still considered the best cell-based alternative to eggs for isolation and propagation of influenza viruses, but concerns over the tumorigenic potential of by-products from this cell line make it less suitable for vaccine production (Gregersen *et al.*, 2011). Since SD-PJEC cells represent a non-transformed, non-tumorigenic cell line, they could be tested as a candidate cell line for use in vaccine production. To support this notion, we present similar growth characteristics, if not improved, when propagation of viruses within SD-PJEC cells compared to MDCK cells; and have observed no mutations in the HA gene upon sequential passage of the A/swine/Texas/4199-2/1998 virus (10 passages) within these cells.

In conclusion, we characterized a swine epithelial cell line, SD-PJEC that is permissive to infection with human and swine influenza A viruses and some avian influenza viruses, but poorly support the growth of human-origin influenza B viruses. The availability of this cell line provides an additional cellular model system for elucidation of the mechanism of influenza virus pathogenesis, especially for those associated with successful reassortment events within the intermediate swine host. Further evaluation of the interactions between this cell line and influenza viruses will allow for identification of relevant virulence factors and, eventually, the development of effective strategies to prevent influenza virus infection.

METHODS

Cell lines and culture conditions. The SD-PJEC cell line was generated by a subclone of IPEC-J2 cells, which appears to be a more homogeneous cell population. For subcloning the IPEC-J2 cells, a confluent cell monolayer was trypsinized with 0.25 % trypsin-EDTA and 100 representative cells were obtained based on haemacytometer counts. These 100 cells were seeded in a 96-well plate to obtain a cell density at approximately 1 cell per well in 250 μl cell culture medium. Cells were allowed to adhere for 24 h, and then washed and replaced with fresh medium. Cell culture medium was changed every other day to allow the cell to grow into a single clone in each well. Each clone of cells grown into a confluent monolayer in 96-well plate was expended into 24-well plates and a proportion of cells in each well were tested further for their permissiveness to influenza virus infection.

Both IPEC-J2 and SD-PJEC cells were grown in Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 (1 : 1) medium (DMEM/F12) (Invitrogen). The DMEM/F12 medium was supplemented with 5 % FBS (Hyclone), 1 % insulin-transferring selenium supplements (Invitrogen), 5 ng epidermal growth factor (Invitrogen) ml^{-1}, 1 %

penicillin–streptomycin (penicillin 10 000 U ml^{-1} and streptomycin 100 mg ml^{-1}; Invitrogen) and 15 mM HEPES. Cell culture media were changed every other day. In addition, MDCK and HEK293T cells were also used in this study, and they were maintained in minimal essential medium (MEM) supplemented with 5 % FBS.

Influenza virus isolates. Two sets of influenza viruses were used in this study. Table 1 lists a total of 25 influenza virus isolates of human, swine or avian origin. Nine of these isolates were obtained from the repository at the St. Jude Children's Research Hospital (Memphis, TN); and two swine-origin influenza viruses were obtained from South Dakota Animal Disease Diagnostic Laboratory (Brookings, SD). The rest of the viruses were isolated from field samples submitted to Newport Laboratories (Worthington, MN). Virus stocks were grown on either SD-PJEC or MDCK cells in MEM supplemented with 0.3 % FBS and L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin (1 μg ml^{-1} for MDCK and 0.1 μg ml^{-1} for SD-PJEC). Infected cells were incubated at 37 °C for 72 h, and observed daily for cytopathic effect (CPE). To confirm the presence of virus, haemagglutination assay with 0.5 % chicken erythrocytes was performed.

Phenotyping of SD-PJEC cells. SD-PJEC cells were stained with antibodies which recognized various epithelial, fibroblast and smooth muscle markers using the protocol as described previously (Kaushik *et al.*, 2008; Rhoads *et al.*, 1994). Briefly, SD-PJEC cell cultures were trypsinized and washed with PBS. Cytospins (1×10^5 SD-PJEC cells) were prepared using a cytofuge (Cytospin 3; Thermo Shandon Inc.), air-dried, fixed in acetone and stored at 4 °C until they are ready for immunoassays. For antibody staining, slides were equilibrated at room temperature, rehydrated in PBS and then incubated with PBS containing 1 % goat serum to block non-specific protein binding. The presence of cytokeratin, vimentin, ASMA and desmin proteins was detected by immunohistochemical (IHC) staining using anti-cytokertain mAb C6909 (IgG2a isotype), anti-vimentin mAb V5255 (IgM isotype), anti-ASMA mAb A2547 (IgG2a isotype) and anti-desmin mAb D1033 (IgG1). mAbs M9144 (IgG2a isotype), M9269 (IgG1 isotype) and M5170 (IgM isotype) were used as irrelevant isotype-matched controls. Cells without primary antibody staining were used as negative control. All mAbs were purchased from Sigma and used at 1 μg ml^{-1} concentration with 1 h incubation. Slides were washed three times (3 ×) in PBS and then incubated with isotype-specific, biotinylated goat anti-mouse IgG2a, IgG1 or IgM antisera (1 : 2000 dilution; Caltag laboratories) for 30 min. Slides were washed 3 × and then incubated in PBS containing 0.3 % hydrogen peroxide to block endogenous peroxidase activity. Antibody labelling was visualized by adding ready-to-use (RTU) HRP–streptavidin solution for 30 min followed by the addition of RTU diaminobenzene (DAB) substrate (Vector Laboratories). Cytospins were counterstained with haematoxylin, dried overnight, contained with a coverslip and examined under the light microscope. Pictures were taken under × 40 magnification using an Olympus AX70 microscope.

Flow cytometric analysis of sialic acid receptor expression. Biotinylated MAL-II specific for Sia2-3Gal and SNA (Vector laboratories) specific for Sia2-6Gal were used to stain both SD-PJEC and MDCK cell lines as described previously (Meroz *et al.*, 2011). Briefly, 5×10^5 cells of each cell type were incubated with biotinylated MAL-II and SNA lectins (final concentration 10 μg ml^{-1}) followed by staining with streptavidin–FITC (1 : 200 dilution). The negative control cells for both cell lines were stained with streptavidin–FITC only. Stained samples were subjected to flow cytometric analysis.

Immunofluorescence microscopy. SD-PJEC cells were infected with influenza virus A/swine/Texas/4199-2/1998 at an m.o.i. of 0.01 for 1 h and virus supernatant was replaced with the growth medium containing 1 μg TPCK-trypsin ml^{-1}. Infected cells were incubated at 37 °C for 24 h and then fixed with methanol–acetone (1 : 1 ratio in volume) at −20 °C for 20 min. The fixed cells were stained with a primary mAb 42-100 to the NP at 37 °C for 1 h. The Alexa Fluor 549-labelled goat anti-mouse antibody (Kirkegaard & Perry Laboratories) was used as a secondary antibody and incubated for another hour. Nuclear staining with DAPI was performed as recommended by the manufacturer (Molecular Probes). Specimens were imaged using a Zeiss LSM510 confocal microscope with a × 63 objective, and images were processed with NIH ImageJ and Adobe Photoshop 6.0 software.

Viral growth kinetics analysis. Growth kinetics of influenza viruses in SD-PJEC cells was compared with those in MDCK cells. Confluent cell monolayers were infected with influenza A/swine/Texas/4199-2/1998 virus at an m.o.i. of 0.01 and incubated at 37 °C for 1 h. The virus suspension was then removed, and the MEM containing 1 μg TPCK-treated trypsin ml^{-1} was added (1 μg ml^{-1} for MDCK and 0.1 μg ml^{-1} for SD-PJEC). Cell culture supernatants were collected at 12 h intervals until 60 h post-inoculation. The virus titre was determined by titration on MDCK cells.

Virus rescue from cloned cDNA. SD-PJEC, 293T and MDCK cells were grown to 100 % confluence in a T75 flask and then trypsinized with trypsin-EDTA (Invitrogen) and resuspended in 10 ml Opti-MEM I (Invitrogen). Cells were counted and seeded into each well of a six-well tissue culture plate (3 ml per well with 1×10^6 cells). For co-culture of 293T with SD-PJEC or MDCK cells, cells were seeded at a 3 : 1 ratio for 293T/MDCK and 3 : 1 ratio for 293T/SD-PJEC. Cells were incubated at 37 °C for 16–18 h, and transfected with 1 μg each plasmid DNA using the Fugene HD reagent (Promega) following the manufacturer's instructions. Six hours post-transfection, the DNA-transfection mixture was replaced by Opti-MEM I. At 30 h post-transfection, 1 ml Opti-MEM I containing TPCK-treated trypsin (1 μg ml^{-1} for MDCK and 0.1 μg ml^{-1} for SD-PJEC) was added to the cells. At 24, 36, 48 and 60 h post-transfection, 200 μl of culture supernatant was collected at each time point. The amount of viruses present in the supernatant was determined by titration on MDCK cells and virus titres were calculated as $TCID_{50}$ ml^{-1}.

Statistical analysis. Statistical analysis was performed using GraphPad InStat version 3.06 (GraphPad Software). Comparison was performed by one-way analysis of variance with Tukey's multiple comparison tests to determine the mean significance. Differences between treatment groups were considered statistically significant at $P<0.05$.

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