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The crucial role of bile acids in the entry of porcine enteric calicivirus

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Replication of porcine enteric calicivirus (PEC) in LLC-PK cells is dependent on the presence of bile acids in the medium. However, the mechanism of bile acid-dependent PEC replication is unknown. Understanding of bile acid-mediated PEC replication may provide insight into cultivating related human noroviruses, currently uncultivable, which are the major cause of viral gastroenteritis outbreaks in humans. Our results demonstrated that while uptake of PEC into the endosomes does not require bile acids, the presence of bile acids is critical for viral escape from the endosomes into cell cytoplasm to initiate viral replication. We also demonstrated that bile acid transporters including the sodium-taurocholate co-transporting polypeptide and the apical sodium-dependent bile acid transporter are important in exerting the effects of bile acids in PEC replication in cells. In summary, our results suggest that bile acids play a critical role in virus entry for successful replication.

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Introduction

Calicivirus is a non-enveloped virus with a diameter of 27–35 nm and possess a single-stranded, positive sense RNA genome of 7–8 kb. The family Caliciviridae consists of five genera: norovirus, sapovirus, lagovirus, vesivirus and nebovirus (Carstens, 2010). Noroviruses and sapoviruses cause gastroenteritis in humans and animals, whereas lagoviruses and vesiviruses mostly infect animals causing a variety of diseases (Green et al., 2001). Norovirus infection usually occurs as epidemic gastroenteritis outbreaks and affects 10–21 million people in all age groups in the U.S. each year (CDC, http://www.cdc.gov/norovirus/trends-outbreaks.html). Therefore, norovirus is recognized as the major etiological agent of foodborne and waterborne infections in humans. Human noroviruses have remained uncultivable to date, and it has been a major hindrance to research on viral pathogenesis and development of vaccines and antivirals for norovirus infection. Cultivable caliciviruses include murine norovirus (MNV) (Wobus et al., 2004), porcine enteric calicivirus (PEC) (Flynn and Saif, 1988), feline calicivirus (FCV) and Tulane virus (primate calicivirus) (Farkas et al., 2008). PEC was first isolated in primary porcine kidney cells (Flynn and Saif, 1988) and subsequently in continuous porcine kidney cell line (LLC-PK cells) in the presence of the intestinal content (IC) (Parwani et al., 1991). Later it was found that bile acids in IC were responsible for PEC replication (Chang et al., 2004). The requirement of IC or bile acids in virus replication in cell culture is a unique phenomenon for PEC and implies that biologically important interactions may occur between bile acids and PEC in the intestines (Flynn et al., 1988). Bile acids were also shown to play important roles in the replication of some viruses propagating in the bile rich organs, such as the liver and the intestines. Bile acids were reported to promote hepatitis B and C virus replication (Chang and George, 2007; Chhatwal et al., 2012; Kim et al., 2010; Scholtes et al., 2008) but to inhibit rotavirus replication (Kim and Chang, 2011). Unlike these viruses which are cultivable without addition of bile acids, PEC replication is completely dependent on the presence of bile acids in the medium. While it was previously reported that protein kinase A (PKA) pathway and/or innate immunity elicited by IC or bile acids is involved in supporting PEC replication in LLC-PK cells (Chang et al., 2002, 2004), the detailed mechanism of bile acids in supporting PEC replication is yet to be determined.

Bile acids are amphipathic molecules which are synthesized from cholesterol in the liver. Bile acids are the active constituents of bile and essential for solubilization and absorption of dietary lipids in the digestive tract (Johnson, 1998). In addition to their role in lipid absorption, bile acids are also involved in various metabolic processes, such as cholesterol and lipid homeostasis, and inflammatory process by acting as signaling molecules (Schaap et al., 2014). Primary bile acids, such as cholic acid (CA) and chenodeoxycholic acid (CDCA) and their glycine and taurine conjugates, are synthesized in the liver and excreted into the intestinal tract (Johnson, 1998). Subsequently, secondary bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA) and their glycine and taurine conjugates, are produced by intestinal bacteria (Johnson, 1998). The total bile acid concentrations range from

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The enterohepatic circulation of bile acids involves various bile acid transporters that include the sodium-taurocholate cotransporting polypeptide (NTCP) and the apical sodium-dependent bile acid transporter (ASBT) expressed in the liver and the intestines (Dawson et al., 2009; Trauner and Boyer, 2003). These bile acid transport proteins are important in maintaining bile acid pool in the enterohepatic circulation and extra-hepatic tissues. Bile acids also bind to specific bile acid receptors to exert various metabolic regulation and bile acid homeostasis (Schaap et al., 2014). These bile acids receptors include a farnesoid X receptor (FXR) and a G-protein-coupled receptor (G protein-coupled bile acid receptor, TGR5), which are involved in glucose and lipid metabolism as well as in inflammation (Schaap et al., 2014). In the present study, we investigated the effects of bile acid on PEC replication cycle using various methods including time-of-addition study, gene knockout and activation study, and a confocal microscopy.

**Results**

**Growth kinetics of PEC in the presence or absence of bile acids**

To determine bile acid-dependent steps in PEC entry and replication, one step replication studies were conducted by infecting LLC-PK cells with PEC at a high MOI. At up to 4 h PI, viral genome copy numbers were comparable between virus-infected cells with or without GCDCA, indicating that viral attachment and uptake is not influenced by GCDCA (Fig. 1A). The viral RNA levels in the cells incubated with GCDCA increased steadily from 4 h to 12 h PI (Fig. 1A). However, there was no indication of virus replication over time in the cells without GCDCA.

Viral titers determined by the TCID<sub>50</sub> method at each time point were in line with the viral RNA levels in all samples (Fig. 1A): viral titers were comparable in the cells incubated with or without GCDCA up to 4 h PI, but viral titers increased from 4.8 to 6.04 log<sub>10</sub> TCID<sub>50</sub>/ml at 8 and 12 h PI, respectively, only in the cells incubated with GCDCA (Fig. 1A). In line with these results, extensive CPE started to appear at 12 h PI (Fig. 1A). However, there was no evidence of viral replication when GCDCA was added at 4 h PI (treatment e) (Fig. 2B). Notably, GCDCA was most efficient in inducing viral replication when it was present during 1 h of viral inoculation (treatment b) (Fig. 2B). We also examined if bile acids and/or low pH have deleterious effects on viral particles. Incubation of concentrated PEC with GCDCA (200 μM) at pH 7.0 and 5.0 did not affect virus replication (data not shown). In addition, pre-incubation of cells with GCDCA (treatment a) for 30 min did not lead to virus replication (Fig. 2A and B).

**Transfection of viral RNA does not require bile acids to produce infectious viruses**

To investigate if bile acids are involved in the virus replication steps following the release of virus genome in the cytoplasm, we transfected LLC-PK cells (permissive) with high concentrations of PEC RNAs and incubated the cells in the presence and absence of GCDCA. In addition, MARC-145 cells (non-permissive) were transfected with PEC RNA in the absence of GCDCA. MARC-145 cells are not susceptible to PEC infection by a usual route of virus infection (incubation of virus in cell culture) regardless of the presence of bile acids, thus described as non-permissive cells for PEC. At 12 h post-transfection, viral protein expression (2AB, POL and VPg) was evident in the LLC-PK cells incubated with or without GCDCA, and also in MARC-145 cells (only LLC-PK cells incubated without GCDCA are shown in Fig. 3B). Virus titration conducted at 16 h post-transfection demonstrated the production of infectious viruses following transfection in both cell lines (Fig. 3A). These results indicate that GCDCA affects the early steps of PEC replication that occur prior to viral replication in the cytoplasm.

**Bile acid is required for PEC escape from the late endosomes into the cytoplasm**

We examined the entry event of PEC in LLC-PK cells in the presence and absence of GCDCA by studying the subcellular localization of virus with a confocal microscope. At 1 h PI, little immunofluorescence staining for PEC was observed in the cells incubated with GCDCA under confocal microscopy (Fig. 4A). However, positive staining for PEC was observed in the cytoplasm in the cells incubated without GCDCA, or cells incubated with GCDCA and chloroquine at 1 h post-transfection (Fig. 4A). In these cells, confocal microscopy analyses demonstrated colocalization of virus with rab7, a late endosomal marker (Fig. 4A, panels h and p). At 4 and 6 h PI, viruses colocalized with rab7 were still observed by confocal microscopy (Fig. 4B). In the next experiments focusing on the first one hour following virus infection, we observed that fluorescent virus particles gradually decreased over time during 5–60 min PI in the cells incubated with GCDCA (Fig. 4C). ImageJ-colocalization (MBF) analysis and JACoP plugin was used for colocalization analysis. The thresholds were set by the Costes’ Auto threshold method and the thresholded Manders’s split colocalization coefficients were determined. The split coefficient for colocalization of virus particles (green) with rab7 (red) in the confocal images were found to be > 0.90 for all experiments indicating a high degree of colocalization of viral particles with rab7. These results demonstrated that PEC is able to reach the late endosomes without the help of bile acids, but viral escape from the late endosomes requires the presence of bile acids. Our finding on the negating effects of chloroquine in bile acid-supported PEC replication was confirmed by virus titration of the cells treated with chloroquine (data not shown).
Bile acid transporters are involved in bile acid-mediated PEC replication

The siRNA-mediated gene silencing was performed using siRNA of NTCP, ASBT or FXR, with maximal silencing of > 90% (Fig. 5A). Transfection of NTCP or ASBT siRNAs significantly reduced GCDCA-mediated virus replication by more than 80%, measured by real-time qRT-PCR (Fig. 5A). When CDCA was used, the level of reduction in PEC replication was similar to that with GCDCA (data not shown). However, transfection of siRNAs of FXR or irrelevant siRNA did not lead to a significant reduction in PEC replication (Fig. 5A). Treatment of cells with individual or combinations of FXR or TGR5 agonists, GW4064 or oleanolic acid, respectively, did not have any effect in PEC replication (Fig. 5B), indicating that FXR and TGR5 are not involved in bile acid-mediated PEC replication.

The siRNA-mediated gene silencing of NTCP or ASBT, followed by virus infection and determination of the virus titers at 1 h PI showed no significant change in virus titers between mock-transfected control cells and siRNA-transfected cells. This result suggests NTCP and ASBT do not function as receptors for PEC, and the reduction of PEC replication by siRNA of NTCP and ASBT was not due to the inhibition of PEC entry.

Discussion

Enteric caliciviruses are important diarrheal agents in humans and animals. In humans, norovirus, an enteric calicivirus in the Norovirus genus, is responsible for the majority of diarrheal outbreaks of food- and water-borne viral gastroenteritis (Green,
However, inability to grow human norovirus in cell culture has been a major obstacle in vaccine and antiviral drug development. PEC is an enteropathogenic calicivirus (Flynn et al., 1988) which requires IC as a supplement in the medium for virus culture (Flynn and Saif, 1988; Parwani et al., 1991). It was previously reported by our group that bile acids are the active factors in IC essential for PEC replication (Chang et al., 2004). The effects of bile acids in enhancing or inhibiting the replication of some viruses in the bile-acid rich organs, such as hepatitis B and C viruses and rotavirus, have been reported, suggesting that bile acids may act as important factors in modulation of viral replication (Chang and George, 2007; Chhatwal et al., 2012; Kim et al., 2010; Kim and Chang, 2011; Scholtes et al., 2008). However, absolute requirement of bile acids for viral replication in cell culture is unique for PEC. In this study, we investigated the effects of bile acids on the replication cycle of PEC by time-of-addition study, gene knockout and agonists study, and confocal microscopy.

First, one-step PEC replication kinetics with high MOI of virus and bile acids was established for virus entry and the time-of-addition studies (Fig. 1). The time-of-addition study showed that the critical time points when bile acids are most effective are the early stages of virus replication (Fig. 2), demonstrated by the increase in viral RNA levels and viral proteins when CDCA or GCDCA was added within 2 h following virus infection. The following PEC RNA transfection study showed that bile acids are not required at the virus replication steps subsequent to the release of virus genome into the cyttoplasm, suggesting that bile acids are involved in the very early stages of virus replication cycle (Fig. 3). It is of note that we previously observed that transfection of LLC-PK cells with PEC RNA did not result in the detection of virus replication when the transfection was performed without bile acids (Chang et al., 2004). We speculate that the discrepancy between the previous and current results may have arisen from different experimental condition. In the previous study, low virus RNA level and multi-cycle virus replication (up to 120 h PI) allowed virus detection by enzyme-linked immunosorbert assay (ELISA) only in the cells incubated with bile acids through subsequent multi-cycle virus replication. In this study, higher virus RNA concentrations and a single round of virus replication were used to determine which virus replication steps require the presence of bile acids. When we repeated the transfection study using low RNA concentrations, we found that virus in the transfected cells incubated without bile acids failed to propagate through multi-cycle virus replication. The results from our single-round virus replication study using PEC-transfected cells suggest that bile acids are not required after virus genome is released into the cytoplasm.

Since our observation indicated that bile acids are involve in the early virus replication steps preceding virus replication in the cytoplasm, we then studied virus trafficking in PEC-infected LLC-PK cells incubated in the presence or absence of GCDCA by confocal microscopy at various time points following virus infection. Without GCDCA in the medium, PEC was observed to remain in the late endosome even at 6 h PI. In contrast, PEC gradually disappeared from the late endosome during the first hour of virus infection (Fig. 4A–C). We did not track the fate of the trapped virus particles in the cells incubated without GCDCA at more than 6 h PI, but it seems that the trapped viruses remain infective for at least 2 h PI, based on our time-of-addition study. Nonetheless, viruses that remain in the late endosomes until they fuse with lysosome would be degraded. We also found that inhibition of endosomal maturation by chloroquine abolished the effects of bile acids in PEC replication, as observed with a confocal microscope (Fig. 4A, panels m–p). This result indicates that endosomal low pH is indispensable for the function of bile acids in PEC escape from the late endosome. Inhibition of endosomal acidification by chloroquine and bafilomycin A1 in cell culture supplemented with bile acid led to significantly reduced PEC replication (at MOI of...
0.1–1 or > 50) compared to control in our laboratory (data not shown), confirming the important role of endosomal pH in the function of bile acids in supporting PEC replication.

There are numerous reports that viruses entering the host cells via receptor-mediated endocytosis must escape from the endosomes before fusion of late endosomes with lysosomes occur. This endosomal escape is critical in the viral entry process and virus replication, and viruses utilize different mechanisms to translocate to the cytoplasm or other cellular compartments to initiate viral genome replication (Grove and Marsh, 2011; Gruenberg and van der Goot, 2006; Marsh and Helenius, 2006). Many enveloped viruses that utilize host cell’s endocytic pathway, such as influenza viruses, coronaviruses and alphaviruses, have a fusion protein which is activated at a low-pH environment of the endosomes and undergoes fusion with the endosomal lipid membranes (Grove and Marsh, 2011). In addition to a low-pH in the late endosomes, some viruses such as severe acute respiratory syndrome (SARS) coronavirus and Ebola virus also require their fusion proteins to be cleaved by cellular proteases residing in the endosomes, such as cathepsin L and B, to expose a putative fusion domain (Chandran et al., 2005; Cote et al., 2011; Grove and Marsh, 2011). Non-enveloped viruses do not undergo fusion but have distinct but less known mechanisms for endosomal escape. For reovirus, endosomal cathepsin activity is important during virus entry, since it removes the outer-capsid protein σ3, exposing the viral membrane-penetration protein M1 for uncoating and endosomal escape (Baer et al., 1999; Ebert et al., 2002, 2004; Johnson et al., 2009). For parvovirus, a lipolytic enzyme (phospholipase A2, PL2A) at the N-terminus of virus structural protein VP1 is implicated in catabolization of phospholipid in the endosomal membrane, leading to translocation of viral genome in the cytoplasm (Farr et al., 2005; Zadori et al., 2001). In this study, our results suggest that bile acids play a crucial role in the endosomal escape of PEC. This is the first report that bile acids are involved in virus trafficking during the entry events.

Bile acids are taken up into the cells by bile acid transporters and exert various biological functions by acting on bile acid receptors (Dawson et al., 2009). The LLC-PK cells we used in this study for PEC infection originated from porcine renal proximal tubular cells and support PEC replication in the presence of bile acids. This cell line expresses mRNAs of NTCP and ASBT, two important sodium-dependent bile acid transporters, and we found that gene silencing of either transporter mRNA led to a significant reduction in PEC replication (Fig. 5A). NTCP and ASBT are expressed in various cell types such as hepatocytes, intestinal enterocytes, renal proximal tubular cells and function to maintain the enterohepatic circulation of bile acids in the body (Dawson et al., 2009; Trauner and Boyer, 2003). These bile acid transporters reside on the cell plasma membrane and recycle back to the cell surface once internalized (Alpini et al., 2005; Grune et al., 1993; Mukhopadhayay et al., 1997; Reymann et al., 1989). Previously, our group reported that PKA signaling pathway is important in bile- or IC-mediated PEC replication (Chang et al., 2002, 2004). Since recycling of bile acid transporters is regulated by cAMP-dependent PKA pathway (Alpini et al., 2005; Grune et al., 1993; Mukhopadhayay et al., 1997; Reymann et al., 1989), it seems plausible that the importance of cAMP-dependent PKA pathway in bile acid-mediated PEC replication stems from its involvement in recycling of bile acid transporters in the cells. However, further study is required to clarify the roles of PKA pathway in bile entry.

![Fig. 3. Transfection of PEC RNA into the permissible and non-permissible cells. (A) One-day old LLC-PK cells or MARC-145 cells were transfected with PEC RNA genome (0.5 μg/well) in the presence or absence of GCDCA (100 μM). At 16 h post-transfection, recovery of infectious virus was determined by the TCID50 method. (B) One-day old LLC-PK cells were transfected with PEC RNA genome or medium (mock) and incubated without GCDCA. At 12 h post-transfection, the cells were fixed and probed with antibodies for viral proteins 2AB, POL and VPg in an IFA assay.](image-url)
Fig. 4. Confocal laser scanning microscopic examination of PEC entry. Confluent LLC-PK cells grown on Lab-Tek II CC² chamber slides were infected either with mock (medium) or PEC (MOI 50) in the presence or absence of GCDCA (100 µM), fixed and stained at 1 h PI (A) or at 4 or 6 h PI (B) and observed under a confocal laser scanning microscope. (A) Top panels (a–d) show the mock-infected cells without GCDCA; middle panels (e to l) show the cells infected with PEC with or without GCDCA; bottom panels (m–p) show the cells infected with PEC in the presence of GCDCA and chloroquine. (B) Top panels (a–d) show the cells infected with PEC without GCDCA and observed at 4 h PI; bottom panels (e–h) show the cells infected with PEC without GCDCA and observed at 6 h PI. (C) Cells were infected with PEC (MOI 50) for 1 h, then treated with GCDCA for 5 min (a–d), 15 min (e–l), 30 min (i–l) or 60 min PI. (A–C) Cells were fixed and probed with rabbit polyclonal anti-Rab7 and swine polyclonal anti-PEC VLP primary antibodies and detected by PerCP-Cy5.5 labelled goat-anti-rabbit antibody (red) and FITC labelled goat-anti-swine antibody (green). Nuclei were stained with sytox orange (5 µM) (pseudo colored blue), and merged images for PEC and Rab7 were prepared. In the merged images, colocalization of PEC (green) and Rab7 (red) appears in white by using ImageJ.
acid-supported PEC replication. In addition to acting as a bile acid transporter, NTCP is also identified as a functional receptor for human hepatitis B and D viruses (Seeger and Mason, 2013; Xiao et al., 2013; Yan et al., 2012). We found that NTCP and ASBT do not function as receptors for PEC, since silencing of these genes did not abrogate the internalization of PEC into LLC-PK cells. We also found that the effects of bile acids in supporting PEC replication is not mediated by bile acid receptors FXR or TGR5. FXR or TGR5 are important mediators of various biological functions elicited by bile acids, including bile acid homeostasis, lipid and carbohydrate metabolism, innate immunity, and inflammation (Dawson et al., 2009), and FXR was reported to be involved in the replication rotavirus (Kim and Chang, 2011).

Based on our findings, we propose a model for bile acid-mediated PEC replication (Fig. 6). In this model, PEC enters the cells through endocytosis and travels to the late endosomes, from where virus or virus genome must escape with the help of bile acids to initiate replication. In the absence of bile acids, virus fails to exit the late endosomes and is degraded later. The mechanism of endosomal escape of PEC and the roles of bile acids in the process remain to be elucidated with further study. This model does not exclude the potential direct or indirect effects of bile acids during later stages of virus replication cycle and host-virus interactions (such as innate immunity). In summary, we demonstrated for the first time that bile acid-supported PEC replication is associated with viral endosomal escape. This novel finding may provide insight into mechanisms of endosomal escape for caliciviruses and other viruses.

Materials and methods

Cells and virus

Cell culture adapted PEC Cowden strain was propagated in LLC-PK cells with Eagle's Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Virus infection was performed in the presence of GCDCA or CDCA at a final concentration of 100 μM (Chang et al., 2004). MARC-145 cells were also maintained in MEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Reagents and antibodies

GCDCA and CDCA were purchased from Sigma-Aldrich (St. Louis, MO) and resuspended in distilled water or dimethyl sulfoxide (DMSO), respectively, at 100 mM. Hyperimmune guinea pig sera raised against recombinant PEC 2AB, polymerase (POL)
and VPg and hyperimmune swine sera raised against PEC virus-like particles (Chang et al., 2005) were used in this study. Rabbit polyclonal anti-Rab7 serum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies including FITC conjugated rabbit anti-guinea pig or anti-pig IgG and PerCP-Cy5.5 labelled goat anti-rabbit IgG were purchased from commercial sources including Sigma-Aldrich and Santa Cruz Biotechnology. Sytox orange was obtained from Molecular Probes (Bedford, MA). Chloroquine, GW4064, and oleanolic acid were also purchased from Sigma-Aldrich. Other basic chemicals for confocal microscopy and other studies were purchased from various sources including Sigma-Aldrich.

PEC replication kinetics with a high multiplicity of infection (MOI)

High concentrations of PEC were obtained by concentrating viruses through a 40% sucrose cushion. Confluent LLC-PK cells supplemented with GCDCA or CDCA (100 µM, final concentration) were inoculated with PEC at an MOI of 50. We used GCDCA or CDCA in our experiments, since they are most efficient among bile acids in supporting PEC replication (Chang et al., 2004). After incubation for 48 h following transfection, cells were infected with PEC at an MOI of 50, and incubated in the presence of GCDCA (100 µM) for 1 h. Virus infected cells were then further incubated following washing with PBS, and total RNA was collected at 12 PI to assess viral replication and gene knockdown by qRT-PCR. Asterisks indicate a significant ($p < 0.05$) difference in the PEC genome levels, compared to mock (PEC). (B) The effects of an agonist of FXR (GW4064) or TGR5 (oleanolic acid) in PEC replication. Individual or combinations of agonists was added at various concentrations, and viral replication was monitored by real time qRT-PCR at 12 h PI.

Determination of PEC replication

Virus titration

The virus-infected cells were harvested by three repeated freeze-thaw cycles at different time points. Ten-fold serial dilutions of each sample were used to infect 3-day old confluent LLC-PK cells in 96-well plate in triplicates in the presence of GCDCA (100 µM). After incubation at 37 °C for up to 5 days, the TCID$_{50}$ values were determined by the Reed–Muench method (Reed and Muench, 1938).

Real-time qRT-PCR

Before extensive cell cytopathic effects (CPE) occur, total RNA was extracted at 5 min, 4, 8 or 12 h PI from the infected cells using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer protocol. Real-time qRT-PCR was performed by using One-Step Platinum qRT-PCR kit (Invitrogen, Carlsbad, CA) using a forward primer, 5'-AATGAGCTCCAGACAGTCA-3', a reverse primer, 5'-CCAGTGACATTTGTTGAG-3', and a probe, 5'-56-FAM/TGGCAACGGCCATTTCCAGA/3IABkFQ-3', targeting the capsid VP1 gene. The qRT-PCR amplification was performed in a Rotor-Gene Q (Qiagen) with the following conditions: 50 °C for 30 min and 95 °C
for 5 min, then 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s and elongation at 72 °C for 30 s. To calculate PEC genome copy number in a sample, the Ct values of a given sample were normalized against β-actin and then plotted against a standard curve, which was generated using 10-fold dilutions of in vitro-transcribed PEC RNA genome from the infectious clone (Chang et al., 2005).

**Immunofluorescence assay (IFA)**

For analysis of viral protein production, confluent LLC-PK cells grown in 96 well plates were infected with PEC (50 MOI) in the presence or absence of GCDCA or CDCA (100 μM). At 12 h post-infection (PI), the infected cells were fixed with chilled methanol for 10 min, washed with PBS, and incubated with primary antibody diluted at 1:200 (100 μl/well) for 2 h at 37 °C. The plates were then washed three times with PBS and incubated with FITC-conjugated rabbit anti-guinea pig IgG antibody (Sigma-Aldrich) (1:100 dilution) for 1 h at 37 °C. After incubation, the plates were washed with PBS and visualized under a fluorescent microscope.

**Time-of-addition assay**

To determine the replication steps of PEC that are dependent on bile acids, time-of-addition assays were performed. Confluent LLC-PK cells were infected with PEC (50 MOI) and incubated with GCDCA or CDCA (100 μM) for different time periods: for 30 min prior to virus infection; for 1 h during virus infection; or GCDCA is added to the cells at 1, 2, or 4 h PI. All cells were washed 3 times with PBS and fresh media were added before and after virus infection. At all times, cells were incubated at 37 °C, and cell lysates were prepared for real-time qRT-PCR at 6 or 12 h PI. The direct effects of GCDCA on PEC infectivity were also investigated at different pH. Concentrated virus suspension (> 10^11 TCID_{50}/ml) was incubated with PBS or GCDCA (200 μM) at pH 5.0 or 7.0 at 37 °C for 2 h. The mixtures were then diluted 100 times to reduce the concentration of GCDCA to below that is required for PEC replication. The diluted mixture was inoculated into LLC-PK cells in the absence or presence of GCDCA (100 μM), and virus replication was determined by real-time qRT-PCR.

**Transfection of viral RNA**

Viral RNA was extracted from the concentrated virus suspension using the RNeasy kit (Qiagen). The extracted viral RNA (0.5 μg/well) was then transfected into one day old LLC-PK cells or MARC-145 cells (non-permissible cells for PEC) in six-well plates using Lipofectamine 2000® (Invitrogen, Carlsbad, CA). As a control, mock-medium was mixed with transfection reagent and the mixture was transfected into the cells. The transfected plates were further incubated at 37 °C with or without 100 μM GCDCA. Viral titers from the transfected cells were determined at 16 h post-transfection using the TCID_{50} method. For IFA, viral RNA-transfected cells in 96-well plates were fixed with cold methanol at 12 h post-transfection and further probed with the primary and secondary antibodies.

**Confocal laser scanning microscopy**

LLC-PK cells were seeded onto Lab-Tek II CC² chamber slide (Fisher Scientific, Pittsburgh, PA) treated with PBS and grown to 90%
The roles of bile acid transporters and receptors in PEC replication

To study the roles of bile acid transporters and receptors, siRNA knockdown assay and mRNA quantitation by real time qRT-PCR were performed. The design of siRNA, and primers and probes of porcine genes for qRT-PCR are based on the following sequences; porcine NTPC: XM_001927695.2; porcine ASBT: NM_001244463.1; and porcine FXR: XM_003481738.1. The siRNA and primers and probes were synthesized by Integrated DNA Technology (Coralville, IA). The sequences for siRNA, primers and probes are listed in Table 1. For siRNA study, one-day old semi-confluent LLC-PK cells were transfected with siRNA of mock (transfection agents), NTCP, ASBT, FXR, or an irrelevant siRNA (Qiagen) (100 μM each), and incubated at 37 °C for 24 or 48 h. The confluent monolayers were infected with PEC at an MOI of 50 with GCDCA or CDCA (100 μM each) and virus replication was determined at 12 h PI with real time qRT-PCR. Down-regulation of each gene was determined by real time qRT-PCR.

To study the roles of FXR and TGR5 in PEC replication, GW4064 and oleanolic acid was used as an agonist for FXR or TGR5, respectively (Maloney et al., 2000; Sato et al., 2007). Various concentrations of GW4064, oleanolic acid or their combinations were added to LLC-PK cells infected with PEC at pre-, during and post-infection, and virus replication was monitored at 12 h PI by real time RT-PCR. Virus titers were also determined at 16 h PI with the TCD50 assay. Since NTCP is also identified as a functional receptor for human hepatitis B and D viruses (Seeger and Mason, 2013; Xiao et al., 2013; Yan et al., 2012), we tested if NTCP or ASBT is utilized by PEC as a receptor. One-day old semi-confluent LLC-PK cells were transfected with siRNA of NTCP or ASBT (100 μM each) and further incubated at 37 °C for 24 or 48 h. The confluent monolayers were then infected with PEC at an MOI of 50 with or without GCDCA (100 μM). After 1 h incubation at 37 °C, cells were treated with trypsin (0.25%) for 5 min at RT to remove viruses on the cell surfaces. Cells were then washed 3 times with PBS and total RNA were isolated and subjected to qRT-PCR for detection of PEC genome.

**Statistical analysis**

All results shown are the means from at least three independent experiments. The effects of bile acids or siRNA treatment on PEC replication were analyzed by Student’s t-test. Results were considered statistically significant when the p value was < 0.05.

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