STUDIES ON ENTRY EVENTS DURING CALICIVIRUS REPLICATION

by

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B.V.Sc. & A.H., KVAFSU, India, 2007
M.V.Sc., KVAFSU, India, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
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Abstract

Caliciviruses are important pathogens of humans and animals. Noroviruses are major causes of foodborne gastroenteritis cases, but their research is hindered due to the inability to grow human noroviruses in cell culture. Detailed studies on entry events of caliciviruses are lacking and may be crucial for development of cell culture models. We examined the entry events of caliciviruses using porcine enteric calicivirus (PEC), feline calicivirus (FCV) and murine norovirus-1 (MNV-1). PEC replication in LLC-PK cells requires bile acid in the medium, but the mechanism is not well understood. Our studies showed that bile acids are required in the early stage of virus replication, and while internalization of PEC is not dependent of them, they are required for endosomal escape and successful replication. Further examination on virus entry, we demonstrated that endosomal acidification and cathepsin L activity are essential in the replication of PEC, FCV and MNV-1. The results showed that inhibition of endosomal acidification or cathepsin L activity led to retention of viruses in the endosomes. Also we demonstrated that recombinant cathepsin L cleaved structural protein of PEC, FCV or MNV-1, which suggests that the enzyme may facilitate uncoating viruses in endosomes. In addition to bile acids, we found that a cold shock treatment during virus entry supported PEC replication by facilitating the endosomal escape. While PEC alone did not induce ceramide formation, bile acids or cold shock treatment induce ceramide formation on endosomes through activation acid sphingomyelinase (ASM), and this event was crucial for virus replication because inhibition of ASM blocked ceramide formation and significantly reduced PEC replication. Incubation of FCV or MNV-1 with cells led to ceramide formation during virus entry, and inhibition of ASM also significantly reduced their replication. Inhibition of ASM led to endosomal retention of PEC,

FCV or MNV-1 during virus entry, which may be the reason for the reduction of viral replication. These studies revealed the important and common events during calicivirus entry for successful replication, including virus endosomal escape, cathepsin L activity and ASM/ceramide formation. This detailed information may provide clues for understanding the replication of fastidious caliciviruses and for potential therapeutic targets.

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Dedication

I would like to dedicate this dissertation to my wife, my parents and my brother for all their love, support and constant encouragement.

Chapter 1 - Literature Review and Significance

1.1 Calicivirus: Introduction and historical perspective

The viruses in the family *Caliciviridae* are non-enveloped viruses with a diameter of 27-35 nm, and possess a single stranded, positive-sense RNA genome of 7 – 8 kb. The name calicivirus is derived from the Latin word –calyx- for its cup shaped depressions on the capsid seen under electron microscopy (Zwillenberg and Bürki, 1966). Caliciviruses belong to class IV of the Baltimore scheme of virus classification (Baltimore, 1971). The vesicular exanthema of swine virus (VESV) which affects pigs, was the first identified calicivirus in California in 1932 (Hopkins, 1958). The feline calicivirus (FCV) was first isolated from feline kidney cells as a contaminant that was inducing a different phenotype in cells during routine infection with feline panleukopenia virus (Fastier, 1957). FCV were initially designated as feline picornaviruses (Bürki, 1965) but was later classified when *Caliciviridae* family was created in 1976 (Fenner, 1976). Rabbit hemorrhagic disease virus (RHDV) was discovered in china in 1984 in an outbreak that killed 140 million domestic rabbits spread over an area of 50000 km^2 (Liu SJ, 1984) and later in several other countries worldwide. Human norovirus was first identified in 1972 from an acute gastroenteritis outbreak in Norwalk, Ohio in 1968 that was then referred to as “winter vomiting disease” (Kapikian, 2000). Human sapovirus was first isolated from a gastroenteritis outbreak in an orphanage in Sapporo, Japan, 1977. Sapovirus is mainly associated with outbreaks of infantile gastroenteritis. Human norovirus outbreaks are commonly encountered in cruise ships, hotels and places such as schools, hospitals and old-age homes. Human noroviruses and sapoviruses are the leading cause of acute gastroenteritis in humans worldwide.

Calicivirus taxonomy and genome organization

The family *Caliciviridae* is classified into five genera: *Vesivirus, Lagovirus, Sapovirus, Norovirus* and *Nebovirus* (ICTV, 2012). The genus *Norovirus* consists of viruses that are genetically and serologically related to Norwalk virus. The genus is named after Norwalk virus which was the first norovirus identified from an outbreak in Norwalk, USA (Adler and Zickl, 1969; Kapikian, 2000) and noroviruses are the major cause of acute gastroenteritis outbreaks in humans (Patel et al., 2009). The proposed nomenclature for enteric caliciviruses is: species infected/virus genus/virus name/strain designation/year of isolation/country of isolation (Atmar and Estes, 2001) . Noroviruses and sapoviruses are further classified into genogroups and further down to genotypes or clusters based on the phylogenetic analysis of partial RNA-dependent-RNA-polymerase and complete capsid sequences of noroviruses and sapoviruses. The nucleotide differences among the strains within a genotype is around 0-15%, between genotypes is around 15-45% , between genogroups is around 46-62% and between genus is 84-86% (Ando et al., 2000; Okada et al., 2006; Schuffenecker et al., 2001; Smiley et al., 2003; Wang et al., 2005b; Wang et al., 2005c; Zheng et al., 2006a). Noroviruses have been reported to cause enteric diseases in animals including cows, pigs and dogs (Di Bartolo et al., 2011; Martella et al., 2008; Mattison et al., 2007; Wang et al., 2005a). Noroviruses are further classified into 5 genogroups (G) based on their nucleotide sequence of capsid protein (Zheng et al., 2006a). The GI is composed of human strains, GII is composed of human and porcine strains and GIII consists of 2 bovine strains, GIV consists of a human strain and GV is represented by murine norovirus-1.

The genus *Sapovirus* derives its name from its representative member Sapporo virus that was first isolated from an human outbreak in Sapporo, Japan in 1977 (Chiba et al., 1979). Sapoviruses are also shown to infect pigs causing enteric diseases (L'Homme et al., 2010). Sapoviruses are also classified into 5 genogroups based on their capsid nucleotide sequences.

Genogroups GI, GII, GIV and GV consist of human strains and GIII consist of PEC/Cowden strain (Farkas et al., 2004; Schuffenecker et al., 2001). The porcine sapovirus has been adapted to cell culture (Flynn and Saif, 1988) but its propagation requires the presence of bile acids (Chang et al., 2004).

The genus *Lagovirus* consists of viruses that infect only lagomorphs from which the genus gets its name. The rabbit hemorrhagic disease virus (RHDV) causes a fatal disease in rabbits with hemorrhagic lesions in the liver and lung and a high mortality rate (Ohlinger et al., 1990). A non-pathogenic form of this virus has been found circulating in rabbit populations in Europe, Australia and New Zealand (Le Gall-Recule et al., 2011) which can cross-protect against the highly virulent form of the disease (Capucci et al., 1996). The European Brown hare syndrome virus also causes high mortality infections (Wirblich et al., 1994). It was first discovered from infected hares in Sweden in 1980s, the lesions were similar to RHDV characterized by lesions in liver with high mortality rate (Gavier-Widen and Morner, 1993).

The genus Vesivirus is named after the vesicular lesions caused by feline calicivirus (FCV) and vesicular exanthema of swine virus (VESV). FCV infections range from mild oral and ocular lesions to fatal systemic infections in cats (Hoover and Kahn, 1975; Pedersen et al., 2000). VESV causes vesicles in the mouth, nose, extremities and ears with fever in pigs (Smith et al., 1973). San Miguel sea lion virus (SMSV), also a member of the Vesivirus causes similar lesions in marine animals (Smith et al., 1973). SMSV infection of swine produced disease similar to VESV (Smith et al., 1973; Smith et al., 1980). Phylogenetic analysis shows that SMSV and VESV are closely related but distinct from FCV (Neill et al., 1995).

The newly identified Nebovirus is named after the town Newbury, UK and Newbury-1 virus, its only member was isolated from the feces of calf with acute enteritis (Bridger et al.,

1984; Woode and Bridger, 1978). The full genome sequencing reveled only two open reading frames (ORFs) and phylogenetic analyses showed the virus clustered as a separate clade (Carstens and Ball, 2009; Oliver et al., 2006).

The genomes of all caliciviruses contain a short nontranslated region (NTR) of 4-75 nucleotides that starts with GU and a part of the NTR is repeated internally near to the transcription start site of subgenomic RNA (Farkas et al., 2008). All calicivirus genomes are covalently linked to a protein termed as virus protein genome (VPg) at their 5' end (Burroughs and Brown, 1978) and are polyadenylated at their 3' end (Ehresmann and Schaffer, 1977). The number of open reading frames in the calicivirus genome varies from two to four and irrespective of which, the ORF1 encodes for non-structural polyprotein which is co- or post-translationally cleaved by the viral 3C-like protease to form mature non-structural proteins. The ORF2 in norovirus and vesivirus encodes the major capsid protein VP1 and ORF3 encodes for the minor capsid protein VP2. The sapovirus and lagovirus genomes are slightly different with VP1 protein produced in frame at 3' end of the ORF1 (Clarke and Lambden, 2001) along with other non-structural proteins and ORF2 codes for the minor capsid protein VP2 (Glass et al., 2000; Sosnovtsev and Green, 2000). A schematic representation of the genome organization of caliciviruses is shown in figure 1-1.

Calicivirus proteins

Despite the differences in genome organization of caliciviruses, they possess a similar protein processing mechanism, with the non-structural proteins produced by autocatalytic cleavage of a polyprotein encoded from ORF1, mediated by the viral 3C-like protease.

NS1/2- the N-terminal protein product of the polyprotein consists of two proteins, NS1 of 5.6 to 16 kDa and NS2 protein of 28-32 kDa depending on the genera: Vesivirus, Lagovirus or

Sapovirus. In case of norovirus its counterpart is referred to as NS1-2 protein (also known as Nterm or p48) (Belliot et al., 2003; Liu et al., 1996). The NS1-2 protein of norovirus is composed of three domains: the hyper variable-N terminal, middle sequence composed of H-box/NC motif that is seen in tumor suppressor proteins and the C-terminal with a hydrophobic region that is predicted to bind to lipid membranes (Fernandez-Vega et al., 2004). The norovirus NS1-2 protein has been shown to be involved in intracellular membrane changes associated with virus replication and also co-localize with markers of Golgi complex.

NS3- The sequence similarity of calicivirus NS3 protein to picornavirus 2C protein suggest a possible NTPase activity to NS3 (Gorbalenya et al., 1990). Transient expression of NS3 protein in RAW 264.7 cells led to changes in intracellular membrane structure with formation of membranous vesicles (Hyde et al., 2009).

NS4- limited information is available on the role of NS4 protein. NS4 protein was found in precursor form and also in cleaved form in calicivirus infected cells (Sosnovtsev et al., 2006). NS4 has been identified as membrane-associated component (Green et al., 2002). NS4 was also shown to interact with NS5 and function to deliver VPg to calicivirus replication complexes (Kaiser et al., 2006).

NS5/VPg- A 13-15 kDa protein that is covalently linked to 5'-end of viral genome and subgenomic mRNA through Tyr-24 residue (Mitra et al., 2004). Proteolytic removal of VPg from viral RNA by protease K treatment of calicivirus RNA led to drastic reduction in translation efficiency and loss of infectivity in RNA transfection assays (Burroughs and Brown, 1978; Dunham et al., 1998; Herbert et al., 1997). VPg is involved in the initiation of viral RNA replication wherein the uridylation of VPg and elongation of RNA is initiated by 3D RNA dependent RNA polymerase (Belliot et al., 2008; Machin et al., 2001; Rohayem et al., 2006).

Norwalk virus and murine norovirus-1 VPg has been demonstrated to bind translational initiation factors eIF3, eIF4G1 and 40S ribosomal subunit suggesting its role in assembly of translation initiation complex (Daughenbaugh et al., 2003; Daughenbaugh et al., 2006).

NS6/3CL protease- A chymotrypsin-like cysteine protease originally identified based on its sequence homology to the picornavirus 3CL protease (Boniotti et al., 1994; Jiang et al., 1993; Smiley et al., 2002) with the active site cysteine-histidine-aspartate/glutamate triad and employ cysteine residue as a catalytic nucleophile (Allaire et al., 1994; Birtley et al., 2005; Matthews et al., 1994). The NS6 is responsible for the cleavage of ORF1 encoded polyprotein into intermediate and individual proteins (Belliot et al., 2003; Blakeney et al., 2003; Liu et al., 1996; Seah et al., 2003; Sosnovtsev et al., 2006). The number of ORF1 encoded individual proteins varies among genera and also proteolytic processing varies for some viruses with stable precursors reported as mature forms in such cases. Two recognized proteolytically active forms of calicivirus protease (NS6-7 precursor and NS6) have been shown to mediate proteolytic processing of ORF1 polyprotein (Belliot et al., 2003; Seah et al., 2003; Sosnovtseva et al., 1999). The proteolytic processing of larger precursor molecules was also demonstrated suggesting the autocatalytic release of nascent protease was not required for polyprotein processing (Belliot et al., 2003; Robel et al., 2008; Sosnovtsev et al., 2002). The norovirus 3CL protease, a cysteine endoprotease possess a Cys-His-Glu catalytic triad with a substrate specificity for –D/E-F-X-L-Q-G-P- sequence corresponding to subsites S_5-S_4-S_3-S_2-S_1-S_1'-S_2', X is H, Q, E or D and cleavage occurs at P_1-P_1' (Q-G) scissile bond. X-ray crystallographic studies of human norovirus protease (Chiba, Norwalk and Southampton strains) showed 3C-like protease folds with two domains, a N-terminal domain and a C-terminal domain. The catalytic triad (composed of Cys, His and Glu) is located in the center of peptide-binding cleft at the interface of two domains

(Hussey et al., 2011; Nakamura et al., 2005; Zeitler et al., 2006). The domain I comprises a short N-terminal α-helix followed by a β-sheet and a large loop connecting to the domain II which is made of six-stranded β-barrel (Hussey et al., 2011).

NS7/3D RNA dependent RNA polymerase- NS7 protein was found as two distinct types in caliciviruses. The NS6-7 precursor found in vesivirus-infected cells is a stable bifunctional enzyme and does not undergo any additional processing (Martin Alonso et al., 1996). Similarly, NS6-7 has also been reported in porcine enteric calicivirus-infected cells (Chang et al., 2005). The NS6-NS7 precursor produced in norovirus infected cells undergoes further cleavage to produce a fully processed NS7 protein along with smaller quantities of NS6-NS7 protein (Konig et al., 1998; Sosnovtsev et al., 2006). The polymerase was also shown to direct plus-strand subgenomic RNA from internal initiation recognition signal in 50-nt sequence upstream of transcription start site (Morales et al., 2004). The calicivirus polymerase has been shown to uridylate VPg protein (Belliot et al., 2008; Machin et al., 2001) and employs the modified VPg to prime RNA synthesis (Rohayem et al., 2006). X-ray crystallographic structure of lagovirus and norovirus polymerase proteins revealed the presence of 'right hand' domain organization with finger, palm and thumb domains (Ng et al., 2002; Ng et al., 2004).

VP1- The major capsid protein VP1 is approx. 530-550aa (58-60kDa) and initial studies have shown that 180 copies of VP1 can self-assemble into virus-like particles (Jiang et al., 1992) with T=3 icosahedral symmetry (Prasad et al., 1999). The VP1 of sapoviruses and lagoviruses are encoded from ORF1 and differs from noroviruses with the VP1 encoded by ORF2. The X-ray crystallographic structure of Norwalk virus capsid shows that VP1 consists of N-terminal arm (10-49aa), the shell (S) domain (50-225aa) made of eight β-strands denoted as B to I and are organized into sheets, BIDG and CHEF with two α helices positioned between C and D, and E

and F strands respectively and forms the inter-subunit interactions for assembly of icosahedral shell and a protruding domain (P) that generates the 'cup-like' depressions on the surface. A flexible hinge connects the S and the P domain (Prasad et al., 1999). The P domain is subdivided into a stem P1A (226-278aa), globular domain P2 (279-405aa), P1B (406-520aa) and P2 forming the outermost segment and is involved in virus attachment (Tan and Jiang, 2005a). The P2 subdomain is an insertion in P1 domain between the amino acid residues 278 and 406 of VP1 protein with a barrel-like structure made of six β strands (Prasad et al., 1999). The S domain is involved in the icosahedral contacts and the P domain is involved in dimeric contacts. The capsid protein adapts to three quasi-equivalent positions referred to as A, B, and C to form icosahedral structure with T=3. The P domains of A and B subunit interact to across the quasi twofold axes and form the dimeric protrusions whereas and the C subunits interact across the icosahedral twofold axes. The A/B and C/C dimers are stabilized by interactions between the side chains of the involved monomers. Significant variation among the different strains was seen only in the P domain and more in the P2 sub-domain but less in S domain (Chakravarty et al., 2005). The P2 sub-domain harbors the hypervariable region (HVR) that plays an important role in receptor binding, immune response and also associated with susceptibility to infection (Tan and Jiang, 2005a). Studies on binding interactions of P domains to oligosaccharides have identified the locations of carbohydrate binding pockets in P2 subdomain (Bu et al., 2008; Cao et al., 2007; Choi et al., 2008). The complex of P domain and H-type 1 complex shows the pentasaccharide binding to a surface-exposed shallow depression on the P domain with terminal residues of the pentasaccharide α-Fuc and β-Gal attached to the binding site and the remaining part of the pentasaccharide projecting outward. The A-type HBGA trisaccharide binds the same site in the P domain with the terminal N-acetyl-galactosamine residue and α-Fuc make all the contacts with

the P domain. Norwalk virus does not bind Lewis blood group antigen A (Le^a) and B-type HBGA since the Le^a differs from other H-type HBGA with the terminal fucose residue missing and the B-type HBGA having a terminal α-Gal instead of GalNAc. The conserved hydrogen bonding along with hydrophobic interaction of Trp-375 with α-Fuc of the H-type 1 or with the acetamido group of GalNAc of the A-type confers the specificity of the interaction (Prasad et al., 1999). The GII noroviruses exhibit different HBGA binding pattern compared to GI noroviruses and this was shown to be resulting from different binding residues compared to the conserved residues in GI noroviruses. The crystal structure of GII P domain binding to the carbohydrate was distinctly different in location and structural characteristics (Cao et al., 2007).

The binding of FCV capsid to α2, 6-linked sialic acid and feline junctional adhesion molecule-1 (fJAM-1) has also been extensively studied (Makino et al., 2006; Stuart and Brown, 2007). FCV is well characterized among the caliciviruses and is the only calicivirus with an identified protein receptor fJAM-A. JAM-A is a member of the immunoglobulin-like superfamily and consists of N-terminal signal peptide, two Ig-like domains (distal D2 and proximal D1), a transmembrane domain and a short cytoplasmic tail (Kostrewa et al., 2001; Prota et al., 2003) The cryo-electron microscopy and three-dimensional image reconstruction of FCV-F9 bound to a soluble fJAM-A revealed that the P2 domain of VP1 binds to D1 domain of fJAM-A, inducing a conformational change characterized by an anticlockwise rotation of the P dimer at AB and CC positions of the capsid and this was hypothesized to be the initial stages of uncoating process (Bhella et al., 2008; Bhella and Goodfellow, 2011). The flexibility at the hinge region between S and P domains allowing movement in the P domain was also observed in murine norovirus and rabbit hemorrhagic disease virus (Katpally et al., 2010). This suggests that

conformational flexibility exists in several genera of *Caliciviridae*. A model of FCV VP1 protein showing the domain organization is illustrated in figure 1-2.

VP2 - A small basic protein encoded by ORF3 in noroviruses and by ORF2 in sapoviruses. It exhibits high sequence variability between the strains (Seah et al., 1999) and due to its basic nature it is believed to be involved in RNA genome packing (Glass et al., 2003). It also has an important role in stability of virus particles (Bertolotti-Ciarlet et al., 2003).

1.2 Calicivirus epidemiology

Enteric caliciviruses in humans cause acute gastroenteritis outbreaks, usually seen in semi-closed communities such as hospitals (Gallimore et al., 2004), retirement homes, nursing facilities (Jiang et al., 1996) and cruise ships (Gallimore et al., 2003) affecting 10-21 million people in all age groups in the U.S. each year (CDC, http://www.cdc.gov/norovirus/trends-outbreaks.html). The severity of the disease ranges from mild vomition and diarrhea to severe fatal dehydration especially in children and the elderly. Each year an estimated 19-21million cases are reported, including 1.7-1.9 million outpatient visits, 400,000 emergency cases, 56,000-71,000 hospitalizations and 570-800 deaths, costing $777 million in health-care costs (Hall et al., 2013). Norovirus outbreaks are frequently associated with consumption of faecally contaminated oysters (Lees, 2000; Potasman et al., 2002). The incubation period is around 24-48 hrs and the disease lasts for 24-60 hrs. The initial discovery of Norwalk virus was from clinical specimens by immune electron microscopy by Kapikian in 1972 (Kapikian, 2000) but later several small round structured viruses (SRSV) identified by electron microscopy which were morphologically similar to Norwalk virus but were antigenically distinct and were reported from several epidemics of gastroenteritis (Kapikian AZ, 1996). Later these SRSV were described as Sapoviruses (Madeley and Cosgrove, 1975) that were occasionally found in sporadic cases of

gastroenteritis. Most cases of gastroenteritis outbreaks are caused by a single strain and although numerous strains co-circulate the Genogroup II.4 noroviruses have been reported to be the predominant cause of outbreaks worldwide (Lopman, 2006; Widdowson et al., 2004). The GII.4 norovirus strains were also associated with higher hospitalization and mortality rates in outbreak cases of acute gastroenteritis (Desai et al., 2012). A systematic analysis of norovirus outbreaks from 1993 to 2011 showed significant association of higher attack rates in foodservice and winter outbreaks and waterborne outbreaks were significantly associated with GI strains, whereas healthcare outbreaks and winter outbreaks were associated with GII strains (Matthews et al., 2012). Further GI compared to GII, were more likely associated with waterborne transmission and GII.4 strains compared to GI were more likely associated with healthcare setting (Lysén et al., 2009; Matthews et al., 2012). Individuals infected with GII strains were shown to shed higher concentrations of virus compared to individuals with GI infection (Chan et al., 2006). Patients with cardiovascular disease, renal transplant and immunosuppressive therapy were considered at high risk with severe complications during norovirus infections (Mattner et al., 2006). Sapovirus genogroups I, II, IV and V are known to infect humans (Hansman et al., 2007a). Incidence of sapovirus infections is generally lower with gastroenteritis outbreaks (0.01-9%) and sporadic cases (0.4-6.6%) and most studies have shown that sapovirus infections are common in young children than adults (Blanton et al., 2006; Hansman et al., 2007a). Few studies have detected human sapovirus in clams, oysters and water suggesting environmental sources of contamination (Hansman et al., 2007b; Hansman et al., 2007c; Ueki et al., 2010).

Extensive epidemiological studies on animal caliciviruses are lacking. The GIII noroviruses infect cattle and a group of GII noroviruses (GII.11, GII.18 and GII.19) are known to infect pigs. Since human noroviruses can replicate and induce an immune response in

gnotobiotic pigs (Cheetham et al., 2006b), pigs can serve as potential reservoirs of human noroviruses. Porcine noroviruses (GII.18) were detected from adult pigs without clinical signs in Japan, Europe and antibodies to these porcine noroviruses were detected in US swine (Sugieda and Nakajima, 2002; Wang et al., 2006). Porcine noroviruses identified in US adult swine were found to be of genotype GII and a potential human norovirus recombinant was also identified (Wang et al., 2005a). Cattle are also shown to harbor bovine strains of (GIII) noroviruses but are less closely related to human strains (Oliver et al., 2003). Human GII.4 strains have been found in fecal samples of swine and cattle and also from retail raw pork sample (Mattison et al., 2007) suggesting a potential for zoonotic transmission of noroviruses to humans. Seroprevalence studies on bovine noroviruses shows 72% prevalence in young veal calves in US (Smiley et al., 2003) and 44% in Germany (van Der Poel et al., 2000). Calicivirus was isolated from a case of gastroenteritis with diarrhea in a dog in U.S. in 1985 (Schaffer et al., 1985) and later in 1990 from a Japanese 2-month old that showed intermittent watery diarrhea (Mochizuki et al., 1993). Two puppies of 1-2 month old, one showing upper respiratory illness and diarrhea and other showing diarrhea were tested positive for canine calicivirus strain 48 by RT-PCR and were negative for other common canine viruses (Mochizuki et al., 2002). FCV is known to infect all members of *Felidae* (Hoover et al., 1975). FCV is widespread in the general cat population. Prevalence in household cats is generally low while cats in shelters have a high chance of getting infected (Coyne et al., 2006; Helps et al., 2005; Wardley et al., 1974).

1.3 Immune responses in calicivirus infections

Noroviruses are highly heterogeneous with 5 different genogroups and at least 25 genotypes (Glass et al., 2009). Resistance to norovirus infection involves genetic factors and also acquired immunity. Studies in human volunteers showed that the immunity is only short term, is

strain specific and not all individuals are susceptible (Parrino et al., 1977; Wyatt et al., 1974). Natural infection does not provide long-term immunity which might be case with vaccines also and due to the absence of cross-protection among the different strains, multivalent vaccines will be required. The duration of immunity to norovirus infections has been shown to be from 6 months to 2 years based on studies with experimental infection of volunteers, but the challenge studies used doses that was several thousand-fold greater than the actual infectious dose required for causing human illness (Teunis et al., 2008). Epidemiological studies show that the population immunity levels to be in the order of 30-45%, but if duration of immunity is <1 year and incidence of infections is 5% then <5% of the population must have acquired immunity. To explain this discrepancy and get better estimate of duration of immunity a mathematical model of norovirus transmission in a community was developed. Results from the mathematical model suggested that the duration of immunity to norovirus infections is 4.1 to 8.7 years (Simmons et al., 2013). Confirmatory studies on duration of immunity to norovirus infections are required to get better understanding of the duration of immunity. Genetic resistance to norovirus infection was seen with studies in human volunteers showing repeated susceptibility or resistance to norovirus infection (Parrino et al., 1977). Studies on binding ability of different strains of Norwalk virus-like particles (VLPs) with histo-blood group antigens (HBGA) showed that binding ability correlated with susceptibility to infection (Hutson et al., 2002; Lindesmith et al., 2003). *FUT-2* gene that codes for the enzyme fucosyl transferase 2 is required for synthesis of HBGAs and mutations in the *FUT-2* gene leading to nonfunctional enzyme (resulting in absence of HBGAs in secretions hence termed "non-secretors") and absence of HBGA expression was associated with resistance to norovirus infection. When these non-secretors were challenged,

they did not shed any virus and also did not show any mucosal or systemic immune responses (Lindesmith et al., 2003).

Vaccine approaches against caliciviruses

VLP based vaccines for Norwalk virus are currently under development. Intranasal or oral delivery of Norwalk VLPs induces mucosal and systemic immune responses in mice and humans but is not strong enough to prevent illness (Herbst-Kralovetz et al., 2010). Several mucosal adjuvants are used to increase the norovirus antigenicity and skew the immune response toward $CD4^+$ T helper type 1 phenotype (Pulendran and Ahmed, 2006). Cholera toxin, monophosphoryl lipid A (MPL) and other TLR agonists are currently evaluated as adjuvants (Periwal et al., 2003; Vinjé, 2010). Humoral immunity induced by alphavirus-adjuvanted murine norovirus-like particle induced robust immune response and protected mice against heterologous challenge (LoBue et al., 2009). A Norwalk virus GI.1 VLP vaccine is in phase 1/2 human trial which was proved safe and provides protection against challenge with homologous virus (Atmar et al., 2011; El-Kamary et al., 2010). In the phase I trial VLP vaccine derived from norovirus GI.I genotype adjuvanted with monophosphoryl lipid A (MPL) and the mucoadherent chitosan was randomly administered intranasally in 2 doses 21 days apart to healthy human subjects. Subjects that received 100µg dosage showed 4.8 and 9.1 fold increase in Norwalk VLP-specific immunoglobulin G and IgA respectively along with development of antibody secreting cells expressing homing molecules to mucosal and peripheral lymphoid tissues (El-Kamary et al., 2010). In a subsequent phase II clinical trial the individuals similarly immunized with Norwalk virus (GI.1) VLP were challenged with Norwalk virus and monitored for infection and gastroenteritis symptoms. Vaccination reduced the frequency of Norwalk virus gastroenteritis (69% in placebo recipients vs. 61% in vaccine recipients) and among the vaccinated individuals

that developed gastroenteritis, delayed onset of symptoms and overall reduction in disease severity was seen; indicating the norovirus VLP vaccine provides protection against homologous challenge (Atmar et al., 2011). A next step ahead of this a bivalent norovirus VLP vaccine containing genotype GI.1 VLP and consensus GII.4 VLP was evaluated in a phase 1 clinical trial. Intramuscular injection of the bivalent vaccine in adult volunteers was shown to be immunogenic with significant increase in IgA and IgG responses to both VLPs by 7 days after first dose and no further boosting after second dose but antibody secreting cell responses were significant with bias towards IgA achieving HBGA-blocking antibody titers of ≥ 200 (Treanor et al., 2014). The efficacy of the bivalent vaccine will be further tested using a new GII.4 NoV challenge pool (Frenck et al., 2012; Ramani et al., 2014). Another study showed intramuscular vaccination with bivalent (GI.1/GII.4) formulation induced greater antibody titers than intranasal route in rabbits (Parra et al., 2012).

The P domain of the capsid protein forms the exterior protrusions and is the major antigenic structure on noroviruses. The production of P domain alone leads to their assembly and formation of complexes that can bind the HBGAs (Tan et al., 2008a; Tan and Jiang, 2005b). P particle based norovirus subunit vaccines are now developed and evaluated. P particle complexes were shown to elicit efficient cellular immunity and humoral responses similar to VLPs (Fang et al., 2013) showing promise as a vaccine development platform. A dominant Th1 immune response with increased IL-2 and IFN-γ levels were observed in specimens from travelers with norovirus infection (Ko et al., 2006). Intramuscular injection of VLPs derived from RHDV is shown to be highly immunogenic in rabbits and confer complete protection from a lethal challenge (Angulo and Bárcena, 2007; Laurent et al., 1994). In case of feline calicivirus, maternally derived antibodies have an average half-life of 15 days (Johnson and Povey, 1983).

FCV-specific IgG and IgA have been detected in saliva during the course of infection (Knowles et al., 1991). Several vaccines are commercially available against FCV and vaccination at 8 weeks and 12 weeks of age protected cats with 100% protection against oral ulcerations from a virulent challenge with FCV after 3 years post vaccination (Gore et al., 2006).

1.4 Antiviral approaches against caliciviruses

Strategies targeting various stages of virus replication cycle have been examined for development of control measures against noroviruses mainly and other caliciviruses in general.

Inhibitors of virus entry

Caliciviruses are known to utilize surface carbohydrates as cellular attachment factors and human norovirus interactions with HBGAs are well characterized with the identification of conserved capsid regions interacting with HBGA molecules (Tan and Jiang, 2011). Development of specific molecules against the interacting surfaces has been focused. Based on the X-ray crystallographic analysis of the interaction of citrate with capsid protruding domains of GII noroviruses, analogs of carbohydrates such as citrate and other glycomimetics that resemble the structure of fucose have been proposed to block binding of human noroviruses to HBGAs (Hansman et al., 2012). Since heparin sulphate was also shown to bind norovirus capsid, analogs of heparin sulphate such as heparin and suramin was also showed to block binding of norovirus VLP to mammalian cell surfaces (Tamura et al., 2004). Using nuclear magnetic resonance (NMR) spectroscopy various compounds that can bind to HBGA binding site on norovirus capsid were identified from a large compound library and its was also demonstrated that high avidity binders can be designed and evaluated as potential inhibitors of norovirus entry (Rademacher et al., 2011). Using docking simulations and virtual screening, hit compounds that can bind to the HBGA binding interfaces on the capsid protein of GII.4 (VA387) were identified.

Twenty compounds out of the 160 hit compounds blocked the binding of P particle dimers to HBGAs with IC_{50} values of <40 μM and the top-5 compounds of which 4 compounds shared a basic structure of cyclopenta [a] dimethyl phenanthren showed an IC_{50} of <10 μM in oligosaccharide- and saliva-based blocking assays (Feng and Jiang, 2007; Zhang et al., 2013). Immunotherapy using norovirus-neutralizing monoclonal antibodies for treatment in high-risk individual was reported. A combinatorial phage Fab display library derived from mRNA of chimpanzee's bone marrow previously immunized with Norwalk virus was prepared and four Fabs that recognized conformational epitopes in the P domain of Norwalk virus were identified and full length IgG with human γ1 heavy chain constant regions were produced. The identified Mabs were able to neutralize Norwalk virus and prevented infection of chimpanzee on challenge demonstrating its therapeutic potential (Chen et al., 2013).

Inhibitors of virus replication

Inhibitors of viral protease- The calicivirus 3C-like protease cleaves the polypeptide produced by translation of ORF1 into matured forms of nonstructural proteins and inhibition of protease activity results in non-availability of mature nonstructural proteins for viral replication. The crystal structures of Norwalk virus protease have been reported and this has allowed the development of new inhibitors based on the conserved substrate recognition specificity. The calicivirus protease shares common structural features of viral proteases in the picornavirus-like supercluster (picornaviruses, noroviruses and coronaviruses) that are known to have the viral 3C or 3C-like protease possessing a Cys residue as active site nucleophile in catalytic triad (or dyad) and preference for Glu or Gln residue at the P1 position in substrate (Matthews et al., 1994; Nakamura et al., 2005; Zeitler et al., 2006). Various series of protease inhibitors were identified and evaluated in enzyme- and cell-based assays. Dipeptidyl aldehyde inhibitors and α-

ketoamides as transition state inhibitors were shown to possess inhibitory activity on Norwalk virus protease in vitro and also inhibit norovirus replication in cell-based replicon system with EC_{50} of the most effective inhibitor in nanomolar ranges (Mandadapu et al., 2013; Mandadapu et al., 2012; Tiew et al., 2011). Tripeptidyl aldehydes with arylalanine at P3 along with P1 glutamine surrogate and P2 leucine was shown to be highly active against noroviruses, human rhinovirus, severe acute respiratory syndrome coronavirus and coronavirus 229E in cell-based assays (Prior et al., 2013). Bisulfite adduct salt of the dipeptidyl aldehyde was synthesized and evaluated for inhibitory activity against 3C and 3CL-protease (Mandadapu et al., 2013). The bisulfite adduct compound was shown to be inhibitory against most caliciviruses, picornaviruses, and coronaviruses belonging to the picornavirus-like supercluster with half-maximal inhibitory concentrations in high nanomolar or low micromolar range and with high therapeutic indices (Kim et al., 2012).

Inhibitors of viral polymerase- inhibitors of polymerase include: Nucleoside analogues that are phosphorylated to nucleotides after entering the cell and are incorporated into growing nucleic acid chain synthesized by the polymerase leading to chain termination. 2′-arauridine-5′-triphosphate (TriLink technologies) and the 3′-deoxyuridine-5′-triphosphate (TriLink technologies) were shown to inhibit human calicivirus at a concentration of 50μM (Rohayem et al., 2010b). Ribavirin, a guanosine analog has been shown to inhibit human noroviruses in replicon cells with EC_{50} of 40μM and also murine norovirus in cell culture (Chang and George, 2007b). 2′-C-methylcytidine (2CMC) and β-D-N(4)-hydroxycytidine with a EC_{50} value of 6.9 μM and 12.7 μM respectively, has been shown to inhibit MNV replication and with a EC_{50} value of 1.3 μM and 1.5 μM respectively reduced Norwalk virus RNA levels and protein expression in replicon cells (Costantini et al., 2012). Favipiravir (T-705) that was developed as an inhibitor of

influenza polymerase was shown to inhibit murine norovirus replication in cell culture (Rocha-Pereira et al., 2012).

Non-nucleoside inhibitors of polymerase activity are also reported for noroviruses. Suramin and NF023 have been shown to inhibit human norovirus and MNV polymerase with IC_{50} values in nanomolar ranges during in vitro polymerization assays (Mastrangelo et al., 2012). Mycophenolic acid, inhibitor of inosine monophosphate dehydrogenase is known to have antiviral activity against noroviruses (Chang and George, 2007b).

Inhibitors of viral RNA translation- Peptide-conjugated phosphorodiamidiate morpholino oligomers (PPMO) directed against first start codon in norovirus ORF1 was shown to inhibit human norovirus and murine norovirus replication in cell culture (Bok et al., 2008). siRNA molecules have also shown to be 50-fold more effective compared to PPMOs in inhibiting FCV replication (Rohayem et al., 2010a). VPg has been known to interact with eIF4F complex (eukaryotic initiation factor 4F) during translation initiation (Daughenbaugh et al., 2003; Daughenbaugh et al., 2006). Eukaryotic initiation factor eIF4E was shown to directly interact with VPg of Norwalk virus (Goodfellow et al., 2005) and as a complex with eIF4A in FCV and MNV (Chaudhry et al., 2006). Hippuristanol, inhibitor of eIF4A, blocked FCV and MNV viral protein translation but the drug was also shown to inhibit cellular protein synthesis (Chaudhry et al., 2006). Panteamine-A interferes with VPg/eIF4F complex and deregulates eIF4F complex inhibiting translation (Bordeleau et al., 2006).

Other ways of inhibiting calicivirus replication

Interferons establish an antiviral state in cells through its interactions with interferon receptors and activation of STAT (signal transducer and activator of transcription) initiating a cascade of events resulting in production of various antiviral proteins. Type I and II interferons

are shown to inhibit the replication of Norwalk virus in replicon cells (Chang and George, 2007b). Interferon responses were also shown to be required for control of MNV infection in vivo and in vitro (Karst et al., 2003). Type I and II IFN block translation of MNV proteins through Protein kinase R (PKR)-independent and dependent mechanisms respectively (Changotra et al., 2009).

The activity of ACAT1 (acyl-coenzyme A: cholesterol acyltransferase-1) was shown to be required for norovirus infection (Chang, 2009). A class of pyranobenzopyrone molecules containing amino, pyridine, substituted quinolones or 7, 8-benzoquinoline nucleus was shown to possess ACAT inhibitory activity and was demonstrated to have anti-norovirus activity in low micro-molar concentrations (Hua et al., 2003; Pokhrel et al., 2012). WP1130, inhibitor of cellular deubiquitinase USP14 leading to induction of unfolded protein response was shown to inhibit replication of MNV in murine macrophages and Norwalk virus in replicon cells (Perry et al., 2012).

1.5 Virus receptor and co-receptor

Virus entry that forms the first step in virus life-cycle is composed of various mechanisms that result in virus being internalized into cells followed by uncoating of virus to release the viral genome. The primary event during virus infection is its interaction with cellular receptor that starts a series of dynamic events leading to entry of virus into cell. The virus-receptor interaction contributes to tissue tropism and also the host range of viruses. Several cell surface components are used by viruses as receptors. For many viruses only one type of receptor has been identified (CD155 for poliovirus (Mendelsohn et al., 1989), low-density lipoprotein receptor for human rhinovirus-2 (Hofer et al., 1994)), but for some viruses more than one

receptor has been identified (angiotensin-converting enzyme or liver-SIGN for SARS coronavirus (Jeffers et al., 2004; Li et al., 2003).

Caliciviruses are shown to interact with polymorphic carbohydrates on host cell surfaces. The human noroviruses recognizes human histo-blood group antigens (HBGAs) in the ABO, Lewis and secretor families, certain sialyl-modified HBGAs and heparin sulphate (Gange et al., 2006; Hutson et al., 2004; Tan and Jiang, 2005a, 2007). The rabbit hemorrhagic disease virus, a *Lagovirus* was shown to bind H type 2 antigen (Ruvoen-Clouet et al., 2000). Further a study of wild rabbits found evidence for the relationship between H type 2 HBGA and susceptibility to RHDV (Guillon et al., 2009). Murine norovirus (Taube et al., 2009) and feline calicivirus (Stuart and Brown, 2007) have shown to bind sialic acid on permissive cells with feline calicivirus also recognizing the junctional adhesion molecule 1 (JAM-1) as a host receptor required for infection (Bhella et al., 2008; Makino et al., 2006; Ossiboff and Parker, 2007). The interaction of human norovirus with HBGAs was found to be diverse and strain-specific (Donaldson et al., 2008; Harrington et al., 2002; Huang et al., 2003; Hutson et al., 2003; Lindesmith et al., 2008; Shirato et al., 2008; Tan et al., 2004; Thorven et al., 2005). Norovirus strains having close genetic relatedness in capsid sequences share similar HBGA binding patterns but similar HBGA binding patterns have also been detected in strains that belong to different genotypes and genogroups. The strain variations in HBGA binding indicate typical carbohydrate-protein interactions with small changes in capsid will significantly change the HBGA binding specificity or affinity (Tan et al., 2008b). Murine norovirus binding to glycolipid and glycoprotein receptors was shown to be strain-dependent. Both MNV-1 and CR3 strains of murine norovirus were dependent on N-linked glycoproteins for binding and only MNV-1 strain was sensitive to O-linked glycoprotein

depletion for binding to macrophages. The differences in glycan binding contributes to tissue tropism in vivo with CR3 strain replicating in large intestine (Taube et al., 2012).

Some viruses use more than one receptor with distinct roles for each and in some cases, binding to a cellular receptor is not sufficient for infection and additional cell surface molecule is required for entry, hence referred to as co-receptors (Human immunodeficiency virus envelope protein gp120 binding to its primary receptor CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) induces conformational changes that facilitate its interaction with the chemokine receptors CXCR4 or CCR5 (members of the G protein-coupled receptor superfamily) which have been identified as coreceptors for T cell line-tropic and macrophage-tropic HIV-1, respectively) which will induce structural changes in gp41 required for fusion activation (Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996). The entry of hepatitis C virus requires several factors. The initial attachment of virus to cells occurs by interaction of viral apoE with cellular low-density-lipoprotein receptor and glycosaminoglycans (Agnello et al., 1999; Germi et al., 2002). In addition to these attachment factors HCV uses other cell surface molecules such as CD81, scavenger receptor class B (SRB1), claudin 1, occluding and Niemann-Pick C1-like 1. SRB1, expressed on hepatocytes functions to transport lipoproteins into cells but can also bind to HCV associated lipoprotein and induce conformational changes in E2 protein to expose regions that will bind to CD81 (Acton et al., 1996; Bankwitz et al., 2010; Scarselli et al., 2002) . CD81 is ubiquitously expressed on human cells and binds HCV E2 protein only after a post-attachment conformational change in E2 (Bankwitz et al., 2010; Bertaux and Dragic, 2006; Petracca et al., 2000). Claudin 1 interacts with CD81 and not with HCV directly to facilitate virus internalization (Evans et al., 2007; Harris et al., 2010). Occludin 1 is a tight junction protein and functions at a post-attachment step in HCV entry (Sourisseau et al., 2013). The adenovirus

serotype 2, 5 and 12 binds to the coxsackievirus-adenovirus receptor (CAR) cell surface protein (Bergelson et al., 1997) but internalization of the attached virus required the binding of the penton proteins to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins (Wickham et al., 1993) and clustering of which activates intracellular signaling, activation of phosphatidylinositol-3-OH kinase which in turn, activates Rac and CDC42 GTPases inducing actin polymerization, leading to local formation of clathrin-coated pits (Lamaze et al., 1997; Li et al., 1998). Group B coxsackieviruses interact with decay-accelarating factor (DAF) leading to activation of Abl kinase, triggering Rac-dependent actin rearrangement required for lateral movement of the virus to the tight junction. The coxsackievirus and adenovirus receptor (CAR) (Bergelson et al., 1997), which is a component of tight junction, then, interacts with the CVB inducing conformational changes in the virus capsid required for virus entry (Coyne and Bergelson, 2006; Milstone et al., 2005).

The human JC polyomavirus, uses N-linked glycoproteins with alpha 2-6-linked sialic acid as its cellular receptor and also the serotonin receptor $5HT_{2A}$ as its co-receptor to enter host cells (Elphick et al., 2004; Liu et al., 1998). The Simian Virus 40 uses sphingolipid GM1 (Tsai et al., 2003) and also MHC-I (Atwood and Norkin, 1989) as its cellular receptor but also uses $\alpha_2\beta_1$ integrins to activate PI3K-AKT signaling networks for actin reorganization and cellular uptake of virus (Stergiou et al., 2013). Rotavirus uses N-acetylneuraminic acid, also known as sialic acid to attach to the cell surface (Delorme et al., 2001) and this interaction has been shown to be dependent on VP4 genotype (Ciarlet et al., 2002). Rotavirus has been shown to bind several integrins through its VP4 and VP7. VP4 binds integrins $\alpha_2\beta_1$, $\alpha_4\beta_1$ and VP7 binds to integrins $\alpha_x\beta_2$ and $\alpha_4\beta_1$ (Hewish et al., 2000). Rotavirus interacts with several receptors sequentially using different domains on its viral protein VP4 and VP7 (López and Arias, 2004). The neuraminidase sensitive rotavirus makes its initial contact through VP8 domain of VP4 with a ganglioside. The

conformational change induced by initial contact allows VP5 to interact with $\alpha_2\beta_1$ integrin. Following the second interaction, atleast one of these interactions will take place: VP5 interacts with heat shock protein HSC 70 (Zárate et al., 2003), VP7 interacts with $\alpha_v\beta_3$ or $\alpha_x\beta_2$ integrins (Graham et al., 2003). Entry of rotavirus is shown to occur through raft-dependent endocytosis that is clathrin and caveolin independent but requires dynamin and cholesterol (Nabi and Le, 2003). Epstein-Barr virus (EBV) major envelope glycoprotein gp350 binds to the B cell surface receptor CD21 (Fingeroth et al., 1984; Nemerow et al., 1987) and this triggers endocytosis of the virion (Tanner et al., 1987). The viral glycoprotein complex of gH, gL and gp42 binding to HLA-DR (MHCII) was shown to be required for membrane penetration and successful infection (Li et al., 1995; Wang and Hutt-Fletcher, 1998), hence EBV utilizes HLA class II protein as a coreceptor for entry (Li et al., 1997). The initial low-affinity attachment of reovirus to mammalian cells is mediated by sialylated glycans (Chappell et al., 2000; Chappell et al., 1997) which is followed by high-affinity binding to JAM-A (Barton et al., 2001) or NgR1 in neuronal cells (Konopka-Anstadt et al., 2014). The sialylated glycans influences the tissue tropism and disease phenotypes (Forrest and Dermody, 2003) while JAM-A was required for reovirus-induced activation of NF-kappaB and apoptosis (Barton et al., 2001). Ebola virus has been shown to use several receptors/coreceptors to enter cells. The T-cell immunoglobulin and mucin domain 1 (TIM-1) binds to phosphatidylserine on the ebola viral envelope and promotes virus internalization into endosomes (Kondratowicz et al., 2011). Cleavage of the ebola glycoprotein by cathepsin in the endosomes exposes a putative receptor binding domain to bind to the cholesterol transporter protein NPC1 (Niemann-Pick C1) that is required for membrane fusion and viral escape from vesicular compartment (Carette et al., 2011; Cote et al., 2011).

1.6 Mechanisms of virus entry

The virus particles released from the cells are in a very stable form made of complex intermolecular interactions. They must be able to resist the harsh conditions in the extracellular environment during the process of transmission to a new cell or host. Despite their stable conformation the viruses must also be able to disassemble at specific sites and at specific time points during the entry process to release their genome into the cells. This is made possible by the metastable conformations of the viral coat proteins so that they can undergo major conformational changes when triggered by specific signals during entry (Steven et al., 2005). Virus entry starts with interactions of viral factors with the cell surface components and end with decondensation of the genome at the site of replication. This process involves penetration, capsid destabilization and uncoating of the genome resulting from conformational changes in metastable virus structure that are triggered by receptor binding, exposure to low pH, reducing environment, proteolytic cleavage or other cellular factors ensuring that each step occurs at the right point in the sequence, at the right time, and at the right place (Earp et al., 2005; Harrison, 2005; Hogle, 2002; Smith and Helenius, 2004).

Viruses generally exploit the existing cellular mechanisms to enter their host cells. Some of them enter through the ongoing endocytic activities while majority of them induce their uptake through various mechanisms. Virus entry through clathrin-mediated endocytosis, macropinocytosis and caveolar-dependent endocytosis are well studied, although there are other clathrin- and caveolin-independent mechanisms that are less well understood. Once internalized the incoming viruses are trafficked through the endosomal network to their sites of replication. The endosomal network consists of early endosomes, late endosomes, recycling endosomes and maturing endosomes (Bonifacino and Glick, 2004). The endosomal network is also connected to

the secretory pathway via the vesicles shuttling between endosomes, *trans*-Golgi network and plasma membrane (Bonifacino and Glick, 2004). The different classes of endosomes are heterogeneous in composition. The early endosomes have a complex structure with long, narrow, often-branched tubes and they are defined by different Rabs and their effectors (Rab5 positive early endosomes, Rab5 and Rab7 positive maturing endosomes, Rab7 positive late endosomes) (Novick and Zerial, 1997).

Viruses exploit cellular signaling pathways to induce changes in the cell to promote virus entry, early cytoplasmic events and also some later events in the replication cycle. These signaling events promotes the cell to initiate endocytosis process (Marsh and Helenius, 2006). The interaction of adenovirus pentons with integrins activates phosphtidyl-inositol 3-kinase which in turn activates Rac and Cdc42 resulting in the polymerization of actin and clathrin-mediated endocytosis (Nemerow and Stewart, 1999). SV40 internalization by caveolar/raft endocytosis is regulated by five different kinases (Pelkmans et al., 2005) and inhibition of tyrosine kinases blocks the internalization reducing infection (Chen and Norkin, 1999; Pelkmans et al., 2002).

Although various pathways are utilized by different viruses to enter the cell, the endocytic pathway is the most widely used by majority of viruses as it offers the benefit of carrying the viruses in endocytic vesicles into the cytoplasm bypassing cellular barriers (Marsh and Bron, 1997). Clathrin-mediated endocytosis is the most common endocytic route used. The viruses along with their receptors are rapidly transported along the early and/or late endosomes and during this transit the incoming viruses use the decreasing pH of endocytic organelles to activate uncoating mechanisms and escape from the endosomes before they reach the lysosomes (Helenius et al., 1980). The site of endosomal escape can be either early endosomes (pH 6.5-6.0)

or late endosomes (pH 6.0-5.5) depending on the virus. Some viruses like ebola virus, SARS coronavirus and nonenveloped mammalian reoviruses enter through clathrin-mediated endocytosis and require acidic pH and proteolytic cleavage of viral proteins by cathepsin L and B for their process of uncoating and endosomal escape (Chandran et al., 2005; Ebert et al., 2002b; Simmons et al., 2005). Several studies have reported the use of clathrin-independent pathways by several viruses. Caveolae-dependent endocytosis is a well characterized mechanism for biological processes such as virus entry, internalization of glycophosphatidylinositol-anchored proteins and certain signaling cascades including p38 kinase, JNK signaling as well as caspases activation. Caveolae are clathrin-independent raft-dependent endocytosis characterized by cholesterol, sphingolipid and caveolin-rich plasma membrane invaginations (Simons and Toomre, 2000). The caveolar pathway bring viruses including SV40, to caveosomes that are pH-neutral, from which they continue and by a second vesicle transport step reach the ER where penetration occurs in the redox conditions (Anderson et al., 1996; Pelkmans et al., 2001; Stang et al., 1997). Echovirus 1 entry by caveolar/raft endocytosis involves protein kinase C and penetration occurs at caveosomes (Pietiainen et al., 2005; Upla et al., 2004). Macropinocytosis requires actin remodeling mediated by Rac-GTPase and its effector p21-activated kinase (Pak-1) producing membrane ruffles and blebs from cell surface that fold/drop back enclosing the target material (Swanson, 2008) and mediate the uptake of extracellular fluid and bulky materials (Mercer and Helenius, 2008; Mercer et al., 2010).

Macropinocytosis occurs constitutively in dendritic cells and is inducible in other cells by tyrosine kinases like EGFR (Swanson, 2008). Attachment of vaccinia virus activates EGFR, Rho-GTPases and actin remodeling initiating macropinocytosis (Mercer and Helenius, 2008; Mercer et al., 2010). Other viruses that are internalized by this process include Kaposi's

sarcoma-associated herpesvirus, adenovirus, echovirus 1, Ebola virus and Vaccinia virus (Amstutz et al., 2008; Liberali et al., 2008; Mercer and Helenius, 2008; Mercer et al., 2010; Nanbo et al., 2010; Raghu et al., 2009; Saeed et al., 2010; Schmidt et al., 2011). In spite of the heterogeneity in the formation of primary vesicles the later steps in intracellular trafficking involve either endosomes or caveosomes and may require cholesterol (Imelli et al., 2004) and depending on the virus and cell type, penetration reactions occur in five locations: the plasma membrane, early and late endosomes, caveosomes, and the endoplasmic reticulum. Various mechanism of virus entry is illustrated in figure 1-3.

1.7 Endosomal proteases involved in virus uncoating

The repertoire of endo-lysosomal hydrolytic enzymes includes proteases, lipases, phosphatases, glycosidases and nucleases. The predominant proteases are cathepsins that are cysteine (cathepsins B, H, L, S, C, K, O, F, V, X, and W) or aspartic proteases (cathepsin D and E) localized in the endocytic pathway (Pillay et al., 2002; Tjelle et al., 1996). The endocytic pathway likely represents distinct proteolytic compartments with early compartments predominated by neutral proteases like cathepsin H and D, and later compartments dominant with acidic cathepsins S, B, L and D (Claus et al., 1998). Cathepsin L, B and S have been shown to be required for entry of several viruses including severe acute respiratory syndrome (SARS) coronavirus (Bosch et al., 2008), murine hepatitis virus (Qiu et al., 2006), reovirus (Ebert et al., 2002a; Mainou and Dermody, 2012), ebola virus (Brecher et al., 2012b; Schornberg et al., 2006), hendra virus (Pager and Dutch, 2005) and nipah virus (Diederich et al., 2012). The spike protein of SARS-CoV spike exposes a loop between S1 and S2 subunits that is accessible to proteases such as cathepsin L and cleavage of the spike protein at this site changes the protein to a metastable state allowing structural changes required for membrane fusion (Follis et al., 2006).

Similar to the SARS-CoV, the murine hepatitis type-2 strain requires cathepsin L and cathepsin B for fusion activation (Qiu et al., 2006). The feline enteric coronavirus (FECV) were shown to dependent on cathepsin B and/or L activity for its entry into host cells (Regan et al., 2008). Paramyxoviruses, hendra virus and nipah virus require endosomal cathepsins for cleavage of their fusion protein during their entry into susceptible cells. Hendra virus fusion protein precursor (F_0) is cleaved to active form (F_1+F_2) disulfide-linked heterodimer by endosomal cathepsin L (Pager and Dutch, 2005). Similarly nipah virus fusion protein was cleaved by cathepsin L and cathepsin B (Diederich et al., 2012). Ebola virus glycoprotein must be cleaved by endosomal cathepsin L or B to expose a receptor binding subunit that is required to bind and probably induce fusion in the acidic endosome (Brecher et al., 2012a; Schornberg et al., 2006). Reovirus disassembly is mainly mediated by cathepsin L and less efficiently by cathepsin B, generating infectious subviral particles that are capable of penetrating membranes to deliver core particles into cytoplasm (Ebert et al., 2002b; Mainou and Dermody, 2012). Further the retrovirus, ecotropic murine leukemia virus entry into host cells was shown require either cathepsin L or B (Yoshii et al., 2009).

1.8 Endosomal escape

The cellular membrane penetrated during virus entry is either the plasma membrane or the limiting membrane of an intracellular organelle like endosomes. Viruses employ different mechanisms to cross the host lipid membrane to enter the mammalian cells. Viruses exploit the cellular processes to gain entry into cells and the mechanisms of entry are dictated by the complex interactions of virus particles and cellular components. Irrespective of the means of uptake, the invading viruses that ends up in endosomes utilize the endosomal environment of low pH and/or protease activity for activating the membrane fusion/penetration to release their

genome into cytoplasm (Kielian and Rey, 2006; Moyer and Nemerow, 2011). The escape from the endosome is vital for their survival from the hazardous environment in the late endosome and lysosome (Dikic, 2006). This phenomenon of endosomal escape has evolved very well in viruses, varying from simple mechanisms to very complex mechanism seen in some viruses. Enveloped viruses generally carry fusion machinery (Class I , II, III fusion proteins) in their enveloped proteins and activation of the fusion proteins leads to conformational changes with formation of hairpin structure and insertion of the fusion peptide into target membrane that promotes fusion of viral envelope membrane with the host target membrane releasing the viral components into cytosol of host cell (Kielian and Rey, 2006). The trigger for fusion could be exposure to low pH (Heinz and Allison, 2000; Skehel et al., 1982), interaction with receptor (Earp et al., 2005) , interaction with receptor and co-receptor (Eckert and Kim, 2001; Feng et al., 1996), endosomal proteolysis (Chandran et al., 2005), or combinations of any these factors like receptor binding and low pH (Matsuyama et al., 2004; Mothes et al., 2000). Influenza virus hemagglutinin (HA) is a class I fusion protein that is activated in low pH and forms N-terminal trimeric alpha helical coiled coil decorated by C-terminal helices from the activated HA_1 and HA_2 polypeptides. Similar to the HA protein of influenza virus are the GP2 protein of ebola virus (Adam et al., 2004), TM protein of moloney murine leukemia virus (Li et al., 1996) and S2 protein of mouse hepatitis virus (Bosch et al., 2003). The class II fusion proteins are primarily β-sheets containing internal fusion peptides as loops at the tips of β-strands. Viruses with class II fusion proteins are E proteins of dengue virus, west Nile virus, tick borne encephalitis virus and E1 proteins in semliki forest virus (Kielian and Rey, 2006). The vesicular stomatitis virus G protein (Heldwein et al., 2006; Roche et al., 2006; Roche et al., 2007) and herpes simplex virus-1 gB protein (Heldwein et al., 2006) have been characterized as class III fusion proteins which

share features of both class I and class II. They are trimers in pre-fusion forms with central α-helical coiled-coil but their fusion loops are found at the tip of extended β-stands (White et al., 2008).

Endosomal escape of enveloped viruses is well understood and is generally achieved by fusion of the viral envelope with cell membrane which is activated by receptor interaction or endosomal pH or proteolytic activity or a combination of factors but the mechanism of endosomal escape of non-enveloped viruses are complex and still remain less understood. The non-enveloped viruses lack the fusion machinery owing to the lack of envelope and its proteins, but utilize lytic factor for membrane penetration. Various cellular factors similar to enveloped viruses trigger uncoating and release of lytic factor leading to membrane disruption (Moyer and Nemerow, 2011). Non-enveloped viruses are generally known to carry membrane lytic factors as components of their capsid to escape the endosomal barrier. There are three major classes of membrane lytic factors: lipid-remodeling enzymatic domains, myristoyl groups and amphipathic α-helices (Moyer and Nemerow, 2011). Canine parvovirus exposure to low pH leads to exposure of the N-terminus of VP1 deploying a phospholipase A2 domain that mediates lipid modification in endosome bilayer leading to endosomal escape (Hogle, 2002; Parrish, 2010). Reovirus entry is associated with auto-cleavage of μ1generating μ1N peptides linked to N-terminal myristoyl group. Production of μ1N leads to the formation of pores and eventually lysis of endosomal vesicles (Agosto et al., 2006; Odegard et al., 2004). The protein VI of adenovirus possesses an amphipathic helix at its N-terminal that is known to induce positive curvature leading to fragmentation of endosomal membrane releasing the large partially uncoated capsid into cytoplasm (Maier et al., 2010; Maier and Wiethoff, 2010; Wiethoff et al., 2005). Additionally Protein Kinase C (PKC) activation through the clustering of integrins was shown to be required

for endosomal escape of adenovirus but the exact role is unclear (Meier et al., 2002; Nakano et al., 2000). Poliovirus contains amphipathic α-helix at the N-terminal of capsid protein VP1 which is externalized by conformational changes following receptor binding. Poliovirus also carries N-myristoylated VP4 which is also involved in pore formation and VP1 has been shown to tether the virus particle to membrane and VP4 is involved in pore formation (Tuthill et al., 2010).

Some viruses follow a complex pathway of entry with special requirements for uncoating. Semliki forest virus requires cholesterol and sphingolipids for its fusion activity (Kielian and Helenius, 1984), similarly dengue virus passes through endosome and fuses with lysosome to gain access to low pH and anionic lipids such as lysobisphosphatidic acid (Zaitseva et al., 2010). Ebola virus envelope protein GP1 cleavage by cathepsins exposes a binding domain for late endosome/lysosome cholesterol transporter Niemann-Pick C1 to be transported across the membrane (Chandran et al., 2005). The site of endosomal escape of different viruses is illustrated in figure 1-4.

1.9 Bile acid

Bile acids are amphipathic molecules that are synthesized from cholesterol in the hepatocytes of the liver. Synthesis of bile acids is the major pathway of cholesterol catabolism, which involves modification of cholesterol ring structure, oxidation and shortening of the side chain, and conjugation with an amino acid (Russell, 2003). There are two pathways of bile acid synthesis: classical (or neutral) pathway and the alternative (or acidic) pathway (Chiang, 2004; Thomas et al., 2008). The enzyme cholesterol 7α-hydroxylase, encoded by gene *CYP7A1*, is the rate –limiting enzyme of the classical pathway (Pullinger et al., 2002). Bile acid synthesis can also occur by "alternative (acidic)" pathway, that is governed by CYP27A1 which converts

oxysterols to bile acids and unlike CYP7A1, CYP27A1 is not regulated by bile acids (Lefebvre et al., 2009). It was estimated that around 6% of bile acid synthesis occurs via the alternate pathway (Crosignani et al., 2007). The initial products, cholic acid (CA) and chenodeoxycholic acid (CDCA) are referred to as primary bile acids, which are further conjugated with glycine or taurine and are the most abundant bile acids in humans. The conjugated bile acids play an important role in solubilization and absorption of dietary lipids and fat-soluble vitamins in the digestive tract. Bile acids are stored in gallbladder at very high concentrations (>300mM) and about 5% of them reach the colon for excretion via feces and 95% of them are reabsorbed back. Further, the bacterial action converts the bile acids into secondary bile acids forming deoxycholic acid (DCA) from CA and lithocholic acid (LCA) from CDCA (Stamp and Jenkins, 2009). These secondary bile acids then pass portal vein and reach the liver, where they are again conjugated and stored in gallbladder. This recycling of bile acids is known as enterohepatic circulation of bile acids (from the gallbladder, to the ileum, to the portal vein, and back to the liver) (Houten and Auwerx, 2004). The process of conjugation increases the aqueous solubility of bile acids and makes them impermeable to cell membranes. This increases the concentration of bile acids in the lumen sufficient to form micelles required for lipid emulsification and absorption. They are also required for activation of certain pancreatic enzymes, absorption of cholesterol, lipid soluble vitamins and some triglycerides and fatty acids from the intestine (lahcevic Z. R., 1996). Bile acids are now shown to act as hormones regulating various metabolic processes (Houten et al., 2006). Bile acids by activation of various signaling pathways regulate their own synthesis, enterohepatic circulation and also triglyceride, cholesterol, glucose and energy homeostasis (Lefebvre et al., 2009). Sequential synthesis of different types of bile acids is illustrated in figure 1-5.

1.10 Bile acid receptors

Bile acids in addition to their role in lipid absorption and cholesterol homeostasis are now shown to be versatile signaling molecules. Bile acids can activate various signaling pathways of which the predominant ones are the TGR5, a GPCR also known as the "membrane bile acid receptor" (Kawamata et al., 2003; Maruyama et al., 2002) and the FXR, which is the "nuclear bile acid receptor" (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Other nuclear receptors for bile acid include pregnane X receptor (PXR) (Staudinger et al., 2001; Xie et al., 2001) and vitamin D receptor (VDR)(Makishima et al., 2002).

Farnesoid X receptor (FXR): nuclear receptor for bile acid

Both conjugated and unconjugated bile acids can activate FXR, with CDCA and its conjugates being the most potent ones (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). It is predominantly expressed in the liver, gut, kidney, and the adrenal cortex. FXR in the liver and intestine is involved in feed-back regulation of bile acid biosynthesis and enterohepatic recycling of bile acids to protect the cells from toxic accumulation of bile acids (Eloranta and Kullak-Ublick, 2005; Zollner et al., 2006). FXR is hence referred to as the "bile acid sensor." In the liver, FXR activation increases conjugation of bile acids (Pircher et al., 2003) with excretion of bile acids from hepatocyte into bile canaliculi (Chiang, 2002; Houten and Auwerx, 2004; Moschetta et al., 2004). In the intestine, it increases the expression of ileal bile acid binding protein (I-BABP)-34, the basolateral bile acid transporters ((organic solute transporter-α (OSTα and β)) (Landrier et al., 2006; Lee et al., 2006) and fibroblast growth factor 19 (Fgf19) (Holt et al., 2003; Inagaki et al., 2005). In both liver and intestine FXR induces expression of the atypical nuclear receptor called short heterodimer partner (SHP) which is known to inhibit activity of several other nuclear receptors. Bile acid-mediated activation of FXR will in turn induce SHP,

which then binds to other nuclear receptors such as liver X receptor-α (LXR-α) to repress the transcriptional activation of *CYP7A1*, which is the rate limiting enzyme in bile acid biosynthesis (Brendel et al., 2002; Lu et al., 2000). FXR activation has also been shown to induce Fgf15 that suppresses *CYP7A1* in a SHP-independent manner (Holt et al., 2003; Inagaki et al., 2005), the feedback inhibition of bile-acid synthesis also underlies the feedback regulation of fatty-acid, triglyceride biosynthesis and VLDL production (Bilz et al., 2006; Watanabe et al., 2004). SHP along with regulation of *CYPA1* expression also regulates sterol regulatory element –binding protein 1c (SREBP1c), which is the master regulator of fatty acid and triglyceride biosynthesis and also the regulator of many genes involved in lipogenesis (Horton et al., 2002).

TGR5: a GPCR for bile acid

TGR5 is a member of the Rhodopsin-like subfamily of G-protein coupled receptors (GPCRs) (Class A) (Katsuma et al., 2005). *TGR5* mRNA is ubiquitously expressed, but with higher expression levels in gall bladder and lower expression in brown adipose tissue, liver and intestine. TGR5 in immune cells has been related to immunomodulatory properties of bile acids (Kawamata et al., 2003). TGR5 in sinusoidal endothelial cells of liver is important in the regulation of nitric oxide production via cyclic AMP-dependent activation of endothelial nitric oxide synthase (eNOS) to scavenge bile acid-induced reactive oxygen species for protecting liver from lipid peroxidation and bile acid induced injury (Keitel et al., 2007). TGR5 modulates metabolism through its influence on incretin production (Katsuma et al., 2005) and mitochondrial energy homeostasis (Maruyama et al., 2006; Watanabe et al., 2006). TGR5 has been linked to epidermal growth factor receptor (EGFR) and c-Jun N-terminal kinase (JNK) signaling, suggesting its role in cell proliferation and apoptosis (Yang et al., 2007; Yasuda et al., 2007). Bile acid treatment of brown adipocytes, and also human skeletal myocytes led to

increase in the activity of the cAMP-dependent thyroid hormone-activating enzyme type 2 iodothyronine deiodinase (D2) and also oxygen consumption indicating energy expenditure (Watanabe et al., 2006). The increase in cAMP stems from the activation of GPCR TGR5 by bile acids (Kawamata et al., 2003; Maruyama et al., 2002).

1.11 Bile acid transporters

Bile acids synthesized in the liver and secreted into the small intestine are reabsorbed by passive absorption across the length of intestine and by active absorption in the ileum (Krag and Phillips, 1974; Schiff et al., 1972). Synthesis of bile acid from cholesterol in liver balances the fecal bile acid excretion which is major route of cholesterol excretion from the body (Dietschy and Turley, 2002; Dietschy et al., 1993). An efficient intestinal reabsorption and hepatic extraction of bile acids mediated by enterocytic and hepatic transporters enables recycling and maintenance of these cytotoxic bile acids to intestinal and hepatobiliary compartments while ensuring a continuous supply of bile acids for absorption of dietary lipids (Hofmann et al., 1991).

Bile acid transporters in the liver

The liver parenchymal cells transport bile acids from the portal blood into bile by an efficient process driven by a unique set of transporters at the sinusoidal and canalicular plasma membranes (Alrefai and Gill, 2007; Trauner and Boyer, 2003). The conjugated bile acids are transported at the sinusoidal membrane mainly (>75%) through the active Na^+-dependent transporter (NTCP), the Na^+ -independent transport is mediated by the organic anion transporting polypeptide (OATP) family transporters. The unconjugated bile acids undergo N-acyl amidation to taurine or glycine (Hofmann and Hagey, 2008) before they are transported by NTCP or OATP. The active transport of bile acids across the canalicular membrane from the interior of hepatocyte is mediated by bile salt export pump (BSEP) (Stieger et al., 1992), some unusual bile

acids such as sulfated bile acids or tetra-hydroxylated forms are transported by ABC transporters including the multidrug resistance protein-1/2 (Akita et al., 2001; Lam et al., 2005; Nies and Keppler, 2007). NTCP is the member of the solute carrier proteins and functions as a sodium-solute cotransporter where it moves 2 or more Na^+ ions per molecule of solute (Hagenbuch and Meier, 1996; Weinman, 1997) and transporting the major glycine and taurine-conjugated bile acid (Boyer et al., 1994; Platte et al., 1996). Unconjugated bile acids are weak substrates for NTCP (Hata et al., 2003; Mita et al., 2006; Mita et al., 2005) hence are mainly transported by OATP transporters (Meier et al., 1997; Meng et al., 2002). NTCP maintains the enterohepatic circulation of bile acids and reduces their concentration in plasma, and is primarily responsible for the hepatic sinusoidal membrane Na^+-dependent bile acid transport (Hagenbuch and Dawson, 2004). NTCP gene transcription is regulated by FXR-dependent and independent mechanisms to prevent cytotoxic accumulation of bile acids in hepatocyte (Eloranta et al., 2006; Karpen et al., 1996). NTCP transport activity is also regulated post-transcriptionally. cAMP can stimulate (in few minutes) Na^+-taurocholate cotransport in hepatocytes (Grune et al., 1993; Mukhopadhayay et al., 1997) by cAMP-induced movement of NTCP from the intracellular compartment to the plasma membrane (Anwer, 2004). Increased cAMP levels activate protein phosphatase 2B which dephosporylates NTCP at serine-226 leading to increased plasma membrane retention of NTCP (Webster et al., 2002). Bile salt export pump (BSEP) is an important ATP-dependent bile acid efflux transporter in the canalicular membrane (Strautnieks et al., 1998; Strautnieks et al., 2008) that can transport unconjugated, taurine-conjugated and glycine-conjugated bile acids (Hirano et al., 2005). Transcription regulation of BSEP expression has been shown to be under the control of FXR.

Bile acid transporters in the intestine

Bile acids secreted into intestine are reabsorbed by passive absorption in the proximal small intestine and by active transport in the distal ileum. The transporters in the terminal ileum responsible for the active transport are the ASBT that shuttle the bile acids across the apical brush border membrane (Hagenbuch and Dawson, 2004; Shneider, 2001). The ileal enterocytes, proximal renal convoluted tubule cells, and cholangiocytes are shown to express the same type of ASBT (Alrefai and Gill, 2007). The internalized bile acids are then effluxed out of the enterocytes into the portal circulation by OSTα-OSTβ (Ballatori et al., 2005; Dawson et al., 2005). The ileal active transport is the major route for uptake of conjugated bile acids and passive transport for the uptake of unconjugated bile acids which represent a small percent of total intestinal bile acids (Dawson et al., 2009). Studies with ASBT null mice have shown the non-ASBT mechanisms contribute little to intestinal bile acid absorption in mouse (Dawson et al., 2003). Other studies have shown a low level of expression of OATP transporters in the intestine contributing a small percent to the intestinal bile acid absorption (Dawson et al., 2003; Oelkers et al., 1997). Once the bile acid enters the enterocyte irrespective of the method of uptake they will ionize at neutral pH in the cytosol of enterocytes there by trapping them inside and requiring efflux carriers for their exit. OSTα-OSTβ is the major efflux transporters of bile acid at the basolateral membrane of not just the ileal enterocyte but also the cholangiocytes and renal proximal tubule cells (Ballatori et al., 2008; Rao et al., 2008). The co-expression and assembly of both OSTα and OSTβ subunits into a complex is required for their membrane trafficking and bile acid transport (Dawson et al., 2005; Li et al., 2007).

1.12 Influence of bile acid on viral replication

Bile acids modulate several signaling pathways and transcription factors in the liver, intestine and immune cells, and viruses that preferentially infect these cells may be modulated by the effects of bile acids. Bile acids inhibit Jak1 and Tyk2-phosphorylation leading to blockade of STAT1-mediated interferon-α and interferon-β signaling in hepatocytes, NK cells, macrophages and lymphocytes. These inhibitory effects of bile acids on interferon signaling restricts the therapeutic effects of interferons against hepatitis B and hepatitis C viruses (Graf et al., 2010). Bile acids both conjugated and unconjugated have been shown to activate EGFR/ERK pathway and extend S-phase of cells correlating with increased Hepatitis C virus genotype 1b and 2a RNA replication (Chang and George, 2007a; Chhatwal et al., 2012; Patton et al., 2011). Hepatitis B virus (HBV) replicates in liver cells using NTCP as entry receptor and also uses the transcription factors hepatocyte nuclear factor 4α and FXR-RXRα heterodimer with two binding sites for them in its enhancer 2 and core promoter region (Reese et al., 2012). Activation of RXRα or FXR alone was shown to be sufficient to induce replication of HBV (Reese et al., 2013). The down regulation of lipid synthesis through FXR activation by bile acids or FXR agonists led to significant reduction of rotavirus replication correlating with the reduction of cellular triglyceride levels in cell culture and also reduced fecal virus shedding in mice with oral administration of CDCA (Kim and Chang, 2011). Porcine enteric calicivirus/Cowden strain of the genus *Sapovirus* grows in LLC-PK cells only in the presence of bile acids in the media. The activation of cAMP/PKA signaling pathway along with down-regulation of STAT1 by bile acids was shown to be required for PEC replication in LLC-PK cells (Chang et al., 2004).

1.13 Ceramide and Sphingomyelinase

Sphingolipids are a major class of lipids that are important components of membranes in eukaryotic cells. Different studies have identified several sphingolipids such as ceramide, sphingosine, ceramide-1-phosphate and sphingosine-1-phosphate which are known to mediate cellular signaling pathways (Kim et al., 2009; Morales et al., 2007; Ogretmen and Hannun, 2004; Zheng et al., 2006b). Of these, ceramide has been identified and studied as an important signaling molecule in cell growth arrest, differentiation, apoptosis and inflammation (Chalfant and Spiegel, 2005; El Alwani et al., 2006; Hannun and Obeid, 2008; Modrak et al., 2006). Ceramide is a simple sphingolipid made of a sphingosine base linked to an acyl chain via its amide group. Ceramide can be produced by de novo synthesis by ceramide synthases or through hydrolysis of sphingomyelin by sphingomyelinases. It is a secondary messenger, a structural component of membranes and also involved in various biological processes such as cell growth, differentiation, apoptosis, and in bacterial and viral pathogenesis (Bollinger et al., 2005; Kolesnick et al., 2000b). Ceramide is highly hydrophobic due to its small polar headgroup and it can exert its biological functions by direct interaction with target proteins (Grösch et al., 2012). The hydrophobic ceramide molecules separate from the lipids in membrane to self-associate and form microdomains that have a tendency to fuse into platforms (Brown and London, 1998; Kolesnick et al., 2000a; Simons and Ikonen, 1997). Ceramide accumulation in the plasma membrane leads to formation of platforms made of lipid-protein domains during internalization of viruses and parasites and in the induction of apoptosis (Gulbins and Grassmé, 2002). The ceramide-enriched domains serve to trap and cluster receptor molecules and facilitate transmission of the trapped molecules' cognate signals across the lipid bilayer and into the cells (Grassme et al., 2003a) and this process might be mediated by direct interaction of protein with ceramide or with ceramide domains acting as a hydrophobic platform. Clustering of receptors

and involved signaling molecules in ceramide-domains results in high receptor density, spatial association of receptors with intracellular molecules, exclusion of inhibitory enzymes and/or transactivation of intracellular enzymes. This phenomenon was demonstrated with CD95 clustering with signal amplification and activation of caspase-8 (Grassme et al., 2003a). Ceramide enriched domains are required for receptor clustering and trapping as seen from studies with Fcγ II(Abdel Shakor et al., 2004), CD40 (Grassmé et al., 2001; Gulbins and Grassmé, 2002), and receptors for internalization of *Neisseria gonorrhoeae* (Grassmé et al., 2007). Ceramide is also shown to directly interact and regulate cathepsin D (Heinrich et al., 1999), phospholipase A_2 (Huwiler et al., 2001), ceramide activated protein serine-threonine phosphatases (CAPP) (Dobrowsky and Hannun, 1993) and protein kinase C isoforms (Huwiler et al., 1998; Muller et al., 1995). Ceramide also regulates the activity of potassium channel Kv1.3 and calcium release-activated calcium channels (Gulbins et al., 1997; Lepple-Wienhues et al., 1999; Samapati et al., 2012). Ceramide due to its negative curvature and tendency to form non-lamellar inverted phases can influence the properties of lipid bilayer with increased lipid flip-flop, bending and vesiculation of membrane especially when ceramide is formed in one leaflet of the bilayer (Contreras et al., 2005; Holopainen et al., 2000). Ceramide has also been shown to form transmembrane channels which are shown to allow passage of proteins initiating apoptosis (Colombini, 2010; Siskind and Colombini, 2000).

Sphingomyelinases are a family of related enzymes that catalyze the hydrolysis of phosphodiester bond of sphingomyelin to ceramide and phosphorylcholine. Several isoforms of sphingomyelinases have been identified and categorized based on their optimum pH requirement: acid, alkaline and neutral sphingomyelinases. Alkaline sphingomyelinases are found in the intestinal tract and bile for digestion of sphingomyelin (Duan, 2006). Neutral

sphingomyelinase (N-SMase) activity was first identified in 1967 by Scheider and colleagues in the tissues of patients with Niemann-Pick disease (Schneider and Kennedy, 1967) and presently four mammalian N-SMases have been identified. These include nSMase1 (gene name: *SMPD2*), nSMase2 (*SMPD3*), nSMase3 (*SMPD4*) and mitochondrial associated-nSMase (*SMPD5*). The catalytic core of N-SMase consist of eight metal-binding residues that bind two Mg^{2+} ions (Ago et al., 2006) and all N-SMases contain a hydrophobic domain that tethers the catalytic domain to the membrane. The N-SMase2 is well characterized and is an important mediator of cellular stress-induced ceramide production and a special role in bone mineralization. Little is known about the other N-SMases beyond their basic properties. N-SMase localizes to the plasma membrane, Golgi and recycling compartments. TNFα, PMA activation through PKC-δ (Clarke et al., 2008) and p38 MAPK-dependent phosphorylation (Clarke et al., 2007) induces its translocation from the Golgi to the inner leaflet of plasma membrane. Several studies have shown that N-SMase with ceramide is key regulator of exosome formation and microRNA secretion (Aubin et al., 2005; Chen et al., 2012; Khavandgar et al., 2011). N-Smase1 was found to localize to nuclear matrix (Mizutani et al., 2001) but the exact role of this is still not understood. (Mitochondria associated) MA-N-SMase was found co-localized with mitochondrial markers and ER markers (Wu et al., 2010). MA-N-SMase has been found to play a role in fertilization (Petcoff et al., 2008) and its highest gene expression was found in testis (Wu et al., 2010). N-SMase3 does not share sequence similarity with any of the N-SMases, found localized in ER and its activity was optimum at neutral pH and require Mg^{2+} or Mn^{2+} (Krut et al., 2006).

The enzyme acidic sphingomyelinase (ASM) (Enzyme Commission Classification number 3.1.4.12) is a lysosomal glycoprotein and also exist as a secretory form (Jenkins et al., 2009). Deficiency of ASM results in lysosomal storage disease, Niemann-Pick-type A and B

(Brady et al., 1966). The optimum pH for ASM activity is 5.0 (Fowler, 1969). The lipid composition of the membrane alters the Km of ASM permitting its activity at higher pH (Schissel et al., 1998a; Schissel et al., 1998b). After certain stimulation the ASM and NADPH-oxidases localize in domains on the outer leaflet of plasma membrane (Xu et al., 2012) indicating ASM is not restricted to lysosomes. ASM is a metalloenzyme with Zn^{2+}-binding motifs hence require Zn^{2+} for activity (Schissel et al., 1998b). Seven different ASM isoforms have been identified that are splice variants from the same gene (Quintern et al., 1989; Rhein et al., 2012). Dependent on the cleavage site of precursor protein the mature protein is either localized to lysosomes or will be secreted form of the ASM (Schissel et al., 1998b). The localization of different forms of ASM is also dictated by the glycosylation pattern on the mature protein (Ferlinz et al., 1994; Newrzella and Stoffel, 1996). Different studies on the activation mechanisms of ASM have reported. In vitro studies using purified ASM have shown direct oxidation of ASM at cysteine-629 leads to dimerization and activation (Qiu et al., 2003) but no studies have reported ASM dimerization inside cells. Caspase-7 has been implicated in activation of ASM (Brenner et al., 1998; Lang et al., 2007). TNF-α binding with its receptor triggers interaction with caspase-7 and resulted in receptor internalization and formation of endosomes (Bertsch et al., 2011). Further, fusion of these endosomes with lysosomes resulted in multivesicular bodies resulting in lysosomal ASM interacting with caspase-7. Caspase-7 cleavage of ASM activates the enzyme, this mechanism of activation seen with TNF-α may not be a general mechanism for other stimuli. PKC-mediated phosphorylation of ASM at serine-508 inside multivesicular bodies has also been shown to activate ASM (Zeidan and Hannun, 2007). Ionizing radiation has been shown to induce sphingomyelinase activation in intact bovine aortic endothelial cells within seconds to minutes and also in membranes devoid of nuclei (Haimovitz-

Friedman et al., 1994). Further studies on radiation effects on tumor vasculature showed they were apoptosis resistant in ASM-deficient hosts and apoptosis competent in ASM wild type ones, this provided the evidence for radiation induced activation of ASM during apoptosis signaling as ceramide-enriched platforms were detected within seconds of cytotoxic radiation (Garcia-Barros et al., 2003). UV-C radiation like ionizing radiation and CD95 also induced ceramide generation and cell death in Jurkat T cells (Rotolo et al., 2005). Till present the exact mechanism of activation of ASM by UV or gamma-irradiation is not fully understood.

1.14 ASM generated ceramide in pathogen invasion of their host cells

Several bacteria and viruses have been shown to induce ceramide formation through activation of ASM. Staphylococcus aureus known to cause sepsis, pneumonia and wound infections was shown to cause apoptosis of human endothelial cells. The mechanism of induction of apoptosis was shown to be result of activation of ASM and ceramide generation leading to subsequent stimulation of JNK signaling, cellular caspases and release of cytochrome c (Esen et al., 2001). CEACAM receptor mediated phagocytosis of Neisseria gonorrhoeae into human neutrophils led to activation of ASM and pharmacological inhibition of ASM activity inhibited its phagocytosis, further reconstitution of ceramide in ASM inhibited cells restored internalization of N. gonorrhoeae. They also showed that CEACAM receptor-initiated stimulation of src-like tyrosine kinases and Jun N-terminal kinases required ASM activity (Hauck et al., 2000). Pseudomonas aeruginosa infection of human nasal epithelial cells or murine tracheal epithelial cell triggered ASM activation, its translocation from intracellular compartment to the extracellular leaflet and generation of ceramide for the formation of platforms within 5-10 minutes of infection which disappeared in next 30 min and this was required to internalize P. aeruginosa and also induce apoptosis (Grassme et al., 2003b).

Acid sphingomyelinase and ceramide-enriched membrane platforms are also been shown to be involved in the infection of human cells with pathogenic rhinoviruses. Rhinovirus infection triggers rapid activation of acid sphingomyelinase with microtubule- and microfilament-mediated translocation of ASM to plasma membrane and generation of ceramide platforms that colocalize with rhinovirus for their uptake into the cells (Grassmé et al., 2005). Measles virus binding to the pattern recognition receptor on dendritic cells causes activation of neutral and acid sphingomyelinases leading to accumulation of ceramide in plasma membrane. This process of activation of ASM and recruitment of ASM to plasma membrane also induced efficient recruitment of CD150 (which is the uptake receptor for measles virus) from the intracellular Lamp1+ storage compartment shared with ASM. This process promotes receptor and signalosome co-segregation into ceramide enriched microdomains and provide favorable environment for membrane fusion and pathogen uptake (Avota et al., 2011). Ebolavirus was found to be associated with sphingomyelin enriched lipid rafts and its binding led to recruitment of lysosomal ASM to the cell surface. The activity of ASM was shown to be required for the attachment and entry of ebolavirus into the cells (Miller et al., 2012). HIV infection was shown to be inhibited by ASM generated ceramide platforms that restrict the lateral diffusion of CD4 (cellular receptor for HIV) but not the coreceptor CCR5 leading to inhibition of fusion process primarily resulting from clustering of CD4 molecules (Finnegan et al., 2007). *Trypanosoma cruzi,* an intracellular protozoan invades large number of different cell types without the requirement of host actin rearrangement (Schenkman et al., 1991) but required recruitment and fusion of host lysosomes to its entry site (Tardieux et al., 1992). The entry of *T. cruzi* into host cells was shown to mimic a process of plasma membrane injury and repair that involves Ca^{2+}-dependent exocytosis of lysosomes that delivers ASM to the outer leaflet of plasma membrane.

The ASM on the outer leaflet leads to generation of ceramide that activates endocytosis and internalizes the membrane lesion along with internalization of *T. cruzi*. Inhibition or depletion of lysosomal ASM blocked plasma membrane repair and markedly reduced the susceptibility of host cells to *T. cruzi* invasion (Fernandes et al., 2011).

ASM regulates vesicular fusion process by modifying the steric conformation of cellular membranes and this has been shown to have implications in phago-lysosomal fusion in macrophages infected with *Listeria monocytogenes*, in exocytosis of secretory lysosomes of lymphocytic choriomeningitis virus-specific cytotoxic T cells and in generation of multinucleated giant cells in granuloma of mice infected with *Mycobacterium avium*. ASM knock-out mice were highly susceptible to *L. monocytogenes* with bacterial loads in liver and spleen exceeding 100-10,000 folds of wild-type mice. The kinetics of phagosomal maturation and subsequent fusion with lysosomes is critical in control of *L. monocytogenes* infection. In ASM knock-out macrophages a prolonged localization of *L. monocytogenes* in late phagosomes and localization of *L. monocytogenes* with Lamp1+ lysosomal compartments occurred with reduced frequency, suggesting phagosomal maturation defects in ASM knock-out macrophages as a results of perturbation in lysosomal fusion process. The inability of ASM knock-out mice to restrict the growth of *L. monocytogenes* was attributed to the impaired transfer of lysosomal hydrolases to late phagosomes in ASM knock-out mice that was required for killing of *L. monocytogenes* in phago-lysosomes before it can escape from the phagosome into the cytosol (Utermöhlen et al., 2008; Utermöhlen et al., 2003). ASM was found to be critical for fusion of secretory lysosomes with the plasma membrane and formation of fusion pore in cytotoxic T lymphocytes and as a result of this, the final degranulation to release cytotoxic effector molecules from granules was impaired in ASM knock-out cytotoxic T lymphocytes

leading to delayed elimination of Lymphocytic choriomeningitis virus in a ASM knock-out mice (Herz et al., 2009; Utermöhlen et al., 2008). ASM localization to the outer leaflet of the plasma membrane was shown to be important in the formation of multinucleated giant cells by fusion of macrophages in granuloma of mice infected with *Mycobacterium avium*. *M. avium* infected ASM knock-out mice survived for up to 120 days without any clinical signs while wild-type mice died between 70-80 days post infection. The bacterial loads in liver and spleen was comparable in wild-type and ASM knock-out mice but histologically wild-type mice showed large multinucleated giant cells with infiltrates of hypertrophic macrophages harboring masses of mycobacteria but in ASM knock-out mice only small granulomas were seen. The formation of giant cells which requires ASM activity is the site for replication of *M. avium* are not formed in ASM knock-out mice restricting the growth of *M. avium* in them (Utermöhlen et al., 2008).

1.15 Studies on calicivirus entry and replication

Recognition of host cell is mediated by interaction with virus specific receptors on the surface of cells. Carbohydrate antigens associated with human histo-blood group antigens (HBGA) and other carbohydrate structure are commonly used by caliciviruses as attachment receptors (see section 1.3). Initial binding studies using Norwalk VLPs showed the VLPs were able to bind Caco-2 cells which express H antigen and only a small proportion (<7%) of the bound particles were internalized (White et al., 1996). Due to the lack of cell culture systems and animal models to study replication cycle of noroviruses, other members of *Caliciviridae* family have been examined to understand their replication cycle. FCV that can be propagated in Crandell-Reese feline kidney (CRFK) cells and feline embryonic fibroblasts has been studied for a long time (O'Reilly and Whitaker, 1969). Murine norovirus-1 was initially identified as a Norwalk-Like virus that was causing mortality in mice deficient in signal transducer and

activator of transcription 1 (STAT1). It was also shown that STAT1 was required for survival against MNV-1 infection since MNV-1 infection was lethal in mice with intact B and T cell compartments ($STAT^{-/-}$), lacking B and T cells ($RAG/STAT^{-/-}$) but $RAG^{-/-}$ mice survived (Karst et al., 2003). The murine norovirus can be propagated in macrophage-like cells and has been suggested as a surrogate model for human noroviruses (Leung et al., 2010; Wobus et al., 2006) although differences in cellular tropism exit between them, with MNV-1 infecting macrophages and dendritic cells and human noroviruses having enteric tropism affecting upper intestinal tract. The porcine enteric calicivirus (PEC), a Sapovirus, has been successfully propagated in cell culture and because it causes an enteric disease similar to human caliciviruses, PEC has been suggested to be a better model for human noroviruses and sapoviruses compared to FCV and MNV-1 (Chang et al., 2002; Vashist et al., 2009).

Feline calicivirus entry into CRFK cells was shown be through clathrin-mediated endocytosis and also required endosomal acidification (Stuart and Brown, 2006). Further FCV entry was shown to permabilize cell membrane for co-entry of toxins such as α-sarcin (Stuart and Brown, 2006). Murine norovirus was shown to readily infect primary murine macrophages, dendritic cells and their derived cell lines (Ward et al., 2006; Wobus et al., 2004; Wobus et al., 2006). It was further shown that entry of MNV-1 into these permissive cells was pH-independent (Perry et al., 2009) and was mediated through non-clathrin-, non-caveolae-, dynaminII - and cholesterol-dependent pathway (Gerondopoulos et al., 2010; Perry and Wobus, 2010). PEC was first adapted to grow in primary porcine kidney cells (Flynn and Saif, 1988) and later in continuous porcine kidney cell lines (Parwani et al., 1991), in the presence of porcine intestinal contents. It was later established that the bile acids in intestinal contents induced PKA and cAMP

signaling pathway (Chang et al., 2002) and subsequently decrease interferon-mediated STAT-1 in LLC-PK cells and allow the growth of PEC (Chang et al., 2004).

Studies on human norovirus has been hampered by the non-availability of a cell culture system to growth them. Various reports have shown that block in norovirus replication is at the level of viral entry and/or uncoating since transfection of Norwalk virus RNA lead to efficient replication and recovery of virus particles from Huh-7 cells expressing human FUT2 gene (Guix et al., 2007). Some of the advances in norovirus research include demonstration of expression of viral proteins and subsequent NV RNA replication including viral subgenomic RNA following the transfection of a full-length cDNA clone of the NV genome (under control of the T7 promoter) into modified vaccinia Ankara (MVA)-T7 infected cells (Asanaka et al., 2005). A DNA polymerase II driven reverse genetics system for MNV-1 was reported demonstrating the recovery of infectious MNV following baculovirus transduction of viral cDNA into human hepatocellular carcinoma cell line G_2 (HepG2) cells or transfection of the pol II promoter viral cDNA construct into HEK293T cells. Using this reverse genetics system genetically modified MNV can be recovered (Ward et al., 2007). Chang et al. reported the generation of a stable Norwalk (NV) virus RNA replicon system that functions in both human Huh-7 cells and hamster BHK21 cells expressing NV proteins and self-replicating RNA without the need for a helper virus, and the altered Norwalk virus replicon RNA codes for neomycin resistance in place of VP1 protein for selection of replicon harboring cells (Chang et al., 2006). The NV replicon system has enabled the identification of potential antiviral molecules. A reverse genetics system for the MNV-3 stain that is known to cause persistent infections in wild-type mice occurring in caecum and colon with infectious virus shedding up to > 10 weeks was reported (Arias et al., 2012). This reverse genetics system for MNV-3 aids in identification of viral sequences that

contribute to persistence of the virus in immunocompetent mice and an acute infection in immunocompromised host. Very recently a reverse genetics system driven by a mammalian elongation factor-1α promoter for human norovirus was reported. This system supported genome replication, particle formation containing a GFP gene inserted in ORF1 of genomic RNA and without the requirement of helper virus. Further it was shown that the RNA from these particles is infectious producing fluorescent puncta in transfected cells indicating replication competence (Katayama et al., 2014). A recent report demonstrated that human and mouse noroviruses were able to infect B cells in the presence of HBGA-expressing enteric bacteria. The GII.4 human norovirus infection of BJAB human B cell line led to replication with 10-fold and 25-fold increase in viral genome copy number at 3 and 5 days post infection respectively (Jones et al., 2014).

Gnotobiotic pigs have been shown to support HuNoV genogroup II (GII) strain replication with mild diarrhea, fecal virus shedding, and immunofluorescent (IF) detection of both structural and nonstructural proteins in enterocytes (Cheetham et al., 2006a). Chimpanzees were proposed to be models for human norovirus infection studies and testing the efficacy of norovirus vaccines instead of using human volunteers. Seronegative chimpanzees inoculated intravenously with Norwalk virus did not show any clinical signs but showed virus shedding in stools and serum antibody responses observed were similar to patterns in humans. Homologous rechallenge of infected chimpanzees 4, 10 or 24 months later showed resistance to infection. Intramuscular vaccination with two doses of Norwalk virus (GI) and MD145 (GII) VLPs protected them from homologous Norwalk virus challenge 1 month and 18 month after second dose but heterologous challenge did not protect them (Bok et al., 2011). Recently, a mouse model for HuNoV infection was reported where BALB/c Rag-γc-deficient mice was able to

support HuNoV replication following intraperitoneal inoculation (Taube et al., 2013) but generalizability and robustness of this model remains to be established.

1.16 Study objectives

None of the previous studies described have identified the factors responsible for the block(s) of human norovirus replication in cell culture system which remains unknown till date. Understanding the entry events and the host factors required for calicivirus entry will contribute to the development of better models for culturing human norovirus in vitro.

The overall objectives of this dissertation:

i) To identify host factors required during entry of caliciviruses. Using the caliciviruses FCV, MNV-1 and PEC we will examine the host factors that are required by caliciviruses for successful entry, trafficking and escape from endosome compartment for productive replication.

ii) To examine the entry events during bile acid dependent PEC replication. Since PEC is an enteric calicivirus with a special requirement of bile acid for its replication, it represents a better model for study of human norovirus. Here we will examine the exact role of bile acids and the mechanism by which bile acids promote PEC replication.

Figure 1-1. Calicivirus genome organization.

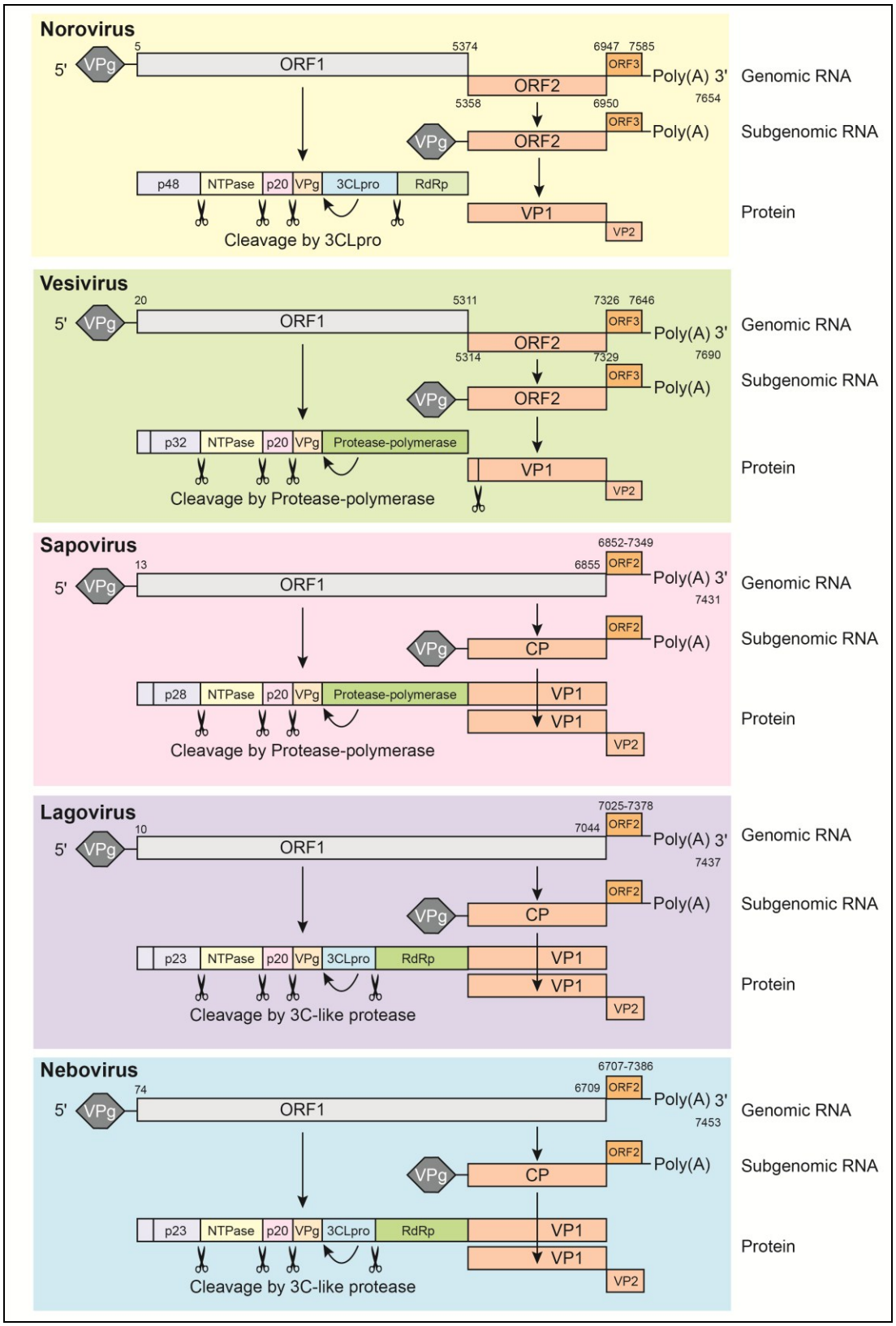


Figure 1-2. Structure of calicivirus major capsid protein (VP1)

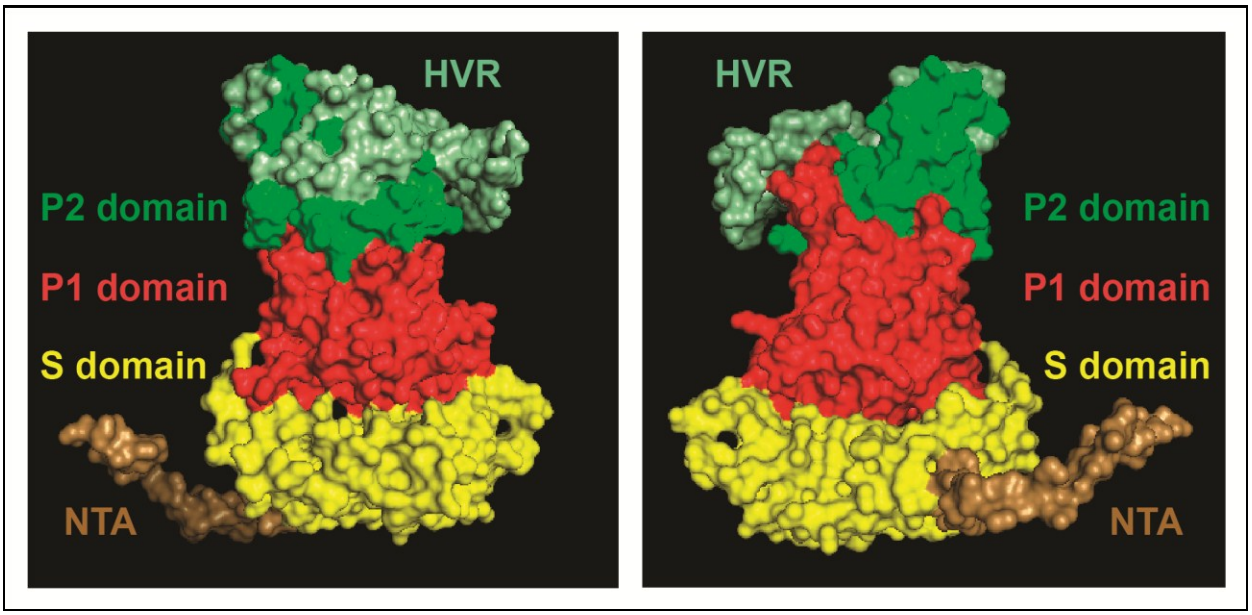


Figure 1-3. Mechanisms of virus entry and trafficking.

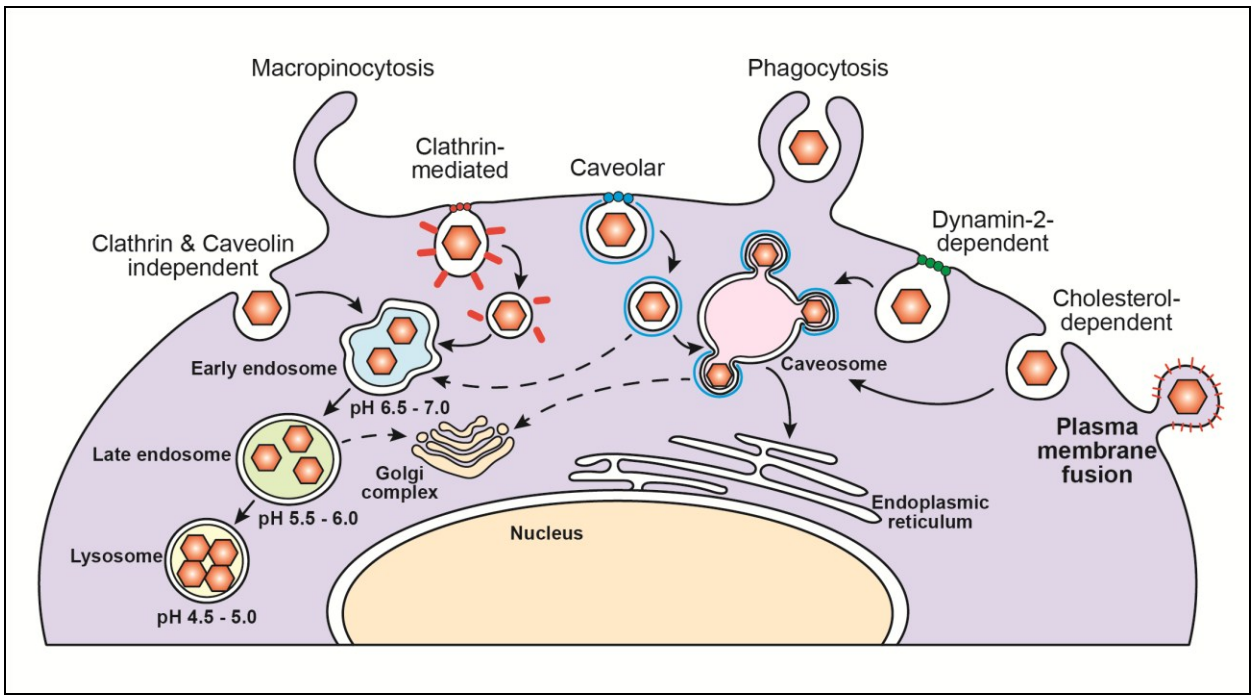


Figure 1-4. Sites of Endosomal escape of viruses.

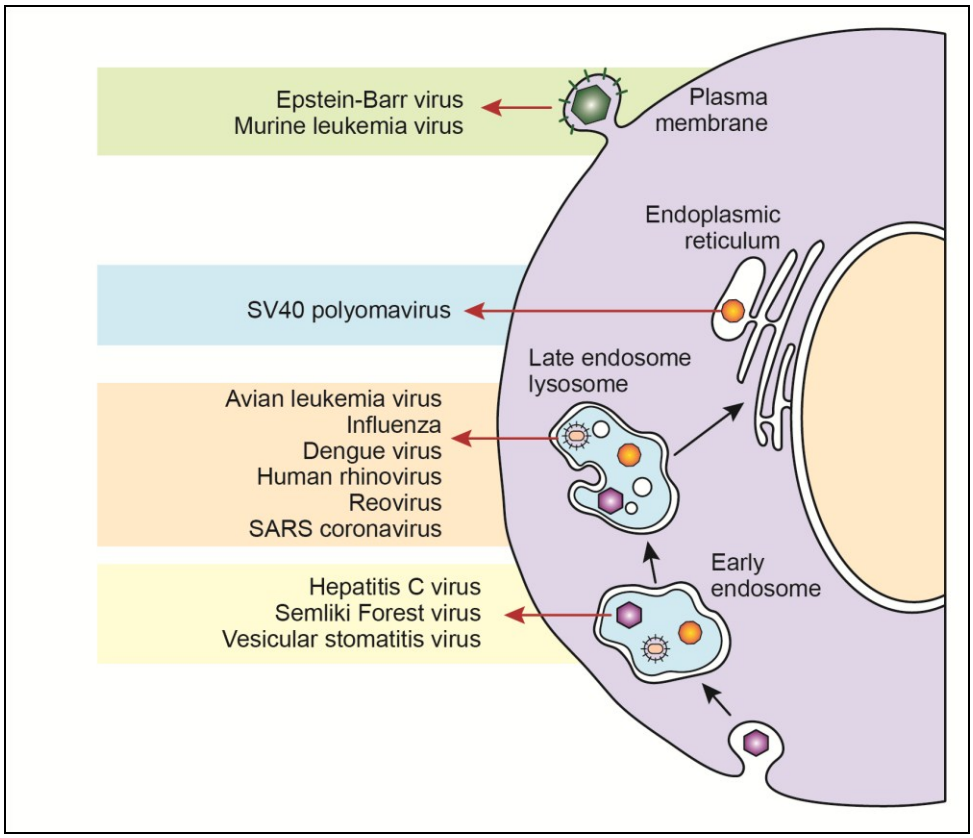
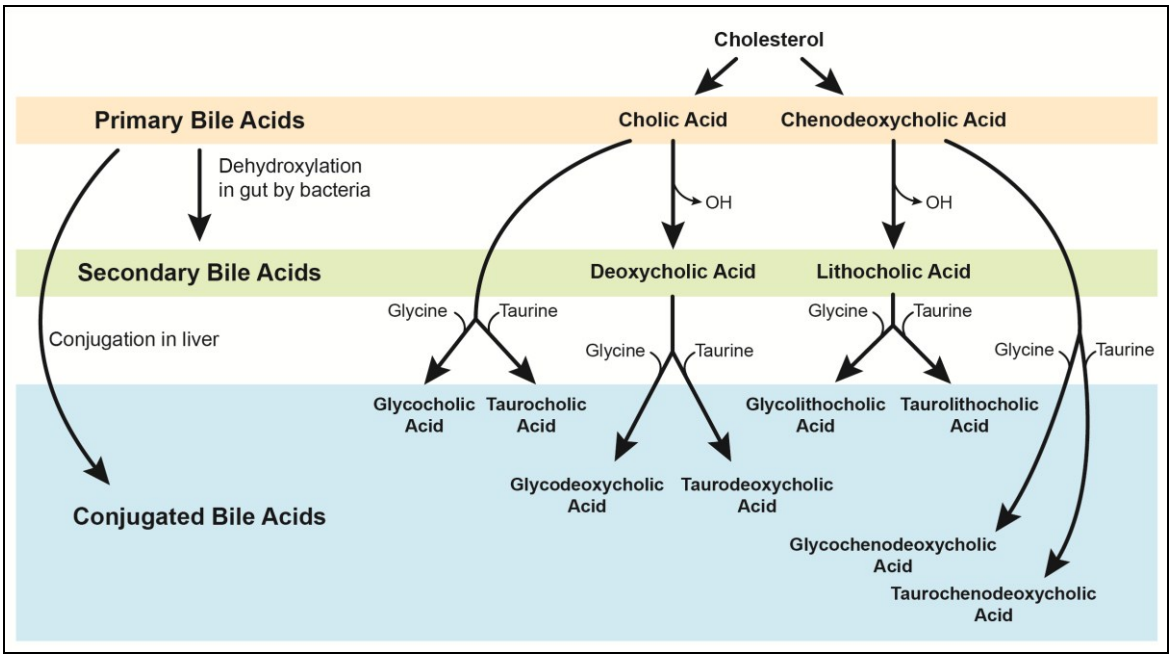


Figure 1-5. Bile acid synthesis and types.



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Chapter 2 - The Crucial Role of Bile Acids in the Entry of Porcine Enteric Calicivirus

2.1 Abstract

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.

2.2 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 four genera: Norovirus, Sapovirus, Lagovirus and Vesivirus (Carstens and Ball, 2009; Green, 2007). Noroviruses and sapoviruses cause gastroenteritis in humans and animals, whereas lagoviruses and vesiviruses mostly infect animals causing a variety of diseases (Green, 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; Chang et al., 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., 2013). 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 2~30 mM in the small intestines (Dowling, 1973; Northfield and McColl, 1973), and majority of bile acids are reabsorbed in the small intestines and returned to the liver (enterohepatic circulation) (Johnson, 1998). PEC replicates primarily in the proximal intestinal tract (duodenum and jejunum) (Flynn and Saif, 1988) where bile acid concentrations are high. In LLC-PK cells, any bile acid, with the exception of hydrophilic ursodeoxycholic acid (UDCA) and its conjugates, support PEC replication (Chang et al., 2004). Among them, glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA) support PEC growth at concentrations as low as 50 μM (Chang et al., 2004).

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., 2013). 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., 2013). 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.

2.3 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 1 h incubation at 37 °C, cells were washed 3 times with PBS, and fresh MEM (2% FBS) containing the same concentration of GCDCA or CDCA, or mock-medium was added to the cells. Virus-infected cells were then further incubated at 37 °C for various time points and virus titers were determined by the 50 % tissue culture infective dose ($TCID_{50}$) method or real-time quantitative RT-PCR (qRT-PCR).

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 Reed-Muench method (Reed and Muench, 1938). **Real-time qRT-PCR.** Before extensive cell cytopathic effects (CPE) occur, total RNA was extracted 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'- AATGAGTCCAGACCAGTCCA-3', a reverse primer, 5'-CCAGGTGACATTGGTGTAGG-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 sec, annealing at 60 °C for 60 sec and elongation at 72 °C for 30 sec. 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 bile acids are 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 RT-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 CC^2 chamber slide (Fisher Scientific, Pittsburgh, PA) treated with FBS and grown to 90% confluency. Confluent cells on the chamber slides were infected with mock (media) or PEC at an MOI of 50, and received various treatments prior to preparation for the confocal microscopy. The treatments included: 1) cells were infected with PEC in the presence and absence of GCDCA (100μM) for 1 h; 2) cells were infected with PEC in the presence of GCDCA and chloroquine (100μM) for 1 h to examine the effect of

endosome maturation in virus replication; 3) cells were infected with PEC in the absence of GCDCA for 1 h, washed with PBS and further incubated at 37°C for 4 or 6 h. In addition, cells were infected with PEC at an MOI of 50 at 37°C for 1 h, and incubated with GCDCA (100μM) for 5, 15, 30 or 60 min before they were fixed for confocal microscopy to trace the virus particles following GCDCA treatment. In a separate experiment, virus titers ($TCID_{50}$ and qRT-PCR) of the LLC-PK cells infected with PEC in the presence of GCDCA and chloroquine (100μM) were determined at 16 PI.

To prepare cells for confocal microscopy, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4) for 15 min at room temperature (RT), permeabilized with 0.1% Triton-×100 (Fisher Scientific) in PBS for 10 min at RT, washed three times with PBS, and incubated in blocking buffer [PBS containing 0.5% bovine serum albumin (Sigma-Aldrich)] for 15 min. The cells were then incubated with primary antibodies specific to PEC (1:200) and Rab7 (1:200) at 37°C to probe PEC and the late endosomes, respectively. After 2 h-incubation, cells were washed three times with PBS and further incubated at 37°C for 2 h with FITC- and PerCP-Cy5.5 labeled secondary antibodies diluted 1:100 in PBS for PEC and Rab7, respectively. The same cells were stained with sytox orange (0.5 μM in 0.9% NaCl) for nucleic acids by washing three times with PBS and incubating for 10 min with sytox orange dye. Coverslips were mounted in Prolong Gold antifade reagent (Molecular Probes), and the cells were scanned with a confocal microscope LSM 510 (Zeiss, Oberkochen, Germany) using a 100× oil-immersion objective. The images were analyzed by using the ImageJ software (1.47 V). Colocalization analysis was done using JACoP and colocalization-MBF plugin for ImageJ. Single channel images were thresholded by Costes' auto threshold method and then Mander's split colocalization values were determined for each experiment.

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 with 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 semiconfluent LLC-PK cells were transfected with siRNA of mock (transfection agents), NTCP, ASBT, FXR, or an irrelevant siRNA (Qiagen) (100 nM 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 $TCID_{50}$ 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 semiconfluent LLC-PK cells were transfected with siRNA of NTCP or ASBT (100 nM) 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.

2.4 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 (Figure 2-1A). The viral RNA levels in the cells incubated with GCDCA increased steadily from 4 h to 12 h PI (Figure 2-1 A). However, there was no indication of virus replication over time in the cells without GCDCA.

Viral titers determined by the $TCID_{50}$ method at each time point were in line with the viral RNA levels in all samples (Figure 2-1 A): viral titers were comparable in the cells incubated with or without of GCDCA up to 4 h PI, but viral titers increased from 4.8 and 6.04 $\log_{10}$ $TCID_{50}$/ml at 8 and 12 h PI, respectively, only in the cells incubated with GCDCA (Figure 2-1 A). In line with these results, extensive CPE started to appear at 12 h PI, and progressed to >90% of cells at 16 h PI only in the virus-infected cells incubated with GCDCA. At 16 h PI, virus titers of PEC-infected cells incubated with GCDCA further increased to 7.5 $\log_{10}$ $TCID_{50}$/ml, while those of virus-infected cells lacking GCDCA remained low at 3.2 $\log_{10}$

$TCID_{50}$/ml (Figure 2-1 B). The expression of viral proteins 2AB, POL and VPg was evident only in the virus-infected cells incubated with GCDCA, as monitored by IFA at 12 h PI (Figure 2-1 C). Similar results were observed with CDCA (100 μM) (data not shown).

Bile acid is required in the early stage of PEC infection.

Addition of GCDCA at 0 h (treatment b), 1 h (treatment c) or 2 h PI (treatment d) resulted in marked viral replication, as determined at 12 h PI (Figure 2-2 A and B), compared to 0 h PI or control (without GCDCA). However, there was no evidence of viral replication when GCDCA was added at 4 h PI (treatment e) (Figure 2-2 B). Notably, GCDCA was most efficient in inducing viral replication when it was present during 1 h of viral inoculation (treatment b) (Figure 2-2 B). 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 (Figure 2-2 A 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 (permissible) with high concentrations of PEC RNAs and incubated the cells in the presence and absence of GCDCA. In addition, MARC-145 cells (non-permissible) were transfected with PEC RNA in the absence of GCDCA. 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 figure 2-3 B). Virus titration conducted at 16 h post-transfection demonstrated the production of infectious viruses following transfection in both

cell lines (Figure 2-3 A). 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 (Figure 2-4 A). However, positive staining for PEC were observed in the cytoplasm in the cells incubated without GCDCA, or cells incubated with GCDCA and chloroquine at 1 h post transfection (Figure 2-4 A). In these cells, confocal microscopy analyses demonstrated colocalization of virus with rab7, a late endosomal marker (Figure 2-4 A, panels h and p). At 4 and 6 h PI, viruses colocalized with rab7 were still observed by confocal microscopy (Figures 2-4 B). 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 min to 60 min PI in the cells incubated with GCDCA (Figure 2-4 C). ImageJ- colocalization (MBF) anlaysis and JACoP plugin was used for colocalization analysis. The thresholds were set by the Costes' Auto threshold method and the thresholded Mander's split colocalisation 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% (Figure 2-5 A). Transfection of NTCP or ASBT siRNAs significantly reduced GCDCA-mediated virus replication by more than 80%, measured by real time qRT-PCR (Figure 2-5 A). 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 (Figure 2-5 A). 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 (Figure 2-5 B), 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.

2.5 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, 2007). 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 (Figure 2-1). The time-of-addition study showed that the critical time points when bile acids are most effective are the early stages of virus replication (Figure 2-2), 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 cytoplasm, suggesting that bile acids are involved in the very early stages of virus replication cycle (Figure 2-3). 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 immunosorbent 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 (Figure 2-4 A-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 (Figure 2-4A, panel's 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 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; Ebert et al., 2004; Johnson et al., 2009). For parvovirus, a lipolytic enzyme (phospholipase A2, PLA2) 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 (Figure 2.5A). 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; Chang et al., 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 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 (Figure 2-6). 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.

2.6 Acknowledgements

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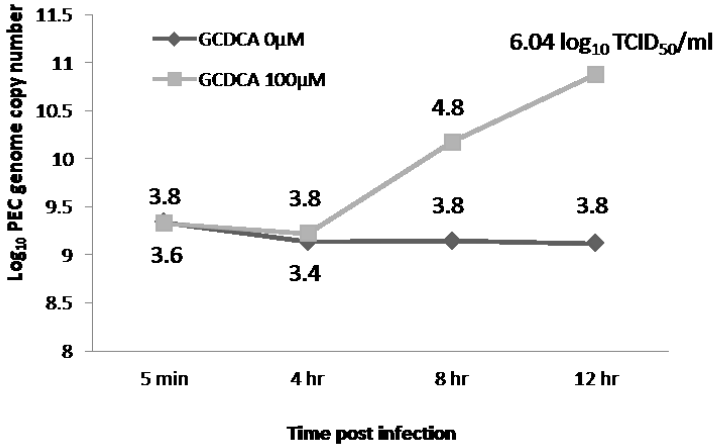
Table 2-1. Sequences of siRNA, and primers and probes for real time qRT-PCR.

Name	Class	Sequences (5' → 3')
pASBT	siRNA	rCrCrArArArCrUrGrGrCrArGrArGrGrArArGrCrCrArArUrArCrArU
		rGrUrArUrUrUrGrGrCrUrUrCrCrUrCrUrGrCrCrArGrUrUrUGG
pASBT	qRT-PCR	Forward: ATTCCAGAGTTGACCCACAG
		Reverse: CTACTGGGTTGATGGCGAC
		Probe: 56-FAM/TGGTATAGATTAAGAGGCACAGCGGC/3IABkFQ
pNTCP	siRNA	rGrUrGrUrUrGrArGrGrArUrGrArUrGrCrCrUrArUrGrGrUrGrCrArG
		rGrCrArCrCrArUrArGrGrCrArUrCrArUrCrCrUrCrArArCAC
pNTCP	qRT-PCR	Forward: CATAGATGCCCCTGGAGTAAAG
		Reverse: CTCTCCAACATCTTCGCTCTG
		Probe: 56-FAM/CATGATGACCACCTGCTCCACCTT/3IABkFQ
pFXR	siRNA	rArCrCrArArUrGrGrUrCrUrGrArUrCrUrGrCrArUrGrCrUrGrCrUrU
		rGrCrArGrCrArUrGrCrArGrArUrCrArGrArCrCrArUrUrGGT
pFXR	qRT-PCR	Forward: AGTGGTACTCTCCTGGCATA
		Reverse: TCCCCTTTTATTCTCCCTGTTG
		Probe: 56-FAM/ATCTCTACTTCCCCAGCCTCTCCC/3IABkFQ

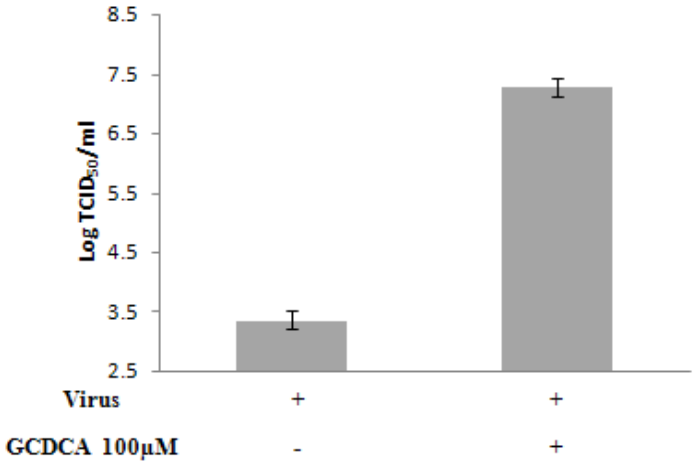
Figure 2-1. The effects of GCDCA on the one-step replication of PEC.

(A) Confluent LLC-PK cells were infected with PEC at an MOI of 50 in the presence or absence of GCDCA (100 μM) at 37 °C for 1h. Viral RNA was extracted from the cells at 5 min, 4 h, 8 h and 12 h PI for real-time qRT-PCR, and genome copy numbers were calculated by plotting Ct values against a standard curve generated using a series of dilutions of in-vitro transcribed PEC RNA genome. The graph shows PEC genome copy numbers in the samples collected at different time points. Numbers above the symbols indicate virus titers determined by the TCID50 method (log10 TCID50 /ml). (B) The TCID50 values of the virus-infected cells incubated with or without GCDCA were determined at 16 h PI. An asterisk indicates a significant difference between the groups ($p < 0.05$). (C) IFA in the cells infected with PEC with or without GCDCA (100 μM). Virus infected cells were fixed at 12 h PI and expression of viral proteins was determined by probing with antibodies specific to viral proteins 2AB, POL and VPg.

A.



B.


8.5
7.5
6.5
5.5
4.5
3.5
2.5
Log TCID50/ml
Virus
+
+
GCDCA 100μM
-
+

C.

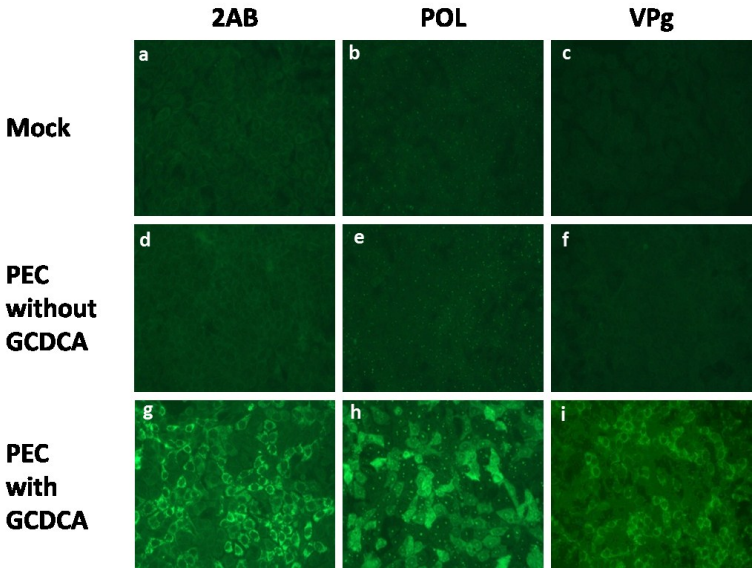
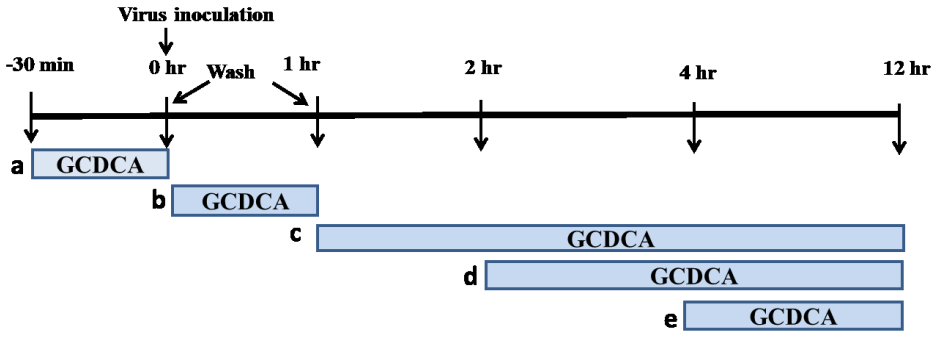

2AB
POL
VPg
Mock
PEC without GCDCA
PEC with GCDCA
a
b
c
d
e
f
g
h
i

Figure 2-2. Effects of bile acids on PEC replication with time-of-addition assay.
Confluent LLC-PK cells were infected with PEC at an MOI of 50. GCDCA (100 µM) was added at different time points during virus replication. (A) A schematic drawing shows various time periods of GCDCA treatments. The bars indicate the time during which GCDCA was present in the media. (B) Virus replication was quantified by real time qRT-PCR at 6 h and 12 h PI. Asterisks indicate significant ($p < 0.05$) difference in virus genome levels, compared to those of PEC infection without GCDCA.

A.



B.

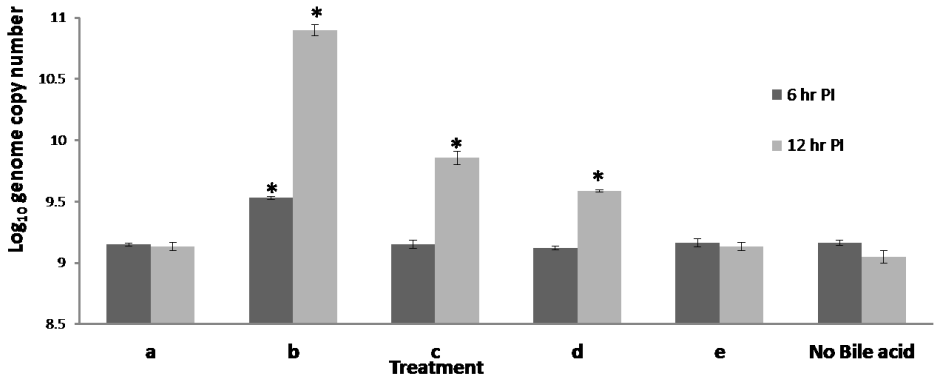
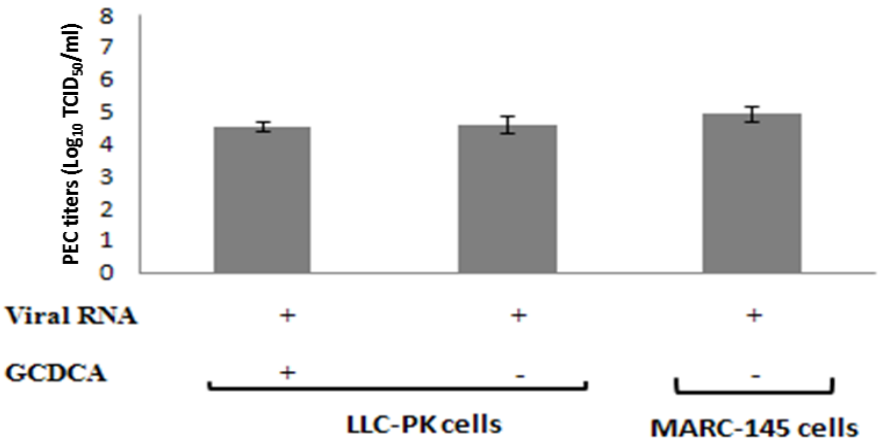


Figure 2-3. Transfection of PEC RNA into the permissible and non-permissible cells. (A) One-day old LLC-PK cells or MARC-145 cells were transfected with PEC RNA genome (0.5 µg /well) in the presence or absence of GCDCA (100 µM). At 16 h post transfection, recovery of infectious virus was determined by the $TCID_{50}$ 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.

A.



B.

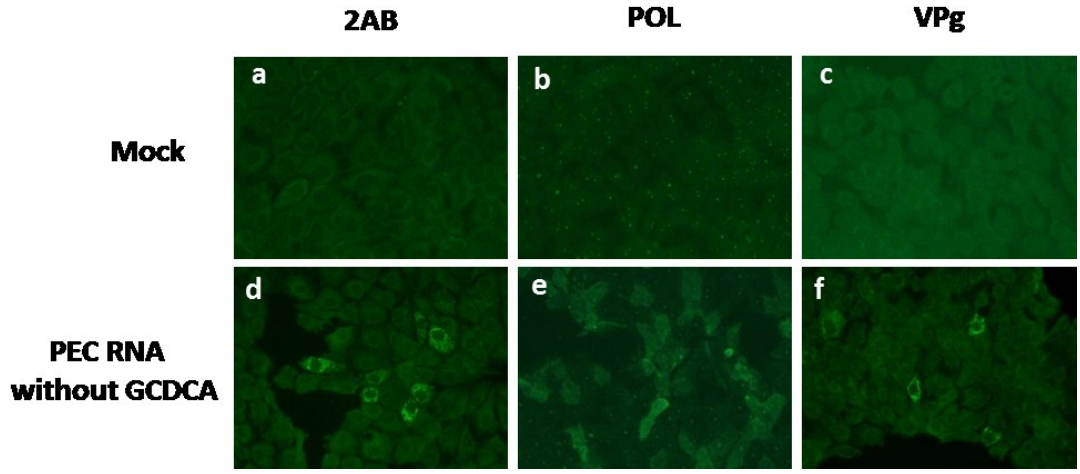
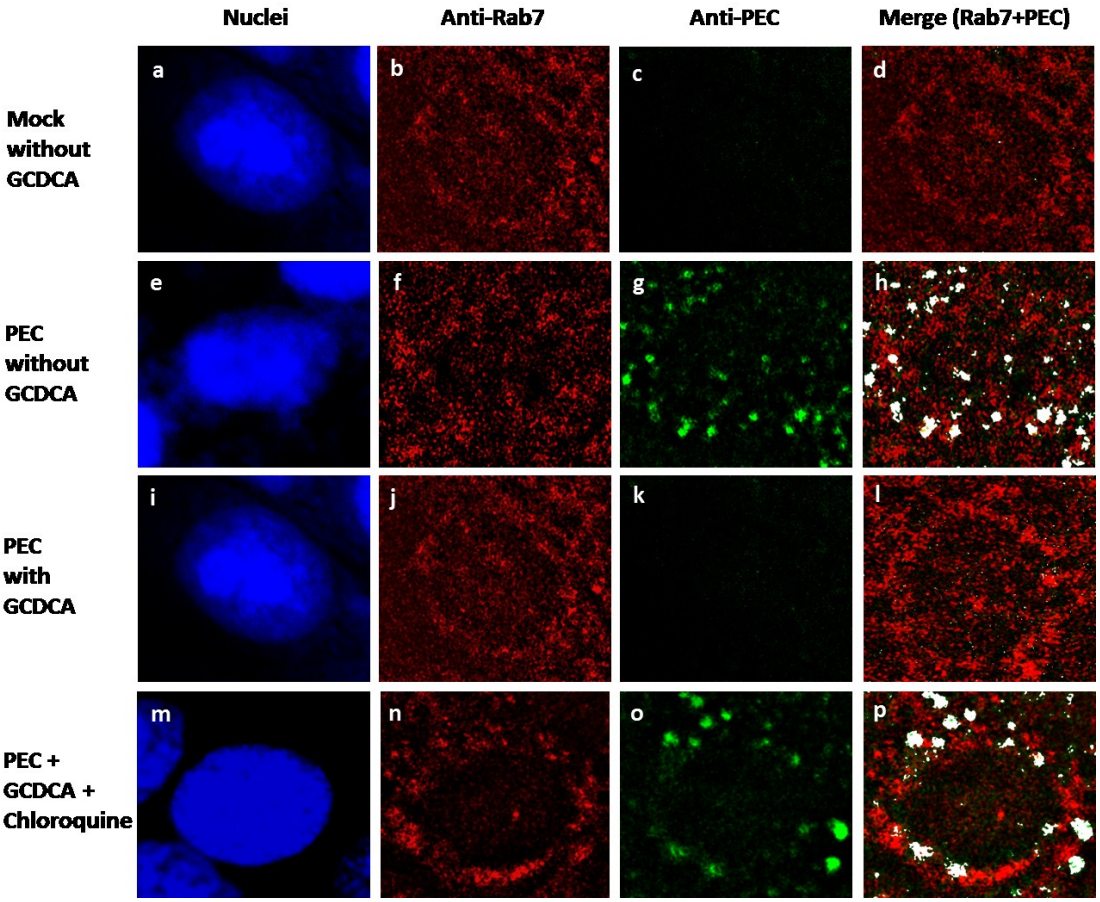
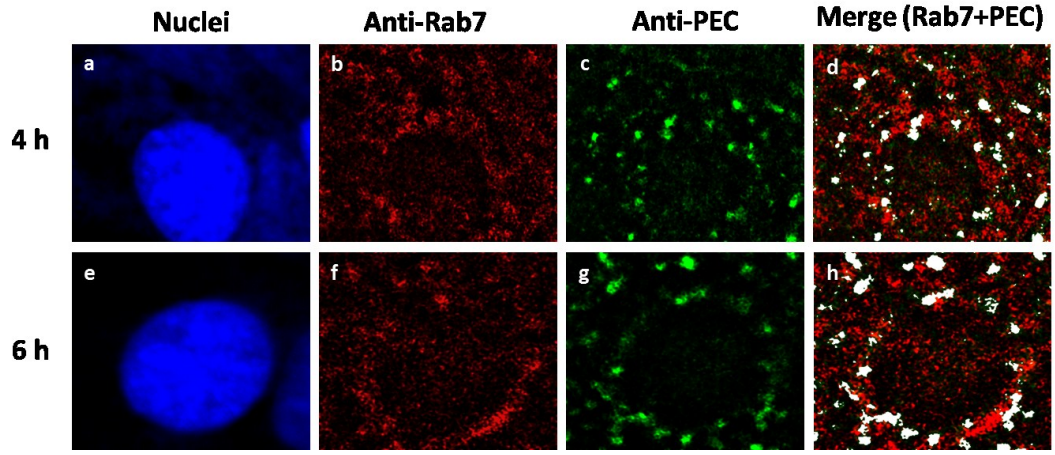


Figure 2-4. Confocal laser scanning microscopic examination of PEC entry.
Confluent LLC-PK cells grown on Lab-Tek II CC^2 chamber slides were infected either with mock (medium) or PEC (MOI 50) in the presence or absence of GCDCA (100μM), fixed and stained at 1 h PI (A) or at 4 or 6 h PI (B) and observed under a confocal laser scanning microscope. (A) Top panels (a to 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 to p) show the cells infected with PEC in the presence of GCDCA and chloroquine. (B) Top panels (a to d) show the cells infected with PEC without GCDCA and observed at 4 h PI; bottom panels (e to 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 to d), 15 min (e to l), 30 min (i to l) or 60 min PI (m to p). (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 (5μM) (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.

A.


Nuclei
Anti-Rab7
Anti-PEC
Merge (Rab7+PEC)
Mock without GCDCA
PEC without GCDCA
PEC with GCDCA
PEC + GCDCA + Chloroquine
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

B.


Nuclei
Anti-Rab7
Anti-PEC
Merge (Rab7+PEC)
4 h
6 h
a
b
c
d
e
f
g
h

C.

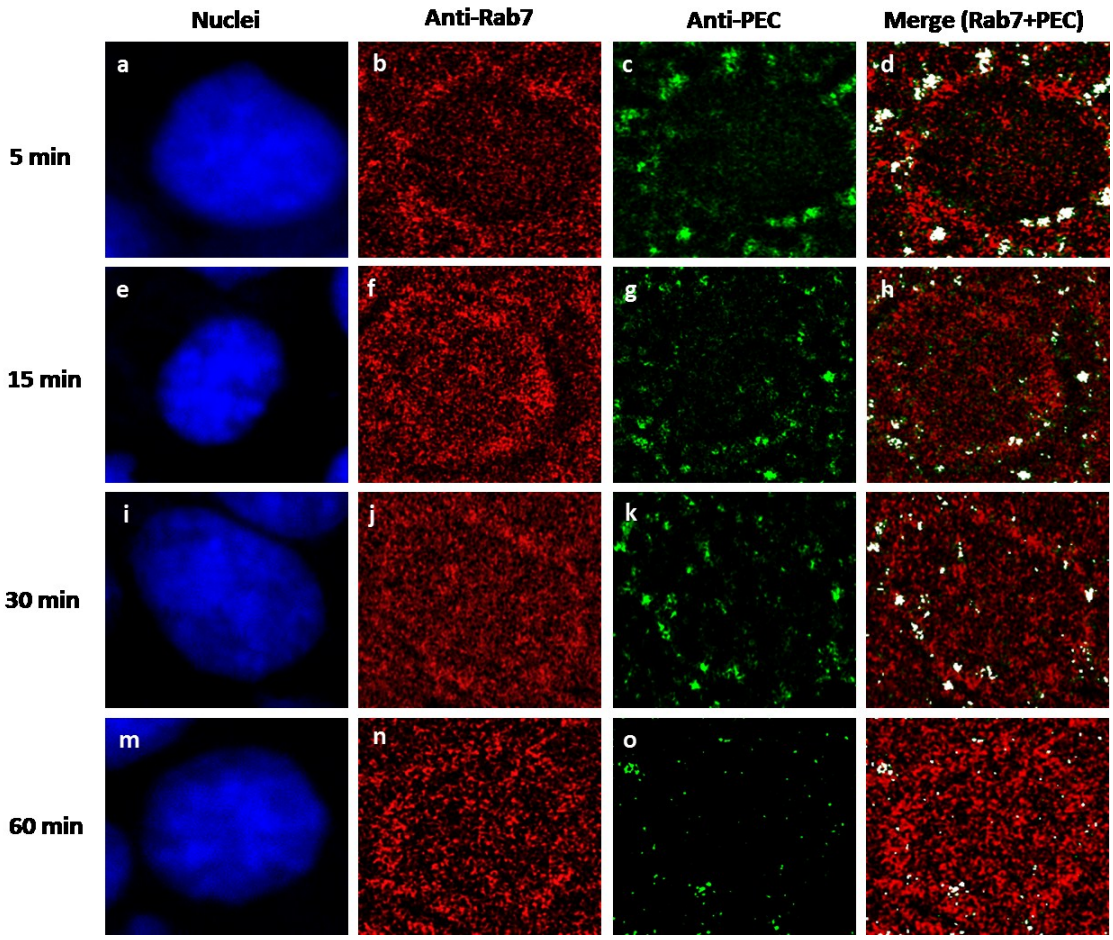
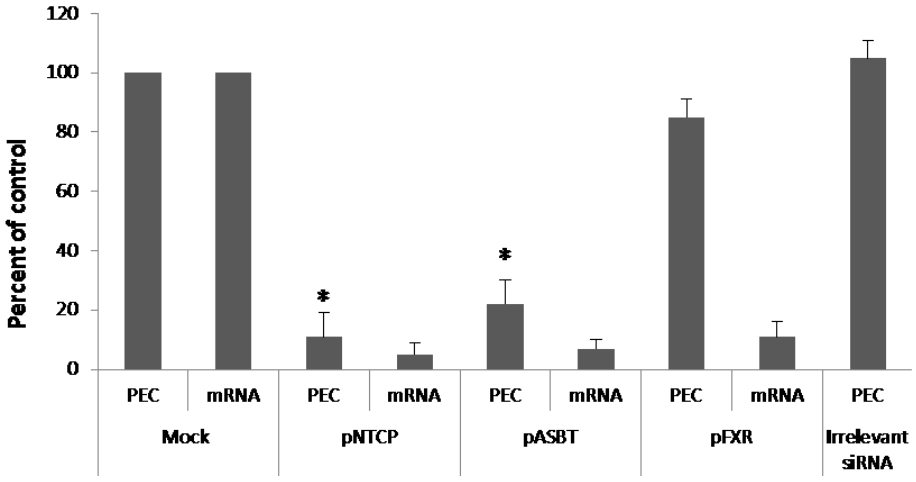

Nuclei
Anti-Rab7
Anti-PEC
Merge (Rab7+PEC)
5 min
15 min
30 min
60 min
a
b
c
d
e
f
g
h
i
j
k
m
n
o

Figure 2-5. Role of bile acid transporters NTCP, ASBT and receptors FXR and TGR5 in PEC replication.

(A) One-day old LLC-PK cells were transfected with siRNA of NTCP, ASBT, FXR, or irrelevant control. 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.

A.



B.

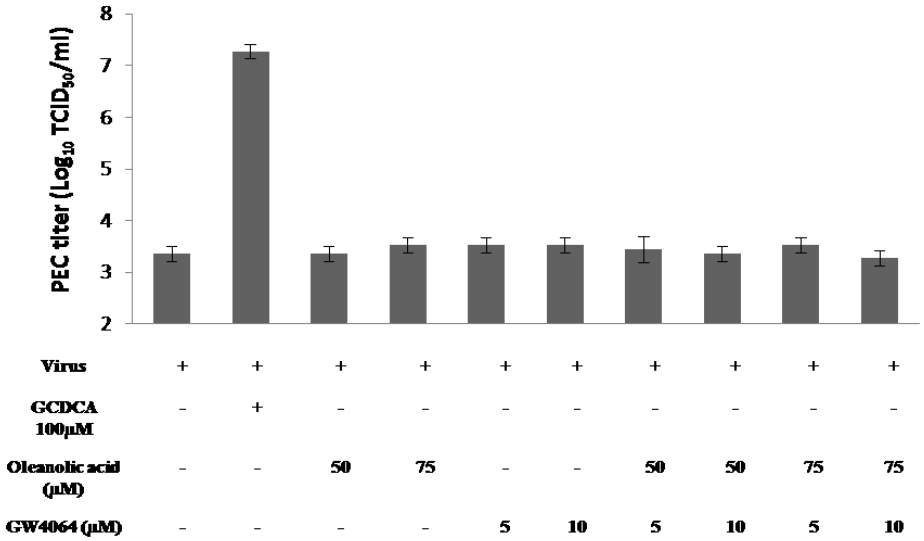
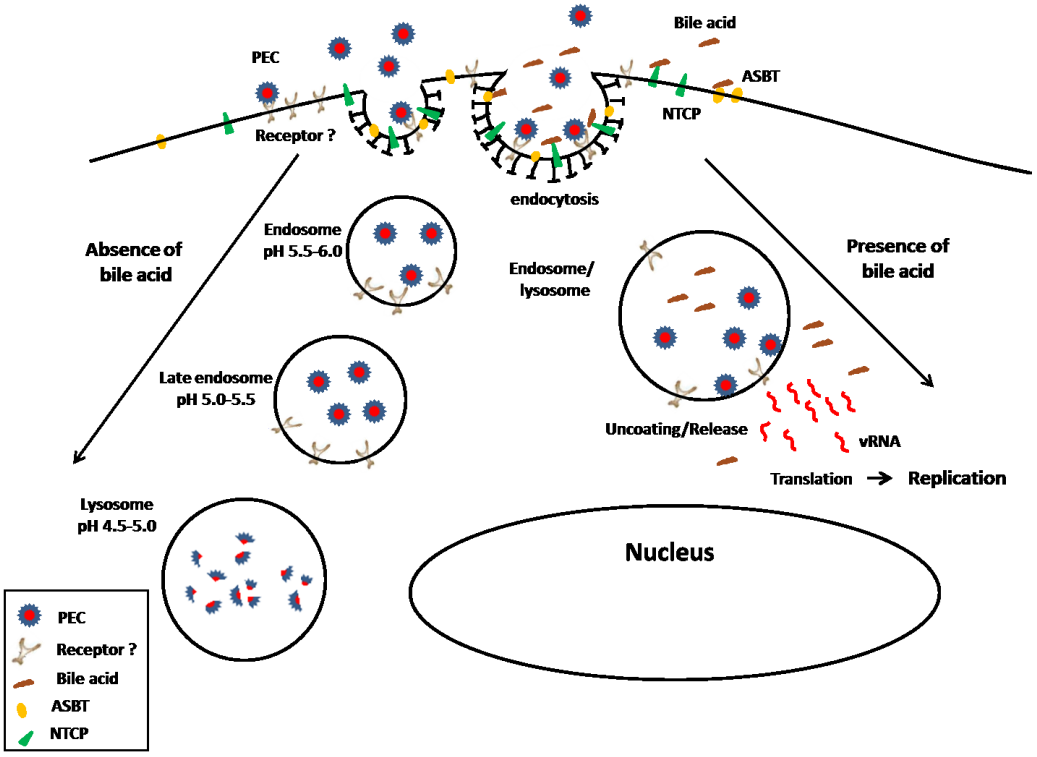

PEC titer (Log10 TCID50/ml)
8
7
6
5
4
3
2
Virus + + + + + + + + + +
GCDCA 100μM - + - - - - - - - -
Oleanolic acid (μM) - - 50 75 - - 50 50 75 75
GW4064 (μM) - - - - 5 10 5 10 5 10

Figure 2-6. A proposed model for bile acid-mediated PEC replication in LLC-PK cells. In this model, PEC enters the cells via endocytic pathway [through unidentified receptor(s)] and reaches the late endosomes. In the presence of bile acids and bile acid transporters, PEC escapes from the late endosomes into the cytoplasm to initiate virus replication. In the absence of bile acids or bile acid transporters, PEC remains in the late endosomes/lysosomes and is destined to be degraded.



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Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., Fu, L., Song, M., Chen, P., Gao, W., Ren, B., Sun, Y., Cai, T., Feng, X., Sui, J., Li, W., 2012. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 1, e00049.
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Chapter 3 - Endosomal acidification and cathepsin L activity is required for calicivirus replication

3.1 Abstract

Although caliciviruses are the causative agents of many important human and animal diseases, their entry process into host cells has not been well understood. In this study, we investigated the role of cellular proteases and endosome maturation in the entry of caliciviruses including porcine enteric calicivirus (PEC), murine norovirus (MNV)-1 and feline calicivirus (FCV). Treatment with chloroquine or cathepsin L inhibitors, but not cathepsin B inhibitors, significantly reduced the replication of PEC, MNV and FCV. Confocal microscopy analysis of PEC and MNV-1 showed that virus particles were retained in the endosomes in the presence of a cathepsin L inhibitor or chloroquine during virus entry. When concentrated PEC, MNV or FCV were incubated with recombinant cathepsin L, the minor capsid protein VP2 of PEC and the major capsid protein VP1 of MNV and FCV were cleaved by the protease based on the Western blot analysis. To further analyze the role of cathepsin L in FCV replication, infectious clones carrying a single alanine mutation at the putative cleavage sites in the conserved region of the P2 domain in VP1 were generated. Virus recovery and replication competency studies using the mutant clones demonstrated that F463 or L472 were crucial for successful virus infection in cell culture. The results of this study suggest the important role of cathepsin L in the replication of caliciviruses, and cathepsin L as a potential therapeutic target for calicivirus infection.

3.2 Introduction

Caliciviruses are nonenveloped viruses of 35 to 40 nm in diameter and possess a single-stranded, positive-sense RNA genome of approximately 7 to 8 kb (Green, 2007). Caliciviruses have a T=3 icosahedral capsid assembled with 90 dimers of VP1 which is composed of three

domains: the N-terminal arm (NTA), the S (shell) and the P (protruding) domains (Ng and Parra, 2010; Prasad et al., 1999). The P domain of VP1 forms the spike on the virion and is composed of P1 and P2 subdomains. The P2 subdomain is located within the P1 subdomain and contains a conserved region flanked by the hypervariable domains (Prasad et al., 1999). The hypervariable domain is mostly involved in receptor binding (Bhella et al., 2008; Bhella and Goodfellow, 2011; Chen et al., 2006). The function of VP2, a minor structural protein, is not well known but it was reported that VP2 is required for the production of infectious feline calicivirus (FCV) (Sosnovtsev et al., 2005), interacts with VP1 to increase stability of norovirus capsid, and is likely to be associated with packaging of RNA genome (Bertolotti-Ciarlet et al., 2003; Bertolotti-Ciarlet et al., 2002).

Caliciviruses belong to the family *Caliciviridae,* which comprises at least five genera including Norovirus, Sapovirus, Lagovirus, Vesivirus, and Nebovirus (Green, 2007). Noroviruses and sapoviruses cause enteric infections in humans and animals (Green, 2001). Lagoviruses and vesiviruses mainly cause oral, respiratory and sometimes systemic infections in animals (Green, 2001). Noroviruses account for 58% of foodborne human illnesses causing about 21 million cases of gastroenteritis and 800 deaths annually in the United States alone (http://www.cdc.gov/norovirus/about/ overview.html). Despite the importance of norovirus in public health, research on understanding norovirus biology and developing antiviral drugs are greatly hindered due to the inability to grow human noroviruses in cell culture. Therefore, easily cultivable murine norovirus (MNV) (Wobus et al., 2004), FCV (Luttermann and Meyers, 2010) and porcine enteric calicivirus (PEC) (Flynn and Saif, 1988), all in the family *Caliciviridae*, have been used as surrogate viruses for noroviruses.

Virus entry is a multistep process that involves consecutive interactions of cellular and viral factors including binding of virus to cellular receptors, virus uncoating and release of viral genome to initiate virus replication (Grove and Marsh, 2011; Marsh and Helenius, 2006). Virus uncoating may occur at the plasma membrane for direct penetration or at the endosomes or other cellular compartments (Marsh and Helenius, 2006). Viruses undergoing uncoating process in the host cells utilize a number of different entry processes, including clathrin-dependent endocytic pathway, clathrin-independent endocytic pathway that involves caveolar or lipid, or other yet poorly defined entry pathways (Mercer et al., 2010). In clathrin-dependent endocytosis, viruses that are endocytosed mediated by clathrin-coated vesicles travel through the endocytic compartments from where virus must escape before the increasingly harsher environment of maturing endosomes irreversibly degrade viruses. The journey of viruses following virus uptake to virus uncoating and genome release is less well understood for viruses that utilize clathrin-independent endocytosis (Grove and Marsh, 2011; Marsh and Helenius, 2006). However, there are reports that subsequent sorting of endosomal cargo such as viruses using different endocytic pathways may overlap in the early or late endosomes (Naslavsky et al., 2004; Sharma et al., 2003).

In the endosomal compartments, host enzymes including cathepsins are reported to be involved in virus fusion and/or uncoating of virus capsid for some viruses (Grove and Marsh, 2011). The cathepsin family of proteolytic enzymes contains several diverse classes of proteases including cysteine (cathepsins B, L, H, K, S, and O), aspartyl (cathepsin D and E) and serine (cathepsin G) proteases (Vasiljeva et al., 2007). Among them, cathepsin L, B, or S have been reported to be associated with successful entry and replication of some viruses including severe acute respiratory syndrome (SARS) coronavirus, murine hepatitis virus, reovirus and Ebola virus

(Bosch et al., 2008; Brecher et al., 2012; Mainou and Dermody, 2012; Qiu et al., 2006; Schornberg et al., 2006). Cathepsin L and B are shown to cleave Ebola virus glycoprotein, leading to exposure of putative fusion domain required for fusion of viral and endosomal membranes (Schornberg et al., 2006). Cathepsin L is also reported to cleave the spike protein of SARS coronavirus for fusion competence (Bosch et al., 2008). In addition to some enveloped viruses that require cathepsin for virus uncoating, some non-enveloped viruses are also shown to rely on host cell proteases residing in the endosomes for uncoating of virus capsid. Reovirus disassembly in the endosomes is reported to be mediated predominantly by cathepsin L and less efficiently by cathepsin B, generating infectious subvirion particles that are capable of penetrating membranes and deliver core particles into cytoplasm (Ebert et al., 2002; Mainou and Dermody, 2012). For caliciviruses, while it has been shown that clathrin-dependent endocytosis and pH-dependent entry is important in FCV replication (Kreutz and Seal, 1995; Stuart and Brown, 2006), virus entry for other caliciviruses including MNV-1, PEC or human norovirus are not well understood to date. For MNV-1, it was reported that MNV-1 entry is not mediated by clathrin or caveolae, but dependent on dynamin and cholesterol (Gerondopoulos et al., 2010; Perry et al., 2009; Perry and Wobus, 2010), but detailed entry mechanism still needs further elucidation.

Here we showed the importance of cathepsin L activity and endosome maturation during the entry stages of calicivirus replication cycle using PEC, FCV and MNV-1. We found that cathepsin L inhibitors, but not B inhibitor, and chloroquine significantly reduced the replication of PEC, FCV and MNV-1 and caused the retention of viruses in the endosomes during virus entry. We also found that recombinant cathepsin L cleaved VP1 of FCV and MNV-1, and VP2 of PEC based on the Western blot analysis, and that mutation of the putative cathepsin L

cleavage sites in VP1 abolished recovery of infectious virus using the reverse genetics system of FCV. Our results suggest the crucial role of cathepsin L in the replication of caliciviruses, and cathepsin L as a potential therapeutic target for calicivirus infections.

3.3 Materials and Methods

Cell culture and viruses

The cell culture adapted PEC Cowden strain was propagated in pig kidney epithelial cells (LLC-PK) in the presence of glycochenodeoxycholic acid (GCDCA, 100 μM) in Eagle's Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. FCV Urbana strain was propagated in Crandell Rees feline kidney (CRFK) cells in MEM containing 5% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). MNV-1 was propagated in RAW 264.7 cells (Wobus et al., 2004) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Each virus was concentrated prior to infection by centrifuging viruses at 27,000 RPM through a 40% w/v sucrose cushion at 4°C for 2 h. The pellet was reconstituted in serum free MEM and stored at -80°C. Cell culture adapted feline coronavirus 1146 strain, which is reported to be susceptible to a cathepsin B inhibitor, was also maintained in CRFK cells with MEM. Insect (sf9) cells for baculovirus growth were maintained in Grace's insect media (Invitrogen, Carlsbad, CA) at 27°C.

Reagents and antibodies

GCDCA and chloroquine were purchased from Sigma-Aldrich (St Louis, MO). Cathepsin L inhibitors, Z-FY-CHO and MDL28170, were purchased from Santa Cruz Biotech (Santa Cruz, CA), and a cathepsin B inhibitor CA074-Me was purchased from Sigma-Aldrich. Recombinant human cathepsin L and cathepsin B were purchased from R & D systems (Minneapolis, MN).

The primary antibodies used in this study were anti-PEC/Cowden antibodies raised in swine (Chang et al., 2005), anti-PEC VP2 antibodies raised in guinea pig (Chang et al., 2005), anti-FCV antibodies raised in guinea pig (Sosnovtsev and Green, 1995), and anti-MNV-1 antibodies raised in guinea pig (Wobus et al., 2004). The secondary antibodies for Western blot analysis included horseradish peroxidase-conjugates of anti-swine Ig and anti-guinea pig Ig antibody (Thermo Scientific, Pittsburgh, PA). The secondary antibodies for confocal microscopy were FITC-labeled anti-swine IgG for PEC, FITC-labeled anti-guinea pig IgG for MNV-1, and PerCP-Cy5.5 labeled anti-rabbit IgG for Rab7. Other basic chemicals for confocal microscopy and other studies were purchased from various sources including Sigma-Aldrich.

Effects of inhibition of cathepsin B or L activities in calicivirus replication

Confluent LLC-PK cells cultured in 12 well plates were pre-treated with inhibitors of cathepsin L (Z-FY-CHO 120 μM; MDL 28170, 100 μM) or cathepsin B (CA074-Me, 120 μM) for 1 h. Cells were then infected with PEC Cowden strain at a multiplicity of infection (MOI) of 50 in the presence of 100 μM GCDCA and a cathepsin inhibitor L or B at 37 °C. After 1 h, cell monolayer was washed twice with PBS and the medium was replaced with fresh medium containing a cathepsin L or B inhibitor at the same concentration. The cells were further incubated at 37°C. At 12 h post infection (PI), cells were subjected to repeated freezing and thawing for virus titration using the 50% tissue culture infectious dose ($TCID_{50}$) assay (Reed and Muench, 1938), or total RNA was extracted from the cells for real time qRT-PCR as described previously (Shivanna et al., 2014)The primers and probe sets used for real time qRT-PCR are listed in Table 1. Viral genome copy number was calculated from an external standard curve that was generated by plotting the Ct values vs. ten-fold dilutions of a known concentration of PEC RNA (Chang et al., 2005).

The effect of each inhibitor on the replication of FCV and MNV-1 was also studied following a similar experimental method. CRFK and RAW267.4 cells were preincubated with Z-FY-CHO (40 μM), MDL 28170 (80 μM), or CA074-Me (40 μM) for 1 h prior to inoculation of FCV (MOI 50) or MNV-1 (MOI 10). Following virus infection for 1 h, cells were washed with PBS and fresh medium containing an inhibitor was added to the cells. In a separate experiment, cells were preincubated with cyclophosphamide (100 μg/ml) for 1 h prior to MNV-1 infection to monitor the levels of internalized virus without further virus replication. At 12 h PI, virus replication was monitored by the $TCID_{50}$ assay (MNV-1 and FCV) or real time qRT-PCR (MNV-1). The inhibitors at the concentrations used in this study showed minimal cytotoxicity at 12 h PI in LLC-PK, CRFK and RAW267.4 cells. Because cathepsin B inhibitor CA074-Me blocks the replication of feline coronavirus (Kim et al., 2013; Regan et al., 2008), feline coronavirus 1146 strain was included in this study as a control and virus replication was monitored by the $TCID_{50}$ assay (Kim et al., 2013).

Effects of inhibition of endosomal acidification in calicivirus replication

Confluent LLC-PK cells cultured in 12 well plates were pre-treated with mock (medium) or an inhibitor of endosomal acidification (chloroquine, 70 μM) for 1 h. Cells were then infected with PEC at an MOI of 50 in the presence of chloroquine or mock at 37°C. Since PEC does not replicate without bile acid, GCDCA (100 μM, final concentration) was added to the media during virus infection and subsequently washed away. Following virus infection for 1 h, cell monolayer was thoroughly washed three times with PBS and the medium was replaced with fresh medium containing the same concentration of chloroquine or mock. In other experiments, chloroquine was added at 6 h PI to determine its effect at later stages of virus replication. Chloroquine at this concentration (70 μM) was minimally cytotoxic in LLC-PK cells. At 12 PI,

cells were subjected to repeated freezing and thawing for virus titration using the $TCID_{50}$ assay, or total RNA was extracted for real time qRT-PCR.

The effect of chloroquine on the replication of MNV-1 was also studied following the similar experimental design using RAW267.4 cells. Cells were pre-treated with chloroquine at 50, 70, 100 or 200 μM at 37 °C for 30 min, and infected with MNV-1 at an MOI of 10 in the presence of chloroquine or mock (medium) at 4°C. Following 1 h incubation, cells were thoroughly washed three times with PBS, and the same concentration of chloroquine or mock was added into the media. When high concentrations of chloroquine (100 or 200 μM) were used, chloroquine was removed at 4 h PI by thoroughly washing the cells with PBS to minimize cytotoxicity (Perry et al., 2009). In all experiments, cells were subjected to repeated freezing and thawing at 12 h PI for virus titration using the $TCID_{50}$ assay and real time qRT-PCR.

Confocal laser scanning microscopy

To study the effects of a cathepsin L inhibitor (Z-FY-CHO) or chloroquine during the entry of PEC or MNV-1, virus trafficking was monitored using confocal microscopy (Shivanna et al., 2014). LLC-PK or RAW267.4 cells were seeded onto a Lab-Tek II CC2 chamber slide (Fisher Scientific) treated with FBS and grown to 90% confluency. Confluent cells on the chamber slides were treated with mock (medium), chloroquine (100 μM) or Z-FY-CHO (100 μM) for 30 min. PEC or MNV-1 was then inoculated to the cells at an MOI of 50 or 10, respectively, and the cells were further incubated at 37 °C for 1 h. In LLC-PK cells, GCDCA (100 μM) was added to the media at the time of virus inoculation. At 1 h PI, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4) for 15 min at room temperature (RT), permeabilized with 0.1% Triton-×100 in PBS for 10 min at RT, washed three times with PBS, and incubated in blocking buffer [PBS containing 0.5% bovine serum albumin] for 15 min. The

cells were then incubated with primary antibodies specific to PEC (1:200), MNV-1 (1:200) or Rab7 (1:200) at 37°C to probe PEC, MNV-1 and the endosomes, respectively. After 2 h incubation, cells were washed three times with PBS and further incubated at 37°C for 2 h with FITC-labeled anti-swine IgG or anti-guinea pig IgG against PEC or MNV-1, respectively. For Rab7, PerCP-Cy5.5 labeled anti-rabbit IgG was used as a secondary antibody. The same cells were also stained with sytox orange DNA stain (0.5 μM in 0.9% NaCl). Coverslips were mounted in Prolong Gold antifade reagent (Molecular Probes), and the cells were scanned with a confocal microscope LSM 510 (Zeiss, Oberkochen, Germany) using a 100× oil-immersion objective. The images were analyzed by ImageJ software 1.47 (http://imagej.nih.gov/ij/), and colocalization analysis was performed using JACoP and colocalization-MBF plugins for ImageJ software. Single channel images were thresholded by Costes' auto threshold method and the Pearson's correlation coefficient for colocalization was then determined for each image.

In-vitro cleavage of viral proteins by cathepsin

Concentrated PEC, FCV or MNV-1 was incubated with or without recombinant human cathepsin L (0.64 μg) or cathepsin B (0.64 μg) in reaction buffer containing 100 mM Na acetate, 1 mM EDTA, and 4 mM DTT at pH 4.0 (for cathepsin L) or pH 5.5 (for cathepsin B), or concentrated viruses were incubated in PBS (pH 7.0) for 2 h at 37°C. The samples were then mixed with 1X LDS sample buffer (Invitrogen) containing 2-mercaptoethanol, and heated to 95°C for 5 min. Following heat treatment, the samples were subjected to SDS-PAGE (12% Tris-glycine) and proteins were transferred to nitrocellulose membranes. Membranes were probed with primary antibodies followed by horseradish peroxidase-conjugated species-specific secondary antibodies. Proteins were visualized by chemiluminescence according to the manufacturor's instructions (Thermo Scientific).

Computational prediction of cathepsin L cleavage sites in FCV VP1

Potential cleavage sites of cathepsin L in FCV VP1 were identified using the PoPS system for Prediction of Protease Specificity (http://pops.csse.monash.edu.au) (Boyd et al., 2005). The cathepsin L cleavage model available in the PoPS database (C01.032>Pike>1.1) was used to generate a position-specific scoring matrix (on a scale of −5.0 to +5.0). The PoPs model was augmented using the knowledge of cathepsin L substrate specificity: the preferential requirement of hydrophobic amino acid phenylalanine (F) or leucine (L) at the P2 site of the substrate. According to the nomenclature of Schechter and Berger (Schechter and Berger, 1967), amino acid residues flanking the scissile bond are designated as P1, P2, etc toward the N-terminal end and P1', P2', etc toward the C-terminal end. The model predicted the cleavage sites on the consensus protein sequence derived from the available FCV VP1 sequences in GenBank including Urbana, 131, Deuce, 2024, Kaos, Jengo and Ari strains. The rankings were determined based on the scores from the augmented PoPs program. The predicted cleavage sites that reside in the conserved region of VP1 and would yield cleavage products of approximately 23 or 36 KDa that were observed in the Western blot assay were selected for mutagenesis.

Generation of FCV infectious clones containing a single mutation at the predicted cathepsin L cleavage sites for virus recovery and replication assay

The P2 residues (F463, L472 and F486) at the predicted cathepsin L cleavage sites on the FCV infectious clone pQ14 (Sosnovtsev and Green, 1995) were mutated to alanine to generate pQ14F463A, pQ14L472A and pQ14F486A clones, respectively, by site directed mutagenesis. In addition, single alanine mutation was also introduced at K464, R474, and K479, where no effect on the putative cleavage is expected, to generate pQ K464A, pQ14R474A, and pQ14K479A, respectively. A GDD motif deletion mutant of viral polymerase was constructed as a replication defective control (pQ14ΔGDD). The primers for the *in vitro* mutagenesis are listed in Table 1.

The parental clone, pQ14 and the mutant clones were verified by sequence analysis and tested for their replication capability, as previously described (Chang et al., 2008). One-day old semi-confluent CRFK cells were infected with vaccinia virus encoding T7 polymerase (MVA-T7) for 1 h, washed with PBS and fresh medium was added to the cells. The clones were then transfected into the cells using Lipofectamine 2000, according to the manufacturer's protocol. Following incubation for 24 h at 37°C, the supernatant from the transfected cells was transferred to a monolayer of CRFK cells. The cells were further incubated at 37°C and observed daily for appearance of cytopathic effects (CPE) for 96 h. For immunofluorescence assay (IFA) for VP1 expression, MVA-T7 infected CRFK cells were transfected with each infectious clone and incubated for 16 h. The cells were then fixed with cold methanol for 10 min, washed with PBS and incubated for 2 h with anti-FCV VP1 antibody, followed by FITC-conjugated secondary antibody for 2 h. VP1 expression was examined under a fluorescence microscope.

Expression of FCV VP1 with F463A or L472A mutation or wild-type VP1 in a baculovirus expression system

To examine if F463A or L472A mutation affects the production of virus-like particles (VLPs), VP1 gene carrying each mutation as well as the wild-type VP1 gene was expressed in a baculovirus expression system. The wild type or mutant FCV VP1 genes was amplified from pQ14, pQ14L472A or pQ14 F463A, respectively, using FCV-VP1-F (AATT*TCTAGA*ATGGATGACGGATCCATAACTGC, XhoI site is in italic and start codon is underlined) and FCV-VP1-R (AATT*TTCGAA*TCATAGTTTAGTCATTGTGCTCCT (XbaI site is in italic and stop codon is underlined) primer sets. The amplified PCR products were then cloned into a pBlueBac4.5 (Invitrogen) baculovirus transfer vector using XhoI and XbaI restriction enzyme sites. Each recombinant baculovirus was generated using the Bac-N-Blue

baculovirus expression system (Invitrogen). Each recombinant baculovirus was plaque-purified in Sf9 cells, and high titer virus stock of a recombinant virus was generated. To produce the wild type or mutant VLPs, sf9 cells were infected with each recombinant baculovirus at an MOI of 5. At 6-8 days post infection, cell culture media was centrifuged at 3000 g for 15 min at 4°C. The supernatant was collected and layered over a 35% sucrose cushion, followed by centrifugation at 4°C for 1.5 h at 100,000 g. The pellets were then collected, resuspended in PBS (pH 7.4) for Western blot analysis. The un-concentrated culture supernatant of sf9 cells infected with each recombinant virus was also prepared for Western blot analysis.

Statistical analysis

The effects of cathepsin or endosomal acidification inhibitors in virus replication were compared to the mock treatment using a two-tailed Student's t-test. P value < 0.05 was considered statistically significant. Data were from at least three independent experiments.

3.4 Results

Inhibitors of cathepsin L, but not cathepsin B, significantly reduced the replication of caliciviruses.

The role of cathepsin B and L in PEC, MNV-1 and FCV replication was studied using inhibitors of cathepsin L and cathepsin B. Our results showed that treatment of cells with inhibitors of cathepsin L (Z-FY-CHO and MDL 28170) significantly inhibited PEC, MNV-1, and FCV replication (Figure 3-1 A-C) in a dose-dependent manner (data not shown). However, cathepsin B inhibitor CA074-Me did not lead to a significant reduction of viral replication (Figure 3-1A-C). As reported previously, CA074-Me significantly reduced the replication of feline coronavirus 1146 strain in CRFK cells (Kim et al., 2013; Regan et al., 2008) (data not shown).

Endosomal acidification is required for calicivirus replication.

Since the maximal activity of cathepsin L requires acidic condition at around pH 5 (Turk et al., 1993; Vasiljeva et al., 2007), we studied the effect of endosomal acidification in calicivirus replication using chloroquine. Our findings indicate that the presence of chloroquine during -1 to 12 h PI resulted in a marked reduction in PEC replication (> 10 fold reduction), determined by real time qRT-PCR (Figure 3-2A) and the $TCID_{50}$ assay (data not shown). However, no significant inhibitory effect was observed when chloroquine was added later during virus replication cycle (at 6 h PI) (Figure 3-2A). Similar inhibitory effect of chloroquine was observed for MNV-1: dose-dependent inhibition of MNV-1 replication was observed either when chloroquine was present during early period (-30 min to 4 h PI) at higher concentrations (100 or 200 μM), or continuously in the media at lower concentrations (50 or 70 μM) (Figure 3-2B and C). However, the effect of chloroquine at 50 μM in MNV-1 replication was not statistically significant compared to the control (Figure 3-2C). The results obtained by real time qRT-PCR also confirmed those with $TCID_{50}$ assay (data not shown). Of note, we found that an extensive and through washing step following virus infection to eliminate any residual virus is crucial to observe the effects of chloroquine.

PEC or MNV-1 accumulated in the endosomes in the presence of a cathepsin L inhibitor or chloroquine.

PEC replication in cell culture is dependent on the presence of bile acids in the media (Chang et al., 2005; Chang et al., 2004). However, the mechanism of bile acid-supported PEC replication has not been known. In our previous report, we demonstrated that absence of bile acid in cell culture media led to accumulation of PEC virus particles in the endosomes and failed

virus replication (Shivanna et al., 2014). In the cells infected with PEC in the presence of GCDCA and incubated for 1 h prior to confocal microscopy, no or little fluorescence staining of PEC VP1 was observed in the cytoplasm, while relatively abundant fluorescence for VP1 was detected in the cells infected by PEC without GCDCA (Figure 3-3). Likewise, abundant fluorescence signals for PEC were detected in PEC- infected cells cultured with Z-FY-CHO or chloroquine, although media was supplemented with GCDCA (Figure 3-3). In RAW267.4 cells infected with MNV-1, only a few viruses were stained in the cytoplasm at 1 h PI (Figure 3-4). However, in the presence of Z-FY-CHO or chloroquine, the fluorescence signal for MNV-1 was high at 1 h PI (Figure 3-4), which is a similar observation made in PEC-infected cells (Figure 3-3). Quantitative colocalization analysis of these images showed that the fluorescence signals for PEC or MNV-1 colocalized with Rab7 (Figure 3-3 and 3-4). Rab7 is a GTPase of low molecular weight and mainly localized in the late endosomes/lysosomes in mammalian cells (Chavrier et al., 1990; Soldati et al., 1995). The Pearson's correlation coefficient for colocalization of virus particles (green) with Rab7 (red) in the confocal images was calculated to be > 0.90 for all images. These results indicate that PEC and MNV-1 failed to escape from the endosomes in the presence of cathepsin L or chloroquine.

Cathepsin L, but not cathepsin B, cleaves calicivirus structural (major or minor) protein.

To study the effect of cathepsin L and B on calicivirus capsid proteins VP1 and VP2, concentrated PEC, MNV-1 and FCV were incubated with recombinant cathepsin L or cathepsin B for 2 h at 37°C. In the Western blots, PEC VP1 and VP2 proteins were detected as a single band of approximately 58 kDa and 18 kDa, respectively, in mock-incubated samples at pH 4 and 7 (Figure 3-5A). The Western blot analysis on the samples incubated with cathepsin L or B

showed that neither enzyme cleaved PEC VP1 (Figure 3-5A). However, a band that corresponds to a full length PEC VP2 (18 kDa) disappeared only in the samples incubated with cathepsin L, suggesting cleavage by the protease (Figure 3-5A). For MNV-1 and FCV, incubation of virus and cathepsin L produced multiple bands in the Western blot analysis: Anti-FCV VP1 antibody detected multiple fragments of approximately 59 kDa (full length VP1), 36 kDa, 34 kDa and 23 kDa (Figure 3-5B), and antibodies against MNV-1 VP1 detected two bands of approximately 59 kDa (full length VP1) and 40 kDa (Figure 3-5B). However, cathepsin B did not cleave FCV and MNV-1 VP1 (Figure 3-5B). FCV and MNV-1 VP1 cleavage by cathepsin L or B was not studied due to unavailability of antibodies to FCV or MNV-1 VP1.

Generation of infectious clones carrying a disrupted cathepsin L cleavage site.

The augmented PoPS program predicted multiple cleavage sites in the conserved region between two hypervariable regions of FCV VP1. Among them, three cleavage sites were chosen based on the cleavage product sizes observed on the Western blot (two major bands, 23 and 36 kDa, Figure 3-5B) for *in vitro* mutagenesis of the FCV infectious clone pQ14. Mutant clones pQ14F463A, pQ14L472A, and pQ14F486A that carry a single alanine mutation at the P2 residues of the putative cathepsin L cleavage sites (F463, L472 and F486) were generated (Figure 3-6 A-C). Additional full length mutant clones, pQ14K464A, pQ14R474A and pQ14K479A, were generated with a single alanine mutation (from a positive charged amino acid) at the sites that are not crucial for cleavage by cathepsin L. Our IFA study using these wild-type and mutant infectious clones revealed that all clones led to positive detection of VP1 in the transfected cells, except for the polymerase-defective pQ14ΔGDD (Figure 3-7A), indicating that they are replication competent. When virus recovery was studied using the wild-type and mutant clones, all clones except for pQ14L463A and pQ14F472A produced infectious viruses,

demonstrated by successful infection of fresh cells following transfer of the media from the transfected cells (Figure 3-7A). The polymerase-defective pQ14ΔGDD clone did not produce infectious viruses, as expected. To study if the mutation in VP1 affected capsid formation, we expressed the VP1 from pQ14F463A, pQ14F472A, and pQ14 in a baculovirus expression system. Based on the Western blot analysis, VP1 was detected in the resuspended pellets, but not cell culture supernatant, from the cells infected with a baculovirus carrying the wild-type VP1 or VP1 with a F473A or F472A mutation (Figure 3-7 C). This result suggests that F463A or F472A mutation did not affect VLP formation.

3.5 Discussion

Virus entry is the first step in virus replication cycle and is characterized by complex interactions of cellular and viral factors that lead to virus uncoating and release of viral genome to initiate virus replication (Grove and Marsh, 2011; Marsh and Helenius, 2006). However, current knowledge of the entry of caliciviruses in general is limited. FCV entry has been relatively well known, but study on the entry of other caliciviruses such as human norovirus, PEC or MNV-1 has been limited (Kreutz and Seal, 1995; Perry et al., 2009; Perry and Wobus, 2010; Shivanna V., 2014; Stuart and Brown, 2006). In this study, we demonstrated that cathepsin L plays an important role in the replication of PEC, FCV and MNV-1 by showing that inhibition of cathepsin L, but not cathepsin B, led to a significant reduction in the replication of PEC, FCV and MNV-1 in cell culture. Cathepsin L is mainly located in the endosomal/lysosomal compartments and has been associated with virus uncoating for some enveloped and non-enveloped viruses (Bosch et al., 2008; Ebert et al., 2002; Huang et al., 2006). However, the function of cathepsin in caliciviruses has not been previously reported. Since cathepsin L requires acidic pH for optimal activity, reflecting their primary location in the late

endosomes/lysosomes (Honey and Rudensky, 2003; Turk et al., 2000), we then determined if endosomal acidification is important in the replication of PEC and MNV-1. It was previously reported that maturation of the endosomes is important in FCV replication (Kreutz and Seal, 1995; Stuart and Brown, 2006), and we confirmed that FCV entry is dependent on pH (data not shown). In our study, chloroquine treatment at the concentrations above 50 μM at early time points significantly reduced the replication of PEC and MNV-1 (Figure 3-2). Previously, MNV-1 entry was reported to be pH-independent (Gerondopoulos et al., 2010; Perry et al., 2009). However, the effect of pH on PEC replication has not been studied. Our finding of the dependency of MNV-1 replication on acidic pH is contrary to previous reports, and the reason for this discrepancy between our finding and others is not clear. We also performed confocal microscopic study and showed that MNV-1 accumulated in the endosomes in the presence of chloroquine (Figure 3-4), in a pattern similar to the one observed with a cathepsin L inhibitor (Figure 3-3). In contrast, no to little viruses were observed in the endosomes in the mock-treated cells at 1 h pi. This result indicates that cathepsin L and acidic pH is important for virus escape from the endosomes, and is also in line with the virus titration results obtained from cathepsin L inhibitor or chloroquine treatment of cells infected with MNV-1. We also found that endosomal escape of PEC is dependent on cathepsin L and endosomal acidification using confocal microscopy.

Since it is a new finding that inhibition of cathepsin L causes endosomal virus entrapment and significantly reduces calicivirus replication, we studied if cathepsin L is capable of cleaving the structural proteins of PEC, FCV and MNV-1. Based on Western blot analysis, cathepsin L cleaved VP1 of PEC VP1 and VP2 of FCV and MNV-1. VP1, a major capsid protein, assembles into a capsid with VP2, a minor capsid protein. The role of VP2 in virus capsid formation is

unclear, but it is speculated that VP2 is involved in the assembly of virus particle by correctly forming capsid and/or increasing the stability of VP1 based on the previous reports using FCV and human noroviruses (Bertolotti-Ciarlet et al., 2003; Di Martino and Marsilio, 2010). Therefore it can be speculated that VP1 or VP2 cleavage by cellular enzymes such as cathepsin L contributes to virus disassembly to allow initiation of virus replication.

In our effort to locate the potential cleavage site(s) on capsid protein by cathepsin L, we used the FCV infectious clone pQ14 to generate the mutant clones that carry a single mutation at the critical enzyme recognition site (S2) in the putative cleavage sites on VP1. The putative cleavage sites based on the cleavage of FCV VP1 and computational prediction, F463, L472 and F486, are located closer to the receptor binding site in the conserved region flanked by hypervariable regions in the P2 domain and exposed on the surface (Figure 3-6 A and B). Thus they are likely to be accessible by endosomal cathepsin L. These putative cleavage sites are also highly conserved among FCV strains (Figure 3-6 C). Transfection study using these clones as well as other mutant clones that were generated by substituting an amino acid non-relevant to cathepsin L recognition and cleavage with alanine showed that all mutant clones, except for a polymerase-defective clone, were replication-competent. However, F463A or L472A mutant clones failed to yield productive infection in the cells. These results indicated that while virus replication was not impaired by F463A or L472A mutation, virus assembly stage including capsid formation or virus entry/uncoating step was disrupted by the mutation. To probe the possibility that these mutation disrupted virus particle formation, we expressed VP1 protein with F463A or L472A mutation using a baculovirus expression system. Based on Western blot, VP1 was detected only in the concentrated pellets, not the supernatant, from the cells infected with a baculovirus carrying VP1 with F463A or L472A mutation, indicating that the recombinant

mutant VP1 formed VLPs. Thus, it may be possible that virus particles with F463A or L472A mutation failed to infect cells due to impaired uncoating events in the endosomes. We plan to study the trafficking of virus particles produced by the mutant FCV clones using confocal microscopy and electron microscopy.

Here we report a new finding that cathepsin L activity is important in virus replication and endosomal escape of caliciviruses, including FCV, MNV-1, and PEC, based on the cathepsin inhibitor study, confocal microscopy, *in vitro* mutagenesis, and capsid cleavage assay. These results allow us to speculate that the entry pathways of many caliciviruses may share a common step involving interaction of virus and cathepsin for virus uncoating, and may also shed an important insight to the entry of other caliciviruses, including human noroviruses which are major public health concerns.

3.6 Acknowledgements

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Table 3-1. The primers and probes used for real time qRT-PCR for PEC and MNV-1, and for a mutagenesis study using a FCV pQ14 clone.

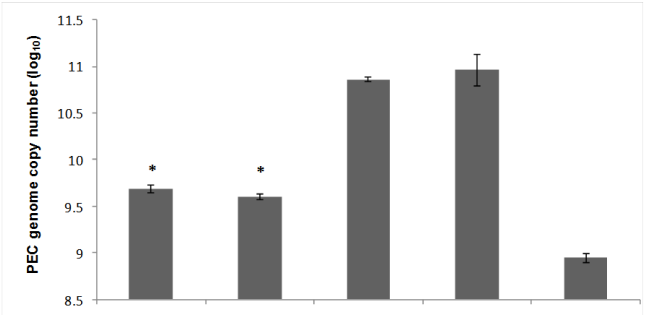
Name	Sequences (5' → 3')
PEC qRT-PCR[a]	F: ATTCCAGAGTTGACCCACAG
	R: CTACTGGGTTGATGGCGAC
	P: 6-FAM/TGGTATAGATTAAGAGGCACAGCGGC/IABkFQ
MNV-1 qRT-PCR	F: CATAGATGCCCCTGGAGTAAAG
	R: CTCTCCAACATCTTCGCTCTG
	P: 6-FAM/CATGATGACCACCTGCTCCACCTT/IABkFQ
FVP1F463A[b]	AGAAACAACACTAACGCGAAGGGCATGTATAT
FVP1L472A	GTATATCTGTGGATCAGCGCAAAGAGCGTGGGGT
FVP1F486A	ATTTCCAACACTGCTGCGATCACCACCGCCACG
FVP1K464A	AACAACACTAACTTCGCGGGCATGTATATCTG
FVP1R474A	GTGGATCACTCCAAGCGGCGTGGGGTGATAA
FVP1K479A	AGAGCGTGGGGTGATGCGAAAATTTCCAACAC

[a] F: forward primer; R: reverse primer; P: probe

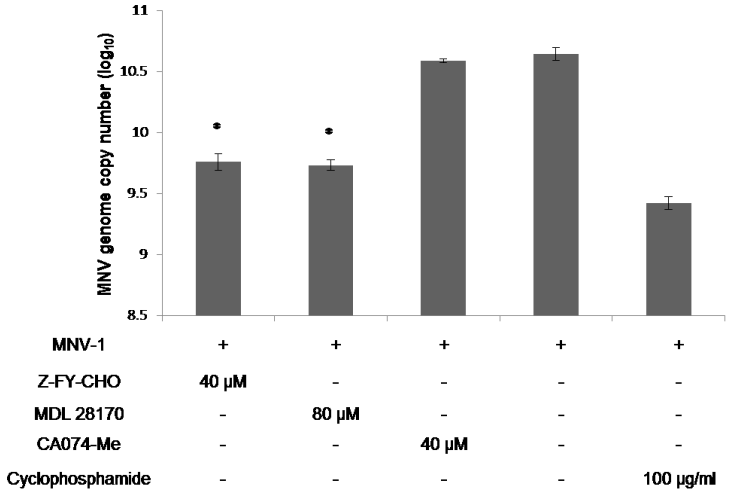
[b] Mutagenesis primers are forward primers only.

Figure 3-1. Effects of cathepsin inhibitors in the replication of PEC, MNV-1 or FCV. LLC-PK, RAW267.4 or CRFK cells were incubated with cathepsin L inhibitors, Z-FY-CHO and MDL 28170, or a cathepsin B inhibitor, CA074-Me, for 1h, then infected with (A) PEC (MOI 50) (B) MNV-1 (MOI 10) or (C) FCV (MOI 50) in the presence of each inhibitor. For PEC, GCDCA (100 μM) was present in the media to support virus replication during virus infection. Following virus infection for 1 h, cells were washed and the media was replaced with fresh media containing an inhibitor. Cells were further incubated at 37°C and collected at 12 h PI. Viral replication was quantified by real time RT-PCR for PEC and MNV-1 or the $TCID_{50}$ assay for FCV. Asterisk indicates that RNA genome level or virus titer was significantly reduced by an inhibitor compared to the control ($P < 0.05$).

A.



B.


MNV genome copy number (log_{10})
11
10.5
10
9.5
9
8.5
*
*
MNV-1 + + + + +
Z-FY-CHO 40 μM - - - -
MDL 28170 - 80 μM - - -
CA074-Me - - 40 μM - -
Cyclophosphamide - - - - 100 μg/ml

C.

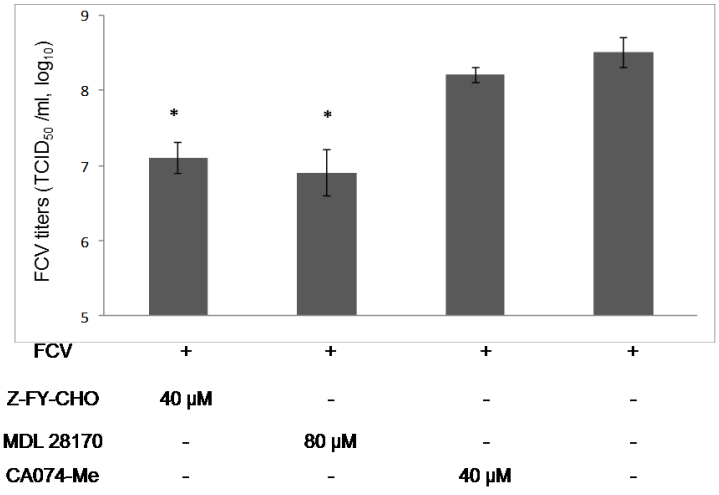
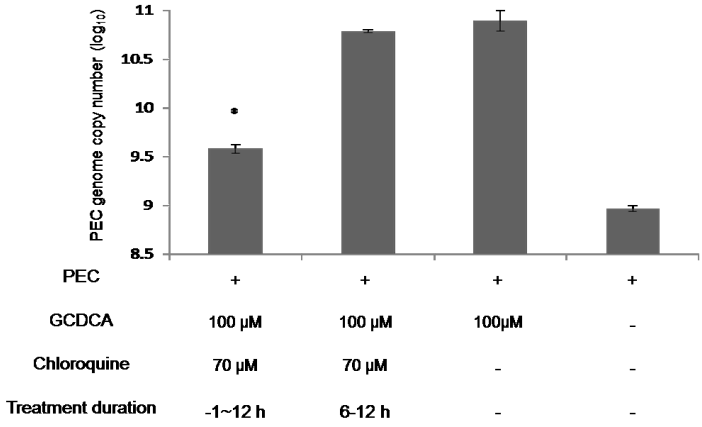
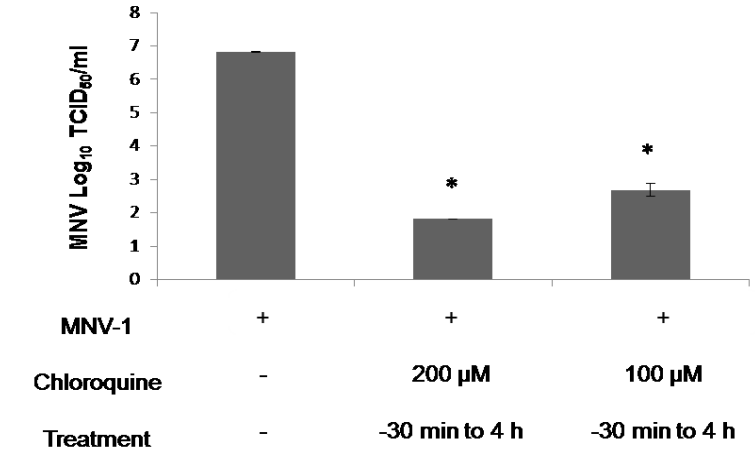

FCV titers ($TCID_{50}$ /ml, log_{10})
9
8
7
6
5
*
*
FCV + + + +
Z-FY-CHO 40 μM - - -
MDL 28170 - 80 μM - -
CA074-Me - - 40 μM -

Figure 3-2. Effects of endosomal acidification in the replication of PEC or MNV-1.
A. LLC-PK cells were pre-treated with mock (medium) or chloroquine (70 μM) for 1h, and infected with PEC (MOI 50) in the presence of mock or cholorquine. In all cells, GCDCA (100 μM) was present in the media to support virus replication during virus infection. Following virus infection, cells were thoroughly washed with PBS and fresh medium containing mock or chloroquine was added to the cells. Cells were then further incubated and collected at 12 h PI. In a separate experiment, chloroquine was added to the PEC-infected cells at 6 PI and cells were collected at 12 h PI for the determination of viral replication by real time qRT-PCR. B. RAW267.4 cells were pre-treated with mock (medium) or chloroquine (200 or 100 μM) for 1h then infected with MNV-1 (MOI 10) in the presence of mock or chloroquine at 4 °C for 1 h. Cells were then washed with PBS and fresh medium containing mock or chloroquine was added to the cells for further incubation at 37°C. At 4 h PI, cells were thoroughly washed again with PBS and fresh medium without chloroquine was added to the cells. Cells were collected at 12 h PI for the determination of virus replication by the $TCID_{50}$ assay. C. Cells were pretreated with chloroquine (50 or 70 μM) for 1 h, and infected with MNV-1 (10 MOI) in the presence of mock or chloroquine at 4°C for 1 h. Cells were then washed with PBS and fresh medium containing chloroquine was added to the cells. Cells were further incubated and collected at 12 h PI for determination of virus replication by the $TCID_{50}$ assay. (A to C) Asterisk indicates a significant reduction of viral RNA genome level or titers in the treatment group compared to the control ($P < 0.05$).

A.


PEC genome copy number (log_{10})
11
10.5
10
9.5
9
8.5
*
PEC + + + +
GCDCA 100 μM 100 μM 100μM -
Chloroquine 70 μM 70 μM - -
Treatment duration -1~12 h 6-12 h - -

B.


MNV Log_{10} $TCID_{50}$/ml
8
7
6
5
4
3
2
1
0
*
*
MNV-1 + + +
Chloroquine - 200 μM 100 μM
Treatment - -30 min to 4 h -30 min to 4 h

C.

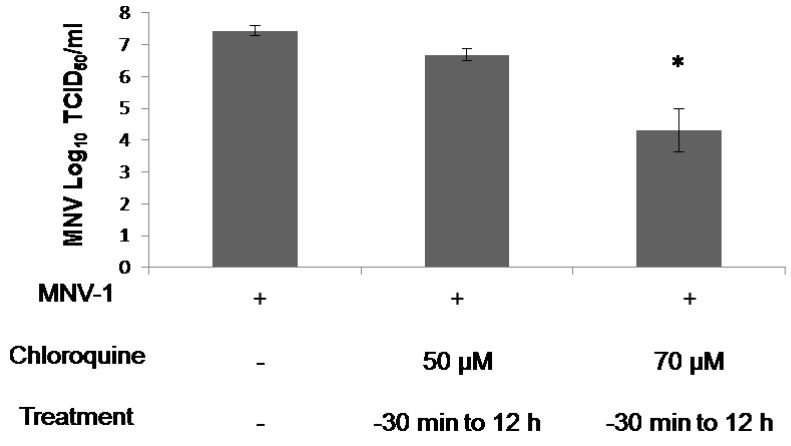

MNV Log_{10} $TCID_{50}$/ml
8
7
6
5
4
3
2
1
0
*
MNV-1 + + +
Chloroquine - 50 μM 70 μM
Treatment - -30 min to 12 h -30 min to 12 h

Figure 3-3. The effects of a cathepsin L inhibitor or chloroquine in PEC entry into the cells. Confluent LLC-PK cells were pre-treated with mock (medium), Z-FY-CHO or chloroquine for 30 min before virus inoculation. Cells were then inoculated with PEC (MOI 50) in the presence of mock, Z-FY-CHO or chloroquine. GCDCA (100 μM) was present in cell culture during virus infection to support PEC replication, with the exception of mock-infected cells and PEC-infected negative control cells. Following virus infection for 1 h, cells were fixed and probed with rabbit polyclonal anti-Rab7 or swine anti-PEC polyclonal antibodies, followed by secondary antibodies of PerCP-Cy5.5 labelled secondary antibody against Rab7 (red, k to o) or FITC-labelled secondary antibody against PEC (green, f to j). Nuclei were stained with sytox orange (pseudo colored blue, a to e). Confocal images on the prepared samples were obtained and colocalization analysis of PEC and Rab7 was performed by ImageJ software. In the merged images (p to t), colocalization of PEC (green) and Rab7 (red) appears in white color.

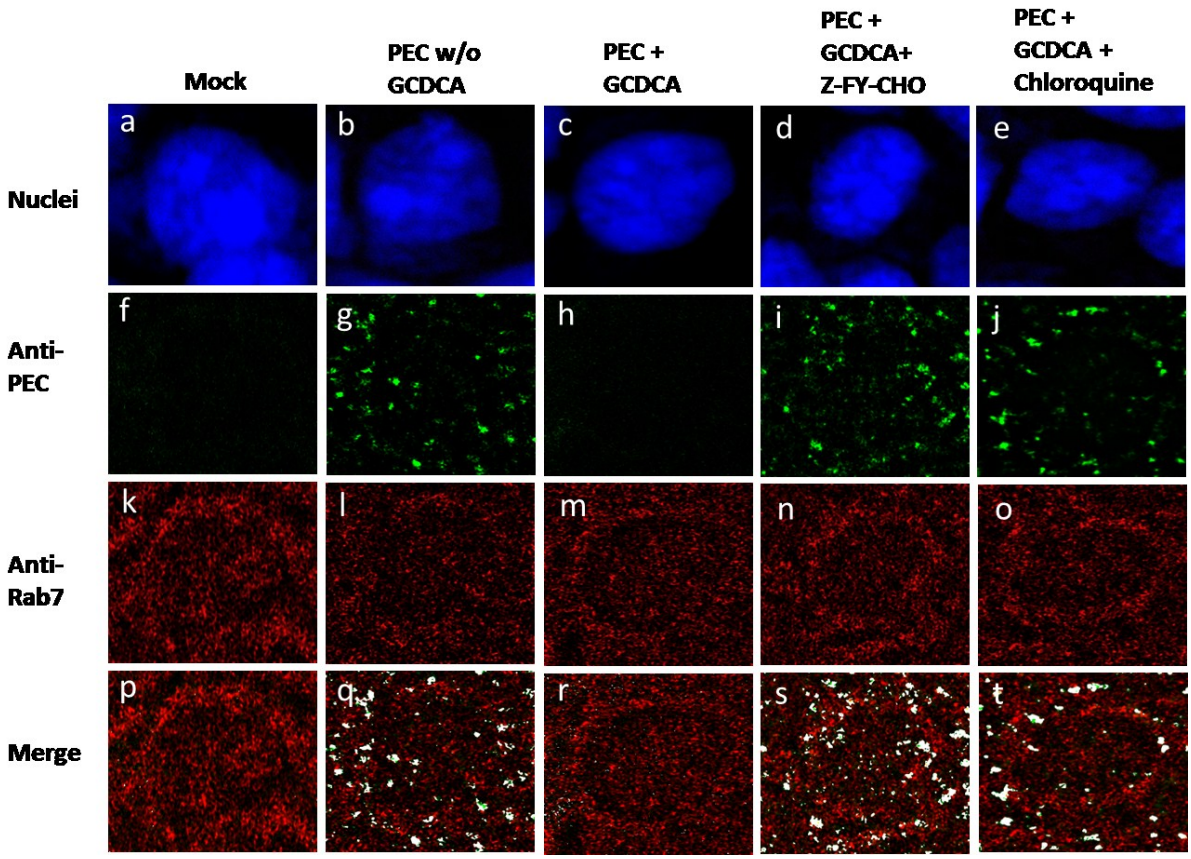

Mock
PEC w/o GCDCA
PEC + GCDCA
PEC + GCDCA+ Z-FY-CHO
PEC + GCDCA + Chloroquine
Nuclei
Anti-PEC
Anti-Rab7
Merge
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t

Figure 3-4. The effects of cathepsin L inhibitor or chloroquine in MNV-1 entry into the cells.

RAW267.4 cells were pre-treated with mock (medium), Z-FY-CHO or chloroquine for 30 min before MNV-1 inoculation (MOI 10). Following virus infection at 37 °C for 1 h, cells were fixed and probed with rabbit polyclonal anti-Rab7 or guinea pig polyclonal antibody against MNV-1, followed by PerCP-Cy5.5 labelled secondary antibody against Rab7 (red, i to l) or FITC-labelled secondary antibody against MNV-1 (green, e to h). Nuclei were stained with sytox orange (pseudo colored blue, a to d). Confocal images on the prepared samples were obtained and colocalization analysis of Rab7 and MNV-1 was performed by ImageJ software. In the merged images (p to t), colocalization of MNV-1 (green) and Rab7 (red) appears in white color.

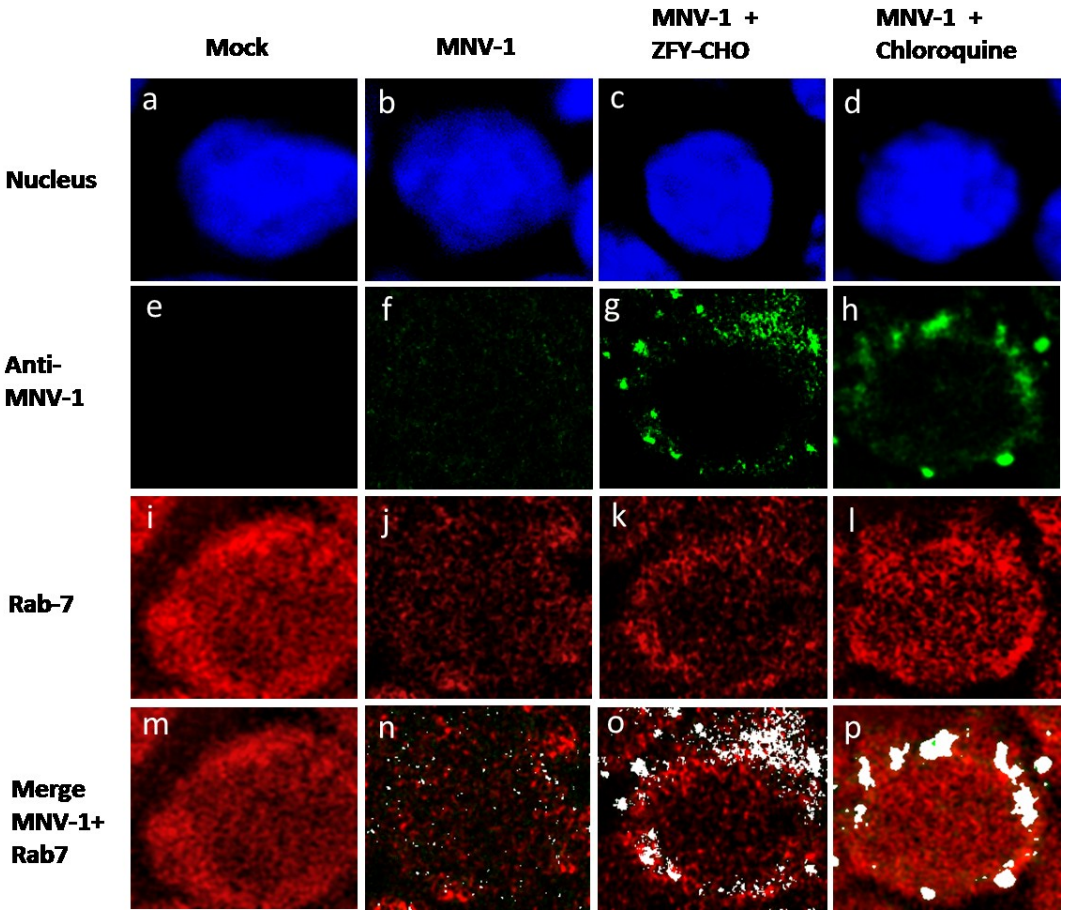
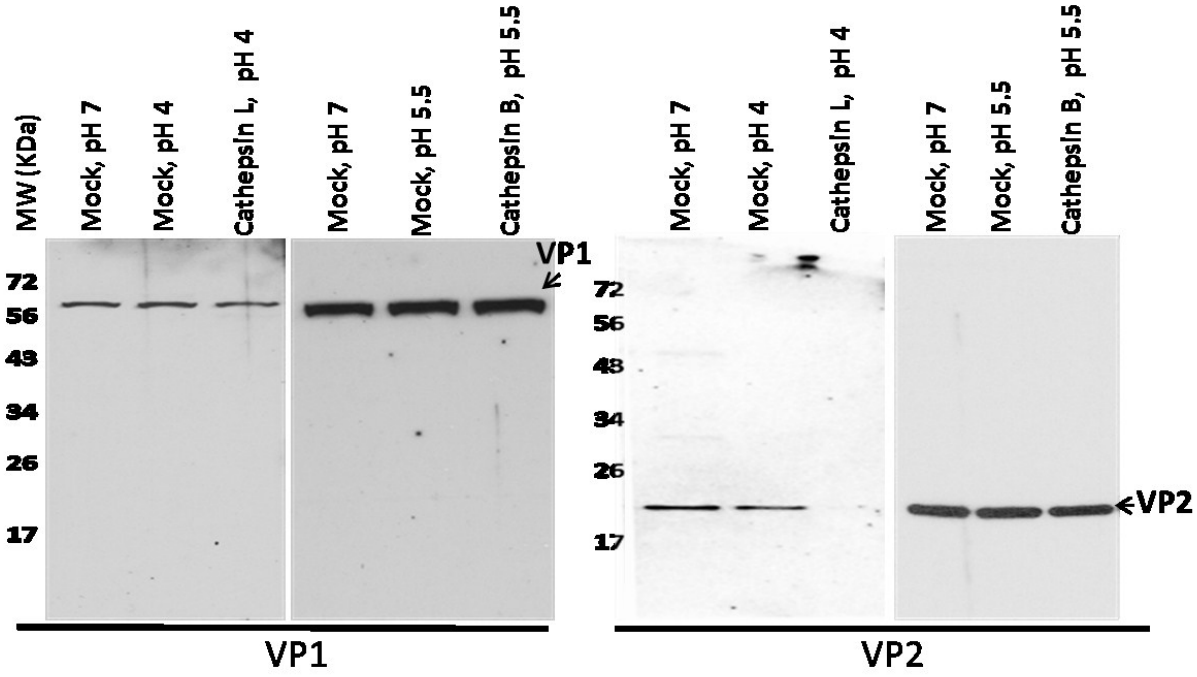


Figure 3-5. The effects of cathepsins on the cleavage of calicivirus capsid proteins. Concentrated PEC (A), FCV (B) or MNV-1 (B) were incubated with a recombinant cathepsin L or cathepsin B in a cathepsin reaction buffer at pH 4.0 or pH 5.5, respectively, for 2 h at 37°C. Mock-treatment samples were incubated in PBS at pH 7.0. The samples were then subjected to SDS-PAGE, transferred to nitrocellulose membrane, probed with antibodies against VP1 (PEC, FCV or MNV-1) or VP2 (PEC) and visualized by chemiluminescence. Uncleaved or cleaved VP1 and VP2 are indicated by arrows.

A.



B.

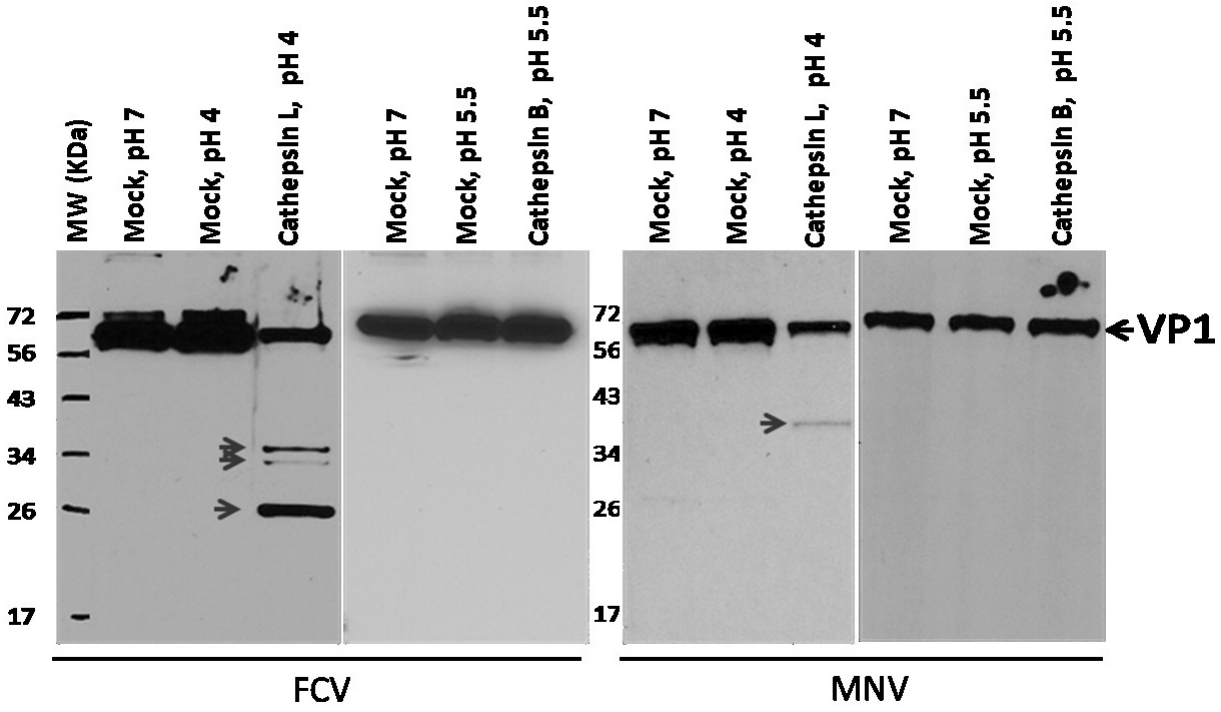
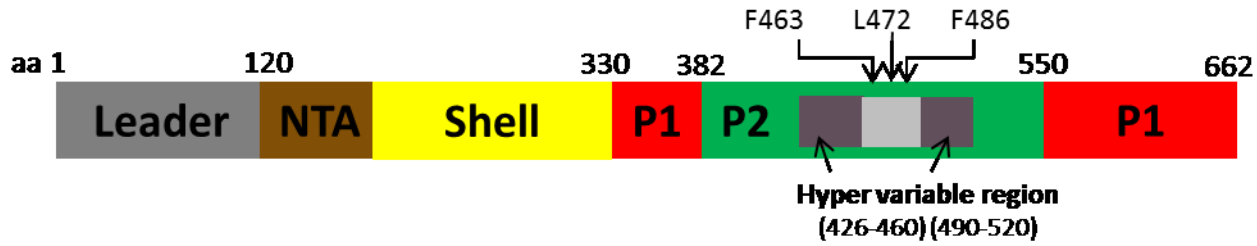

MW (KDa)
Mock, pH 7
Mock, pH 4
Cathepsin L, pH 4
Mock, pH 7
Mock, pH 5.5
Cathepsin B, pH 5.5
Mock, pH 7
Mock, pH 4
Cathepsin L, pH 4
Mock, pH 7
Mock, pH 5.5
Cathepsin B, pH 5.5
72
56
43
34
26
17
72
56
43
34
26
17
VP1
FCV
MNV

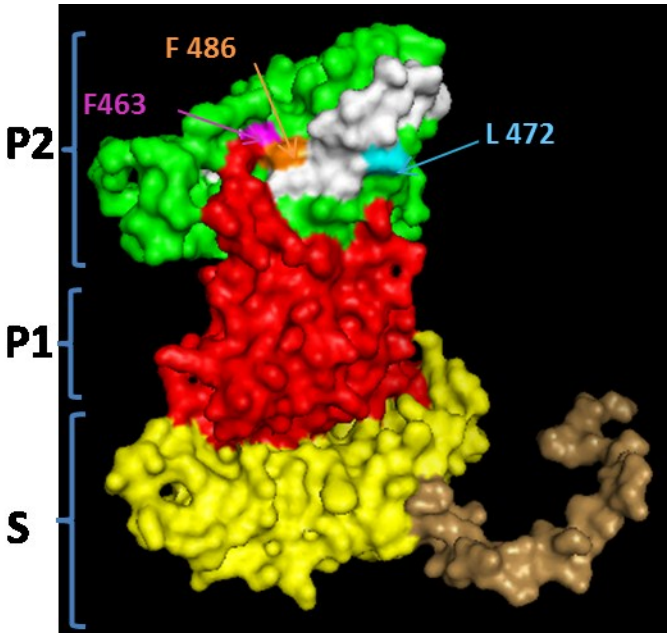
Figure 3-6. Putative cathepsin L cleavage sites in FCV VP1.

A) A schematic representation of FCV VP1 organization. The S2 sites (F463, L472 and F486) of the putative cathepsin L cleavage sites are indicated in the conserved region (light gray region) located between the hypervariable regions (dark gray regions) in the P2 domain. B) A 3D structure of a FCV VP1 monomer model (PDB 3M8L) showing the S, P1 and P2 domains was generated using the PyMOL Molecular Graphics System. Colors in the figure correspond to those in figure 6A. The S2 site of the putative cathepsin L cleavage sites on the P2 domain is shown. C) Sequence alignment of the conserved region (460-490 amino acid) of the VP1 protein from various FCV strains was generated. The black arrow indicates the amino acid at the S2 site in the putative cathepsin L cleavage site, and the red arrow indicates where the cleavage may occur between the S1 and S1' amino acids. The source of each sequence is: FCV-Urbana, L40021.1; FCV-131: DQ910787.1; FCV-Deuce, DQ910789.1; FCV-2024, AF479590.1; FCV-kaos, DQ910795.1; FCV-jengo, DQ910793.1; FCV-Ari, DQ910794.1.

A.


F463
L472
F486
aa 1
120
330
382
550
662
Leader
NTA
Shell
P1
P2
P1
Hyper variable region
(426-460) (490-520)

B.


F 486
F463
L 472
P2
P1
S

C.

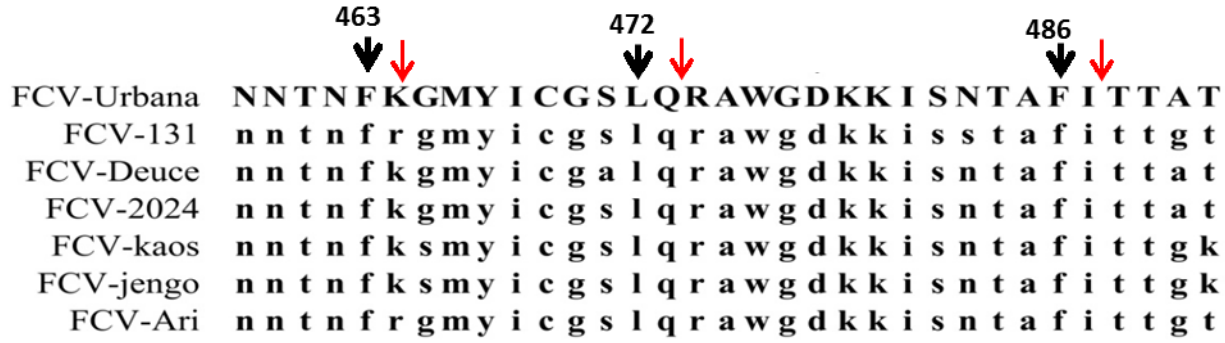

463
472
486
FCV-Urbana NNTNFKGMYICGSLQRAWGDKKISNTAFITTAT
FCV-131 nntnfrgmyicgslqrawgdkkissstafittgt
FCV-Deuce nntnfkgmyicgalqrawgdkkisntafittat
FCV-2024 nntnfkgmyicgslqrawgdkkisntafittat
FCV-kaos nntnfksmyicgslqrawgdkkisntafittgk
FCV-jengo nntnfksmyicgslqrawgdkkisntafittgk
FCV-Ari nntnfrgmyicgslqrawgdkkisntafittgt

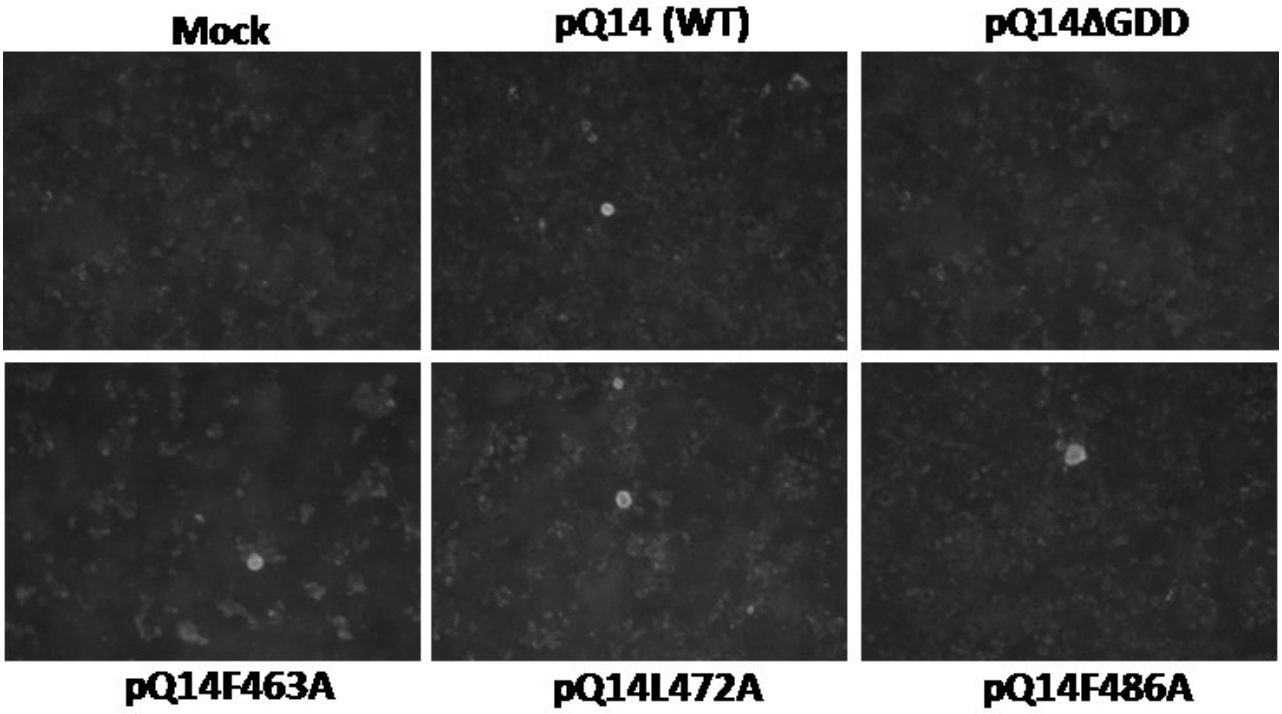
Figure 3-7. Virus recovery and replication competence of pQ14 and the mutant clones and the expression of recombinant VP1 from the wild type and the mutant clones in a baculovirus expression system.

A) A summary of virus recovery and replication competence of pQ14 and the mutant clones. The pQ14 and pQ14 ΔGDD clones served as controls. The mutant clones with a single alanine mutation at the P2 site in the putative cathepsin cleavage sites are underlined. B) IFA detection of FCV VP1 after transfection of MVA/T7-infected CRFK cells with each clone. Cells were fixed at 16 h after transfection for IFA staining. C) Western Blot analysis of the wild type or the mutant VP1 expressed in a baculovirus expression system. Each sample was the supernatant of sf9 cells infected with a recombinant baculovirus or the resuspended pellet obtained after ultracentrifugation of the supernatant. Each sample was probed with antibody against FCV VP1. Concentrated FCV was used as a control in the Western blot.

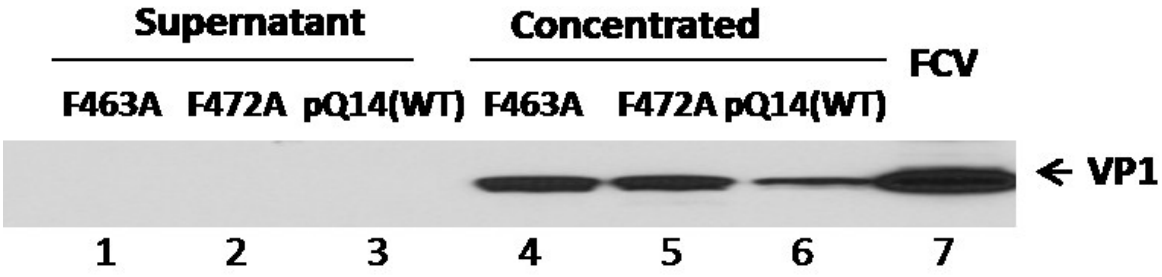
A.

	Recovery	Replication competence
pQ14 (WT)	+	+
pQ14F463A	-	+
pQ14L472A	-	+
pQ14F486A	+	+
pQ14K464A	+	+
pQ14R474A	+	+
pQ14K479A	+	+
pQ14ΔGDD	-	-

B.


Mock
pQ14 (WT)
pQ14ΔGDD
pQ14F463A
pQ14L472A
pQ14F486A

C.


Supernatant
Concentrated
FCV
F463A
F472A
pQ14(WT)
F463A
F472A
pQ14(WT)
VP1
1
2
3
4
5
6
7

3.7 References

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Chapter 4 - Acid Sphingomyelinase Mediated Ceramide Formation Facilitates Endosomal Escape of Caliciviruses

4.1 Abstract

Replication of porcine enteric calicivirus (PEC) in LLC-PK cells is dependent on the presence of bile acids in the medium. Our recent results demonstrated that bile acids facilitate virus escape from the endosomes into cytoplasm during PEC entry for the successful replication. Understanding the mechanism may yield valuable information regarding the replication of fastidious caliciviruses such as human noroviruses. In this study, we report a novel finding that cold treatment during PEC entry results in the same viral escape and replication, and that this event by cold treatment or bile acids is associated with ceramide formation by acid sphingomyelinase (ASM). ASM catalyses hydrolysis of sphingomyelin into ceramide in the endosomes/lysosomes, which is known to destabilize lipid bilayer and promote membrane fusion. Treatment of LLC-PK cells with bile acids or cold led to ceramide formation, while PEC alone did not, and small molecule antagonists or siRNAs of ASM blocked ceramide formation and significantly reduced PEC replication. Interestingly, incubation of feline calicivirus (FCV) or murine norovirus-1 (MNV-1) alone with the target cells resulted in ceramide formation, and inhibition of ASM also significantly reduced ceramide formation as well as viral replication. Finally, inhibition of ASM resulted in the retention of PEC, FCV or MNV-1 in endosomes during the entry in correlation with reduction of viral replication. These results suggest the importance of viral escape from endosomes for calicivirus replication and a common mechanism utilized by caliciviruses during virus entry to cells.

4.2 Introduction

Viruses in the *Caliciviridae* family are small non-enveloped viruses of 27-35 nm diameter with a single-stranded, positive-sense RNA genome of 7-8kb. There are at least five genera in the *Caliciviridae* family are established: norovirus and sapovirus cause enteric infections in humans and animals, whereas lagovirus, vesivirus and nebovirus cause a range of diverse infections mainly in animals (Green, 2007). Human norovirus infections account for about 60% of non-bacterial gastroenteritis cases and cause 21 million cases and 800 deaths annually in the United States alone (Hall et al., 2012; Scallan et al., 2011). However, there is a severe limitation to study human noroviruses because they do not grow in cell culture (Duizer et al., 2004; Herbst-Kralovetz et al., 2013). The inability of growing human noroviruses in cell culture may be related to the early stage of viral replication such as receptor binding and/or uncoating process, because the transfection of norovirus RNA into cultured cells was shown to result in virus replication with release of viral particles into the medium (Guix et al., 2007). Virus-receptor interactions are crucial for the replication at cell membrane levels, and after the initial contact, viruses further interact with specific cellular components to uncoat and release their genome to subcellular locations where viral replication begins (Hogle, 2002). Many viruses utilize host endosomal trafficking system for entry into host cells and specific sequential events lead to viral escape from endosomes (Kielian and Rey, 2006; Moyer and Nemerow, 2011). Enveloped viruses generally carry fusion machinery in their enveloped proteins and its activation leads to interact with membranes and release the viral genome into cytosol of host cells (Kielian and Rey, 2006). The activation of fusion machinery could be mediated by low pH (Heinz and Allison, 2000; Skehel et al., 1982), interaction with receptor (Earp et al., 2005) , interaction with receptor and co-receptor (Eckert and Kim, 2001; Feng et al., 1996), endosomal proteolysis (Chandran et al., 2005), or combinations of any these factors (Matsuyama et al., 2004; Mothes et

al., 2000). The non-enveloped viruses lack the fusion machinery, but some viruses are known to utilize lytic factors to membrane disruption and penetration (Moyer and Nemerow, 2011).

Acid sphingomyelinase (ASM) catalyzes the hydrolysis of sphingomyelin to ceramide which has emerged as a biochemical mediator of stimuli such as ionizing radiation, UV light, heat, CD95 as well as infection with some pathogenic bacteria and viruses (Gulbins and Kolesnick, 2003; He et al., 2003; Montes et al., 2008; Stancevic and Kolesnick, 2010). ASM activity has been shown to be required for entry of measles virus, rhinovirus, Japanese encephalitis virus and Ebolavirus (Avota et al., 2011; Grassmé et al., 2005; Miller et al., 2012; Tani et al., 2010). Ceramide formation alters the biophysical properties of membranes with the hydrophobic ceramide molecules forming small rafts that fuse together to form large ceramide-enriched membrane platforms, changes in membrane fluidity and permeability (Montes et al., 2002; Siskind and Colombini, 2000), facilitates membrane fusion (Basáñez et al., 1997), or promotes macropinocytosis (Gulbins et al., 2004; Gulbins and Kolesnick, 2002). It may also lead to formation of channels large enough for proteins to cross membranes or cause lipid flip-flop (Contreras et al., 2009; Samanta et al., 2011). Our previous reports have shown that bile acids facilitate the endosomal escape of porcine enteric calicivirus (PEC), but the exact mechanism involved was not well understood. In this study, we demonstrated that cold-shock treatment during virus entry of PEC resulted in the same viral escape and replication even in the absence of bile acid. Further we found that while PEC alone did not lead to ceramide formation, bile acids or cold-shock treatment resulted in ceramide formation via ASM activity in LLC-PK cells. The blocking ceramide formation significantly reduced PEC replication in the cells. Interestingly, feline calcivirus (FCV) or murine norovirus-1 (MNV-1) could induce ceramide formation in the target cells, and blocking ceramide formation by inhibiting ASM activity

significantly reduced the replication of both FCV and MNV-1. We further showed that inhibition of ASM activity lead to retention of PEC, FCV and MNV-1 in endosomes by the confocal microscopy, which suggests the crucial role of ceramide formation by ASM in viral escapes from endosomes during the early stage of virus replication.

4.3 Materials and methods

Cells and viruses

PEC Cowden strain was propagated in LLC-PK cells in the presence of glycochenodeoxycholic acid (GCDCA, 100 μM) in Eagle's Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. FCV Urbana strain was propagated in CRFK cells in MEM containing 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. MNV-1 was propagated in RAW264.7 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultured viruses were concentrated by centrifuging at 27,000 rpm through a 40% w/v sucrose cushion at 4°C for 2 h in a SW27 rotor. The obtained pellet was resuspended in serum free MEM and stored in -80°C.

Reagents and antibodies

GCDCA was purchased from Sigma-Aldrich (St Louis, MO). AY9944, an ASM inhibitor was purchased from Santa Cruz Biotech (Santa Crus, CA). Fluoxetine, desipramine, chlorpromazine were purchased from Sigma-Aldrich. For confocal microscopy, the primary antibodies including anti-PEC/cowden antibodies raised in swine (Chang et al., 2005), anti-FCV antibodies raised in guinea pig (Sosnovtsev and Green, 1995), anti-MNV-1 antibodies raised in guinea pig (Wobus et al., 2004), mouse IgM anti-ceramide antibody from Enzo Life Sciences, Inc (Farmingdale, NY) and rabbit polyclonal IgG anti-Rab7 antibodies purchased from Santa

Cruz Biotech were used. The secondary antibodies including PerCP-Cy5.5 anti-rabbit from Santa Cruz Biotech, FITC anti-swine IgG and FITC anti-mouse purchased from Kirkegaard and Perry Lab (Gaithersburg, MD) and FITC-anti-guinea pig IgG from Sigma-Aldrich were used in this study. Additional PEC antibodies including anti-2AB, RdRp or VPg were described previously (Chang et al., 2005).

Infection and replication of PEC

Confluent LLC-PK cells were inoculated with PEC at a MOI of 50 in the absence or presence of GCDCA (100 μM) and incubated at 4 °C or 37 °C for 1 h. After the initial incubation for 1 h, the plates were immediately transferred to 37°C, and incubated for additional 1 h. Then, virus infected cells were washed twice with PBS, replenished with MEM containing 5 % FBS, and further incubated at 37 °C for 12 h or other indicated time points. Viral replication was quantified by real-time qRT-PCR (Shivanna et al., 2014a) or by $TCID_{50}$ assay (Reed and Muench, 1938). For real time qRT-PCR, total RNA was extracted from cells (before extensive cell lysis) and used for the templates. For $TCID_{50}$ assay, cells disrupted with 3 cycles of freezing/thawing, and supernatant was used for virus titration.

Inhibition of ASM by siRNA transfection or small molecule inhibitors

To knockdown and assay ASM expression in LLC-PK cells, siRNA and primers/probes for qRT-PCR were designed targeting the porcine ASM gene (GenBank accession No. XM_005656607), respectively. The siRNA, primers and probes were synthesized from Integrated DNA technology (Coralville, IA). The porcine ASM siRNA was prepared using sequences of 5'-rArUrUrCrGrGrUrArArUrArArUrUrUrCrCrArG and 5'-rGrCrUrGrGrArGrCrUrGrGrArUrUrArU. For siRNA studies, mock (transfection agent), irrelevant siRNA (negative control from Qiagen) or ASM siRNA was transfected to one-day old

LLC-PK cells and incubated for 48 h at 37 °C before infection or any treatment. To monitor ASM levels after the transfection, ASM specific mRNA levels were quantified by real-time qRT-PCR using primers: F-5'-CCTTCGCACCCTCAGAATC, R- 5'-CAGAAGTTCTCACGGGAACAA, and Probe: 5'- 56-FAM/ATTGAGAGA/ZEN/GATGAGGCGGAGGC/ 3IABkFQ/ -3'. The RT-PCR products were also run on agarose gels for assessing ASM mRNA levels. After 48 h post transfection, cells were inoculated with PEC at a MOI of 50 GCDCA (100 μM, 37 °C for 1 h) or cold treatment (4 °C for 1 h). After washing with PBS, virus infected cells were further incubated for 1 h (for confocal study) or 12 h (viral replication study). Inhibitors of ASM including AY9944, fluoxetine, desipramine and chlorpromazine were also used to examine their effects on ceramide formation and viral replication. For the study, each ASM inhibitor was treated with LLC-PK cells for 1 h, and then, cells were inoculated with PEC at a MOI of 50 with GCDCA (100 μM, 37 °C for 1 h) or cold treatment (4 °C for 1 h). After washing with PBS, virus infected cells were further incubated with the same inhibitor for 1 h (for confocal study) or 12 h (viral replication study).

BEI inactivation of FCV and MNV-1

The harvested virus suspension was centrifuged at 3000 rpm for 15 min to remove cell debris. The resulting supernatant virus suspension was mixed with fresh BEI in the ration 9:1 (0.1 M solution of bromoethylamine hydrobromide in 0.2N NaOH cyclized to BEI by incubating at 37 ^{0}C for 1h in a water bath). The treated virus suspension is stirred for 24 h at 37 ^{0}C. BEI is then inactivated by adding fresh filter sterilized sodium thiosulfate (1.0 M solution) at the rate of 30 ml per liter treated fluids to 0.03 M concentration. Mix for 2-4 h at 37 ^{0}C, adjusts the pH to 6.6-7.2 with 4 N HCl. The inactivated virus suspension is then concentrated by spinning at 27000

rpm through a 40% sucrose cushion in a SW27 rotor. The resulting pellet was resuspended in MEM and stored at 4 ^{0}C. To confirm their inactivation status, the inactivated FCV and MNV-1 were used to infect CRFK and RAW264.7 cells respectively and examined for their inability to produce any cytopathic effects.

Infection with FCV or MNV-1

To study ceramide formation by FCV or MNV-1 infections, confluent CRFK and RAW267.4 cells were inoculated with mock-medium (Mock) or high MOI of FCV (50 MOI) or MNV-1 (20 MOI), respectively, and incubated at 37°C for 1 h. Inactivated FCV or MNV-1 was also tested to CRFK or RAW267.4 (both target and non-target cells) at the equivalent amount of each virus. To test ASM activity on ceramide formation, desipramine was pretreated with CRFK and RAW267.4 cells at 37°C 1 h, and the same amount was added during virus inoculation. Ceramide formation was determined by confocal microscopy. Inhibitors of ASM including AY9944, fluoxetine, desipramine and chlorpromazine also examined for the replication of FCV or MNV-1 with the similar procedures as PEC study. Briefly, confluent CRFK and RAW267.4 cells were pretreated with each ASM inhibitor (AY9944 45 μM, fluoxetine 35 μM, desipramine 45 μM, chlorpromazine 7.5 μg/ml) for 1 h. Each compound did not show any toxicity at the concentration. Then cells were inoculated with FCV (MOI 50) or MNV-1 (MOI 30) in presence of the same inhibitor for 1h. Virus infected cells were washed twice with PBS and replenished with fresh MEM containing the same concentration of ASM inhibitors. After additional 12 h, the replication of FCV or MNV-1 was determined by $TCID_{50}$ assay (Reed and Muench, 1938).

Confocal microscopy

For confocal microscopy, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4) at room temperature (RT) for 15 min, permeabilized with 0.1% Triton-×100 in PBS for 10 min at RT, washed three times with PBS, and incubated in blocking buffer (PBS containing 0.5% bovine serum albumin) for 15 min. Also fixed cells without permeabilization were tested as a control. The cells were then incubated with primary antibodies specific to the capsid protein of PEC (1:200), MNV-1 (1:200) or FCV (1:200), or Rab7 (1:200) at 37°C to probe PEC, MNV-1, FCV or endosomes, respectively. After 2 h incubation at 37°C, cells were washed three times with PBS and further incubated at 37°C for 2 h with appropriate secondary antibodies. Cellular DNA was stained with sytox orange (0.5 μM in 0.9% NaCl). Coverslips were mounted in Prolong Gold antifade reagent (Molecular Probes), and the cells were scanned with a confocal microscope LSM 510 (Zeiss, Oberkochen, Germany) using a 100× oil-immersion objective. The images were analyzed by ImageJ software 1.47 (http://imagej.nih.gov/ij/), and merged images were prepared. For the colocalization of ceramide and Rab7, colocalization analysis was performed using JACoP and colocalization-MBF plugins for ImageJ software. Single channel images were thresholded by Costes' auto threshold method and the Manders split correlation coefficient for colocalization was then determined for each image.

Statistical analysis

All the results shown are mean of at least three independent experiments. The effects of ASM siRNA and ASM inhibitors were analyzed by two-tailed Student's *t*-test. P value <0.05 was considered statistically significant. Confocal images shown are representative of at least three independent experiments.

4.4 Results

Cold treatment induces PEC replication without bile acids

To synchronize PEC entry, confluent LLC-PK cells were infected with high MOI (50) PEC, incubated at 4°C for 1 h and then immediately transferred to at 37°C. After additional incubation for 1 h, cells were washed with PBS, replenished with fresh media and further incubated at 37°C. Using the high MOI, the replication of PEC by the cold treatment was evident by virus titration and IFA assay (Figure 4-1). Virus titers by real time qRT-PCR or $TCID_{50}$ method at various time points indicated efficient viral replication by cold treatment comparable to bile acid-mediated replication (Figure 4-1 A). There was no difference in the virus replication kinetics measured by real time qRT-PCR at 1, 4, 8, and 12 h pi by cold treatment, bile acids or combination of two (Figure 4-1B). While PEC RNA levels were unchanged without any treatment, they were approximately 10 fold increased by each treatment during 4-8 h, and reached up to 100-fold at 12 h by the treatment (Figure 1B). When used lower MOI of 5 or 1, similar replication kinetics were observed, but with MOI below 1, detection of viral replication was not consistent probably due to the single replication cycle by the cold treatment (data not shown). Viral replication induced by cold treatment was also examined by immunofluorescence assay (IFA). Using 50 MOI of PEC and incubation for 12 h, the expression of viral proteins such as 2AB, polymerase or VPg was easily detected in the virus infected cells with cold treatment. However, without any treatment, even 50 MOI infection did not yield any evidence for the expression of the viral proteins (Figure 4-1C).

We have previously reported that bile acids facilitate viral escape from the endosomes for successful replication of PEC (Shivanna et al., 2014a), here we examined if cold treatment could

also be involved in this phenomenon using the confocal microscopy. For this, mock-medium (Mock) or PEC was inoculated with LLC-PK cells at high MOI (50) with no treatment, GCDA (100 μM, incubated at 37 °C for 1 h) or cold treatment (incubated at 4 °C for 1 h), and incubated at 37 °C for additional 1 h, and cells were fixed and viral trafficking was probed. As demonstrated in our previous report (Shivanna et al., 2014a) , without any treatment, PEC was accumulated in endosome like compartments. However, with GCDCA or cold treatment, PEC was not detected in the cytoplasm, suggesting successful escape from endosomes in the conditions (Figure 4-1 D). Furthermore, PEC replication by bile acids or cold treatment may share the same mechanism during the early stage of viral replication.

Bile acids or cold treatment induce ceramide formation through activation of ASM

Hydrophobic bile acids and temperature stress have been reported to induce ceramide formation in lipid membranes in different systems (Gupta et al., 2004; Montes et al., 2008), and ASM is a major mechanism for ceramide formation by catalyzing the breakdown of sphingomyelin to ceramide and phosphorylcholine (Gulbins, 2003). Using confocal microscopy, we first examined if bile acids or cold-shock induce ceramide formation. Without any treatment, little ceramide detected in LLC-PK cells (Figure 4-2 A a-c), and so did the incubation of PEC (50 MOI) at 37°C for 1 h (Figure 4-2 A d-f). However, treatment of cells with GCDCA (100 μM) or cold treatment (4 °C for 1 h) induced significant levels of ceramide formation in the cytoplasm (Figure 4-2 A, g-l). The ceramide formed by bile acids or cold treatment was co-localized with endosomal marker Rab7 (red) with > 0.90 Manders split colocalization coefficients (Figure 4-2 B), suggesting ceramide localized on the membranes of early or late endosomes. Ceramide formation on the cell membranes was not observed because non-permeabilized cells showed little fluorescence signals for ceramide with any treatment (data not shown). To confirm that

ceramide was formed through the activity of ASM, we used an ASM inhibitor, desipramine (Kornhuber et al., 2010) or specific ASM siRNA, and examine their effects on GCDCA or cold treatment-induced ceramide formation using the confocal microscopy (Figure 4-3). Both desipramine and ASM siRNA significantly inhibited ceramide formation by the treatment with GCDCA or cold in LLC-PK cells (Figure 4-3).

ASM activity is required for productive calicivirus replication

To examine the requirement of ASM activity in PEC replication in correlation with ceramide formation, several ASM inhibitors or ASM siRNA were examined for viral replication. First, the transfection of various concentrations of ASM siRNA significantly reduced ASM mRNA levels in LLC-PK: up to 90% reduction was observed by siRNA transfection (Figure 4A). The replication of PEC induced by GCDCA or cold treatment was significantly reduced by transfection with ASM siRNA up to 8~13 or 7~9.3 fold reduction in genome copy number of PEC, respectively (Figure 4-4 B and C). However, transfection with irrelevant siRNA did not show any effects on PEC replication. Similar inhibitor effects were observed by various ASM inhibitors including AY9944, fluoxetine, desipramine and chlorpromazine: the treatment of each ASM inhibitor led to the reduction of PEC replication induced GCDCA or cold treatment by 9.2~22.5 or 7.2~12.6 fold genome copy number at 12 h pi, respectively (Figure 4-4 D and E).

FCV or MNV-1 can induce ceramide formation in the target cells and inhibition of ASM significantly reduced their replication

Since FCV or MNV-1 does not require bile acids for their replication, we hypothesized it can induce ceramide formation during the virus entry for successful replication. When high MOI of FCV (MOI 50) or MNV-1 (MOI 30) was incubated with CRFK or RAW267.4 cells at 37°C for 1 h, the ceramide formation was evident in the permeabilized cells (Figure 4-5 A). However,

there was no ceramide detected when incubated to non-target cells or non-permeabilized cells with or without virus incubation. Interestingly, BEI inactivated FCV or MNV-1 could also induce ceramide formation to the target cells when incubated with concentrated equivalent amount of each virus to the high MOI (Figure 4-5 B, a –f). The inhibition of ASM with desipramine also blocked ceramide formation by FCV or MNV-1 in CRFK or RAW264.7 cells, respectively (Figure 4-5 B, g–l). The reduction of ceramide formation by the ASM inhibitor was correlated with the reduction of FCV or MNV-1 replication. When the same set of ASM inhibitors were used to inhibit ASM activity in FCV or MNV-1 replication in CRFK cells or RAW267.4, each inhibitor significantly reduced viral replication: 100~375.1 or 100~211.3-fold reduction in $TCID_{50}$ titers of FCV or MNV-1, respectively (Figure 4-6 A and B).

Ceramide formation in cells is required for endosomal escape of calicivirus

Because bile acids are required for viral escape from endosomes and ceramide is known to trigger membrane destabilization, we hypothesized that ceramide formation may facilitate the calicivirus escapes from endosomes during virus entry. To test this hypothesis, we used the confocal microscopy to trafficking PEC, FCV or MNV-1 in the target cells with various treatments. In the presence of GCDCA or cold treatment resulted in successful viral escape from endosomes by absence of detectable accumulation of PEC capsid protein as seen in Figure 4-1D (f-k). However, when ASM was inhibited by an ASM inhibitor or ASM siRNA, there was significant reduction of viral escape from endosomes induced by GCDCA or cold treatment (Figure 4-7 A). This phenomenon was correlated with the reduction of PEC replication by the same treatment (Figure 4-4 B-E). When the entry events of FCV or MNV-1 were examined by similar confocal study as PEC, most of both viruses were successfully escaped from endosome in 1 h (Figure 4-7 B, a-c for FCV and g-i for MNV-1). However in the presence of desipramine,

significant accumulation of FCV or MNV-1 capsid proteins in endosomes in CRFK or RAW 267.4 cells, respectively, was observed (Figure 4-7 B d-f or j-l), suggesting the failure of viral escape in the condition. This observation was also correlated well with the reduction of FCV or MNV-1 replication by ASM inhibitors (Figure 4-6). These results suggest the requirement of ceramide formation (on endosomal membranes) by ASM for successful viral escape from endosomes for three caliciviruses.

4.5 Discussion

The early stage of viral replication was suggested to be related to the inability of growing human norovirus in cell culture, because it was shown that transfection of norovirus RNA into cultured cells led to virus replication with release of viral particles into the medium (6). For PEC, we demonstrated that viral RNA was infectious but without bile acids there was no viral replication was detected and internalized viruses entrapped in endosomes without initiating viral replication (Shivanna et al., 2014a). We also demonstrated that bile acids facilitate viral escape from endosomes for successful replication (Shivanna et al., 2014a). To study further details of calicivirus entry process, we used cultivable related caliciviruses including PEC (with or without bile acids), FCV and MNV-1 (Kreutz and Seal, 1995; Perry and Wobus, 2010; Shivanna et al., 2014a, b; Stuart and Brown, 2006). While PEC requires bile acids, FCV or MNV-1 does not require any supplement for the replication, and comparative studies among these viruses and understanding the molecular basis of viral endosomal escape mediated by bile acids could lead to the development of better cell culture models for noroviruses and other uncultivable enteric viruses. Here we report that ASM mediated ceramide formation is crucial in the endosomal escape of caliciviruses for successful replication.

Our initial attempts to synchronize PEC entry using high MOI (> 10) into LLC-PK cells by incubation at 4^0C and then at 37^0C resulted in unexpected PEC replication even in the absence of bile acid (Figure 4-1). Later, we found out that PEC replication could be initiated with simple cold shock treatment even with low MOI. However, with low MOI, viral replication with cold shock was not easily detected because it leads to a single replication cycle. Literature reviews showed that bile acids can induce ceramide formation by activating ASM (Gupta et al., 2004), and the hot-cold hemolysis with membrane breakdown was due to the same event (Montes et al., 2008). Thus, we hypothesized ceramide and ASM may play important roles in cold shock- or bile acids mediated PEC replication. Using the confocal microscopy, we found that treatment of cold-shock or bile acids induced ceramide formation and co-localizing with Rab7 on endosomal membranes, whereas PEC alone had little effect on ceramide formation (Figure 4-2 A). Using ASM inhibitors and siRNA of ASM, we found that the activity of ASM was required for ceramide formation in LLC-PK cells (Figure 4-3). Since FCV and MNV-1 do not require bile acids for their replication, we examined if ceramide is formed by high MOI of each virus. Incubation of high MOI of FCV or MNV-1 in CRFK or RAW267.4 cells, respectively induced ceramide formation (Figure 4-5). In addition, viral replication is not required for ceramide formation because inactivated FCV or MNV-1 also induced ceramide formation (Figure 4-5 B). These results suggested that ceramide formation was crucial in the replication of caliciviruses, and FCV or MNV-1 may trigger ceramide formation by simple interaction with its receptor(s).

It has been shown that ASM can be activated by many viruses Sindbis virus (Jan et al., 2000), Rhinovirus (Grassmé et al., 2005), Japanese encephalitis virus (Tani et al., 2010), and Ebolavirus (Miller et al., 2012), and the activation is important in virus entry. For Sindbis virus, virus entry requires sphingomyelin as a cofactor for virus-cell fusion, and virus–cell fusion at the

endosomal membranes was shown to trigger ASM activation and subsequent ceramide formation (Jan et al., 2000). Ebolavirus infection recruits ASM to plasma membrane to form ceramide macrodomains and macropinosome for successful virus entry (Miller et al., 2012; Nanbo et al., 2010). Measles virus binding to DC-SIGN on dendritic cells which activates ASM and recruitment of ASM to plasma membrane along with CD150, and co-clustered CD150 and DC-SIGN on cell surface in ceramide-enriched microdomains facilitates virus-cell membrane fusion (Avota et al., 2011). Similar to measles virus, ASM was recruited to cell surface by rhinovirus binding to receptors LDLR or ICAM-1 during its entry into cells (Grassmé et al., 2005). While there is no information available for the cellular receptor(s) for PEC or MNV-1 to date, the specific cellular receptor of FCV was identified as fJAM-A (feline junctional adhesion molecule). Although there is little information of direct interaction between fJAM and ASM or ceramide, it has reported that JAM-A associated with PKC isoforms for its functional formation (Iden et al., 2012). Because some PKC isoforms are known to activate ASM, we speculate that FCV and fJAM-A interaction may activate ASM through a PKC isoform, and this activation is crucial for virus entry. In addition, MNV-1 and its receptor interaction may also be sufficient for ceramide formation and subsequent viral replication.

Next, the requirement of ASM activity for calicivirus replication and virus trafficking during virus entry was analyzed by using siRNA and functional inhibitor of ASM. First, inhibition of ASM significant reduced replication of PEC, FCV and MNV-1 suggesting a common requirement of ASM among the caliciviruses (Figure 4-4, 4-6). Second, caliciviruses trapped in endosomes by ASM inhibitors during the early events of viral replication by the confocal microscopy (Figure 4-7). For PEC, virus was still found in endosomes without any treatment at 1 hr PI, whereas no detectable virus was found in the endosomes in presence of bile

acid or cold-shock treatment (as reported in our previous paper, Figure 4-1 D). However, when ASM was inhibited by siRNA or an inhibitor, PEC was trapped in endosomes even in the presence of bile acids or cold-shock treatment (Figure 4-7 A). Similar results were found with FCV and MNV-1 showing the accumulation of virus in endosomes when ASM activity was inhibited (Figure 4-7 B). In the previous report, we demonstrated that calicviruses including PEC, FCV and MNV-1 required low pH and cathepsin L activity for successful entry (including uncoating) (Shivanna et al., 2014b). And in this report, we demonstrated that activation of ASM by bile acids (PEC) or virus (FCV or MNV-1) would lead to ceramide formation on the endosomal membranes. Since formation of ceramide platforms was shown to alter membrane fluidity and permeability (Montes et al., 2002), form large channels (Samanta et al., 2011), cause lipid flip-flop (Contreras et al., 2009) along with its hydrophobic protein interaction site (Krönke, 1999), we speculate this may lead to release of viral genome into cytoplasm.

4.6 Acknowledgements

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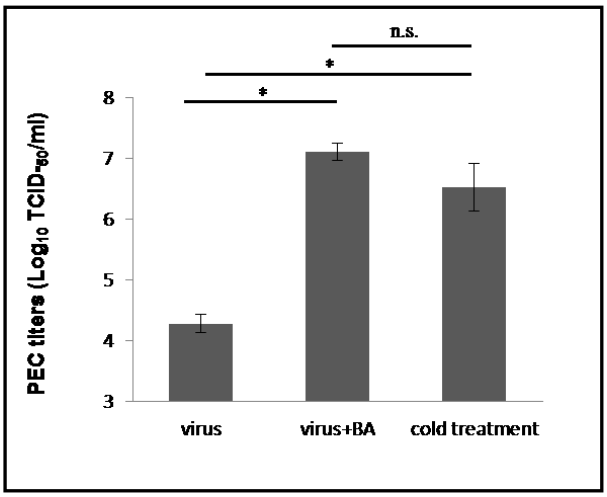
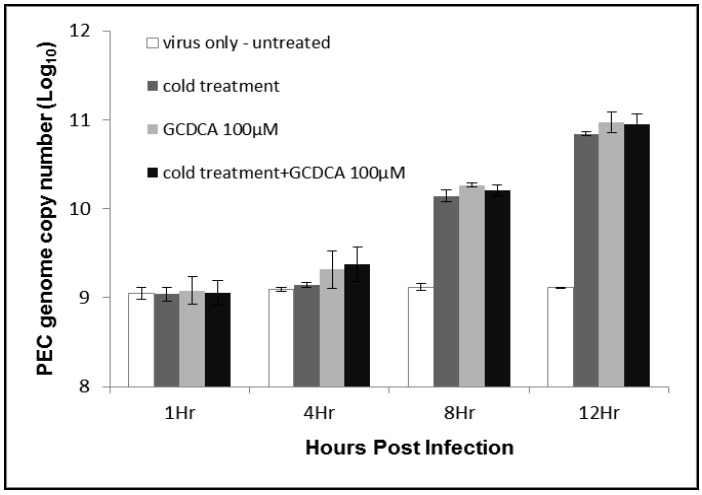
Figure 4-1. Cold treatment during PEC infection resulted in viral replication by facilitating viral escape from endosomes.

A) Confluent LLC-PK cells were infected with PEC (MOI 50) and incubated at 37 °C with (virus + BA) or without 100 μM of GCDCA (virus), or 4°C for 1 h then at 37°C (cold treatment). Virus infected cells were incubated for 16 h, following freezing/thawing 3 times, and virus replication was determined by $TCID_{50}$ method. An asterisk or n.s. indicates a significant difference ($p < 0.05$) or no significance between the groups. B) PEC viral RNA was quantified by qRT-PCR at 1, 4, 8 and 12 h after LLC-PK cells were inoculated with 50 PEC at a MOI of 50 and various treatments as indicated. C) Confluent LLC-PK cells were inoculated with PEC (MOI 50) by incubated at 4°C (cold treatment) or 37°C (no treatment), and cells were further incubated at 37°C for 12 h. Virus infected cells were fixed and expression of viral proteins was determined by probing with antibodies against viral proteins 2AB, polymerase (RdRp) or VPg. D) Confocal laser scanning microscopic examination of PEC entry by various treatments including no virus + treatment (Mock), PEC + no treatment (PEC), PEC + GCDCA (100μM) or PEC + cold treatment (4°C for 1 h). LLC-PK cells grown on Lab-Tek II CC^2 chamber slides were infected either with mock (medium) or PEC (MOI 50) with the various treatments, and incubated for additional 1 h before cells were fixed for confocal microscopy. Nuclei were stained with sytox orange (5μM) (pseudo colored blue), and PEC was probed with swine polyclonal anti-PEC VLP primary antibodies and detected by FITC labelled goat-anti-swine antibody (green). Merged images for nuclei and PEC were prepared.

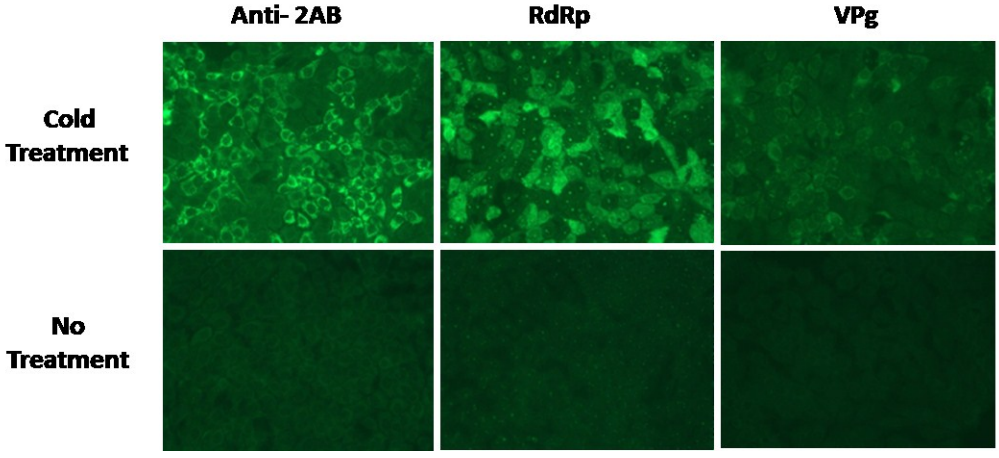
A.


n.s.
*
*
PEC titers (Log10 TCID50/ml)
8
7
6
5
4
3
virus
virus+BA
cold treatment

B.


virus only - untreated
cold treatment
GCDCA 100µM
cold treatment+GCDCA 100µM
PEC genome copy number (Log10)
12
11
10
9
8
1Hr
4Hr
8Hr
12Hr
Hours Post Infection

C.


Anti- 2AB
RdRp
VPg
Cold Treatment
No Treatment

D.

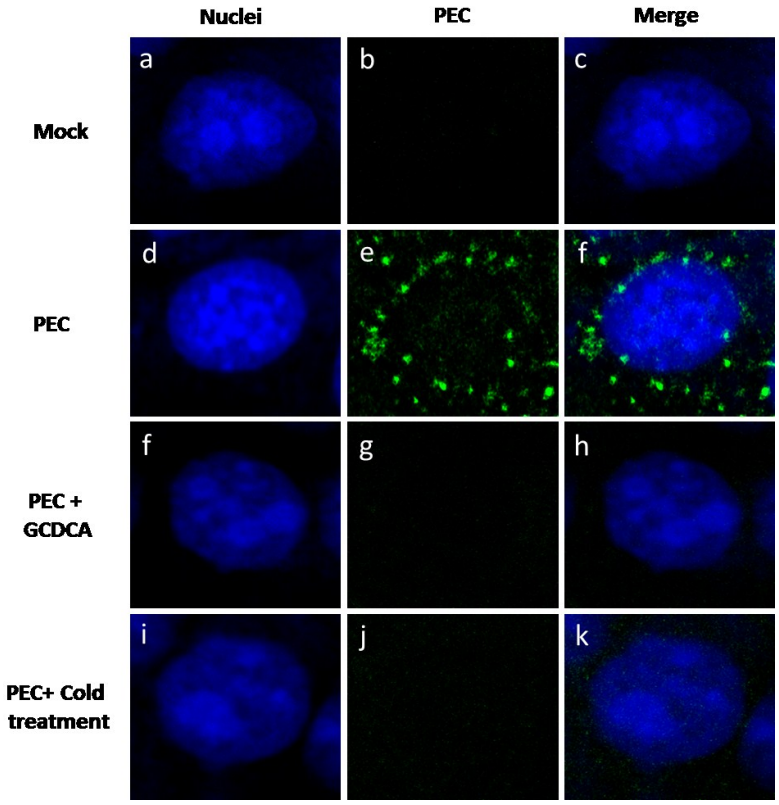
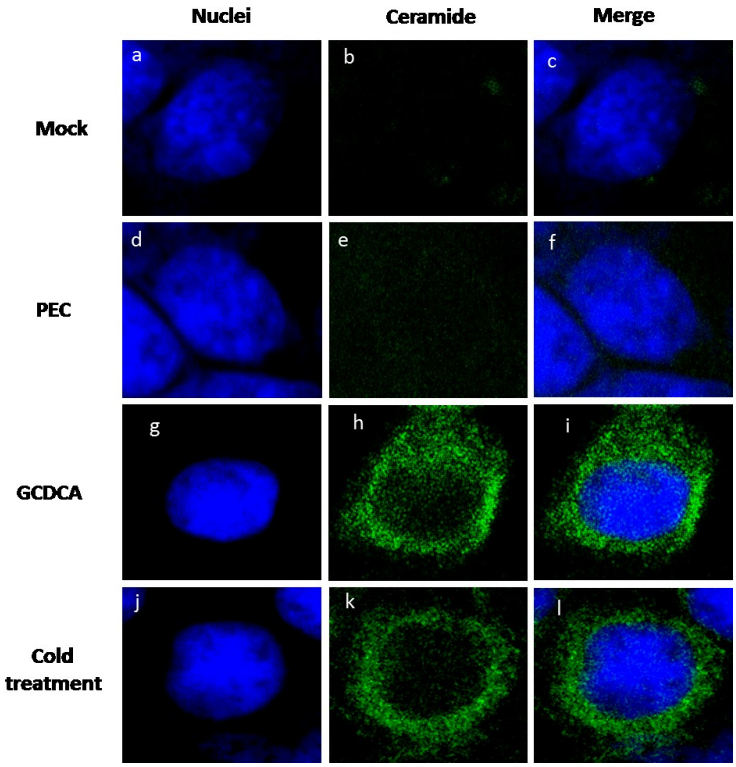

Nuclei
PEC
Merge
Mock
PEC
PEC + GCDCA
PEC+ Cold treatment
a
b
c
d
e
f
f
g
h
i
j
k

Figure 4-2. Bile acids or cold treatment induce ceramide formation in LLC-PK cells.

A) LLC-PK cells grown on Lab-Tek II CC^2 chamber slides were treated with mock medium (Mock) (a-c), PEC (MOI 50) (d-f), 100 μM GCDCA or cold treatment (4°C for 1 hr). The treatment of Mock, PEC or GCDCA was done at 37°C for 1 h, and cells were fixed for confocal microscopy. Ceramide was probed with mouse IgM anti-ceramide antibody with FITC-anti-mouse antibody (green), and merged images for nuclei and ceramide were prepared. B) LLC-PK cells were treated with Mock, GCDCA or cold treatment as above (A), and colocalization of ceramide (green) with Rab7 (red) was analyzed using ImageJ. The images were prepared from cross-sections above nuclei in cells for the colocalization analysis. Rab7 was probed with rabbit polyclonal anti-Rab7 with PerCP-Cy5.5 labelled goat-anti-rabbit antibody (red). Colocalization of ceramide (green) with Rab7 (red) appears in white in the merged images.

A.



B.

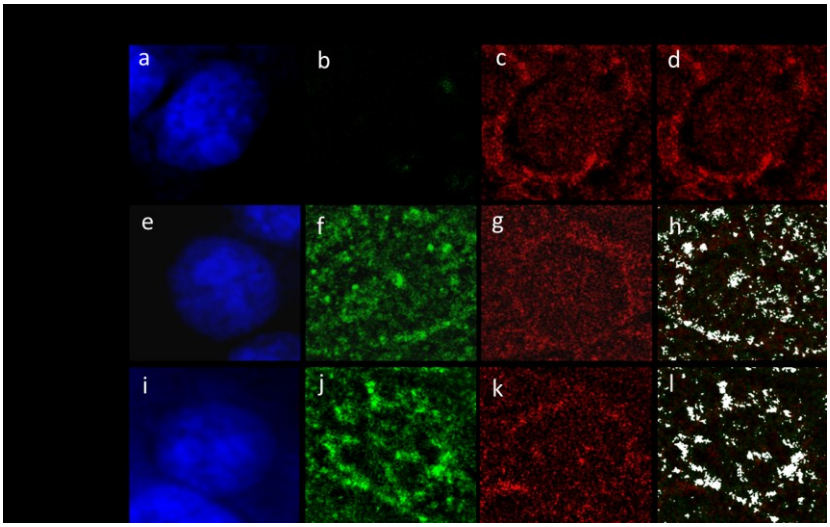


Figure 4-3. Ceramide formation by GCDCA or cold treatment is dependent on ASM in LLC-PK cells.

Cells were inoculated with PEC (50 MOI) with either GCDCA (100 μM, 37 °C for 1 h) or cold treatment (4 °C for 1 h) in the presence or absence of ASM inhibitor (desipramine, 40 μM) or ASM siRNA (200 nM, transfected at 48 h prior virus infection) as indicated in the Figure. After additional incubation at 37 °C for 1 h, cells were fixed for confocal microscopy as described above. Individual or merged images for nuclei and ceramide were prepared.

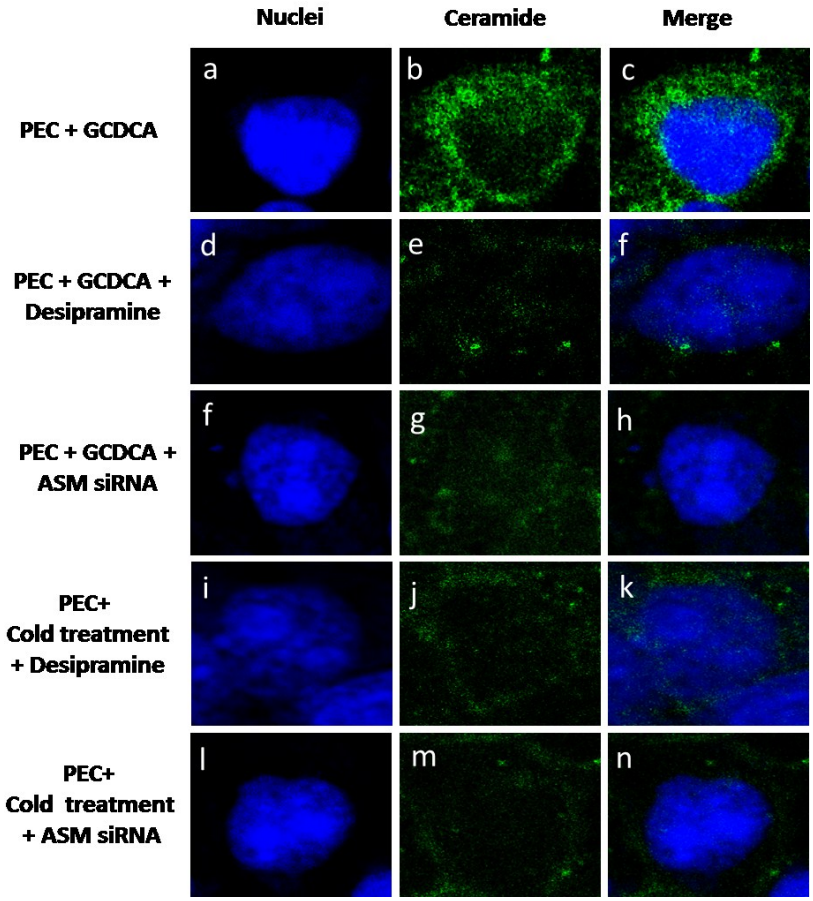
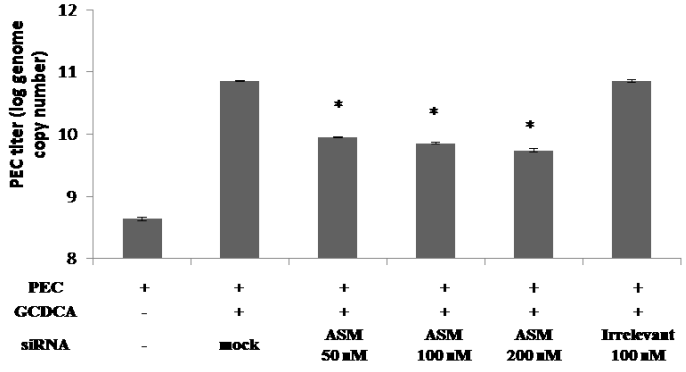


Figure 4-4. Inhibition of ASM with small molecule inhibitors or siRNA significantly reduced PEC replication.

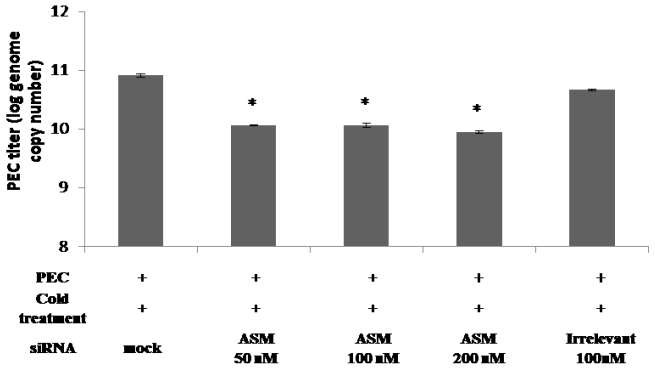
A) The levels of ASM mRNA by the transfection specific or irrelevant siRNA with various concentrations were quantified by real time qRT-PCR, and the products were also analyzed by agarose gel electrophoresis. B-C) One day old LLC-PK cells were transfected with mock or specific or irrelevant siRNA with various concentrations, and after 48 h of incubation, PEC was inoculated at a MOI of 50 with GCDCA (100 μM, 37 °C for 1 h) or cold treatment (4 C for 1h). After 12 h, viral replication was determined by real time qRT-PCR. D-E). Confluent LLC-PK cells were pretreated with various ASM inhibitors at different concentrations as indicated in the Figure for 1 h, and the inoculated with PEC (MOI 50) with GCDCA (100 μM, 37 °C for 1 h) or cold treatment (4 °C for 1h). After the inoculation, cells were treated with the same inhibitors for additional 12 h, and viral replication was assessed with quantified by real time qRT-PCR. An asterisk indicates a significant difference between the groups ($p < 0.05$)

A.

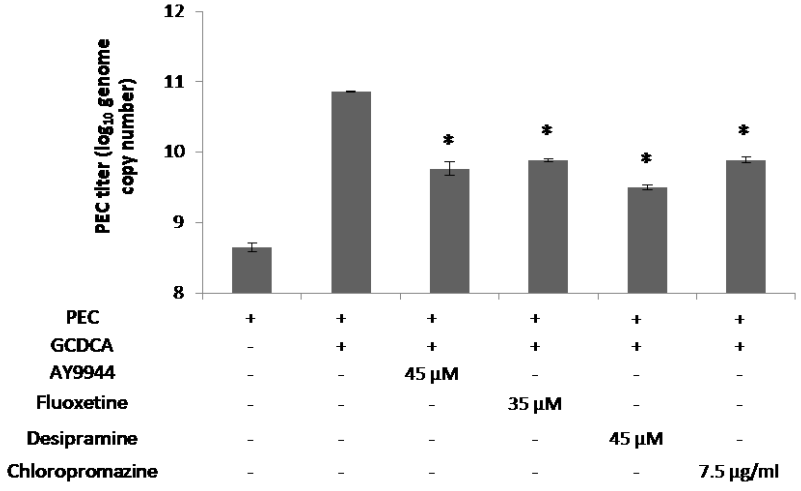
B.



C.


PEC titer (log genome copy number)
12
11
10
9
8
*
*
*
PEC + + + + +
Cold treatment + + + + +
siRNA mock ASM 50 nM ASM 100 nM ASM 200 nM Irrelevant 100nM

D.


PEC titer (log_{10} genome copy number)
12
11
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8
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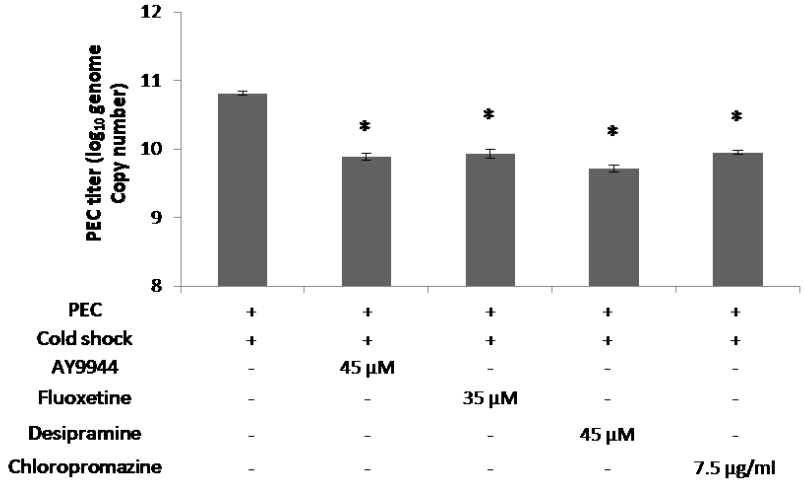
*
PEC + + + + + +
GCDCA - + + + + +
AY9944 - - 45 µM - - -
Fluoxetine - - - 35 µM - -
Desipramine - - - - 45 µM -
Chloropromazine - - - - - 7.5 µg/ml

E.


PEC titer (log_{10} genome Copy number)
12
11
10
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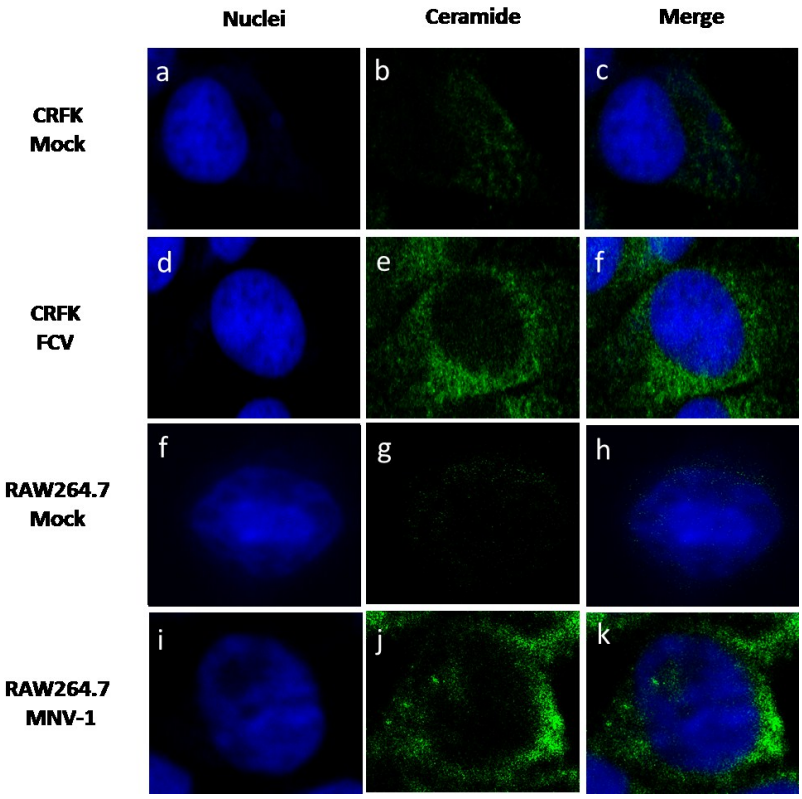
*
PEC + + + + +
Cold shock + + + + +
AY9944 - 45 µM - - -
Fluoxetine - - 35 µM - -
Desipramine - - - 45 µM -
Chloropromazine - - - - 7.5 µg/ml

Figure 4-5. FCV or MNV-1 can induce ceramide formation in CRFK or RAW267.4 cells, respectively, through ASM.

A) CRFK or RAW264.7 cells grown to confluency on Lab-Tek II CC^2 chamber slides were inoculated with mock-medium (Mock, a-c or f-h), FCV (50 MOI, d-f) or MNV-1 (30 MOI, i-k) at 37 °C for 1 h. Then cells were fixed for confocal microscopy. B) CRFK or RAW264.7 cells were inoculated with inactivated FCV (50 MOI equivalent, a-c), inactivated MNV-1 (30 MOI equivalent, d-f), or FCV (50 MOI) or MNV-1 (30 MOI) in the presence of desipramine (45 µM) at 37 °C for 1 h. Cells were fixed and probed with mouse IgM anti-ceramide antibody and goat-anti-rabbit antibody (green) for ceramide or sytox orange (5 µM, pseudo colored blue) for nuclei. Merged images of ceramide (green) with nuclei (blue) were prepared.

A.



B.

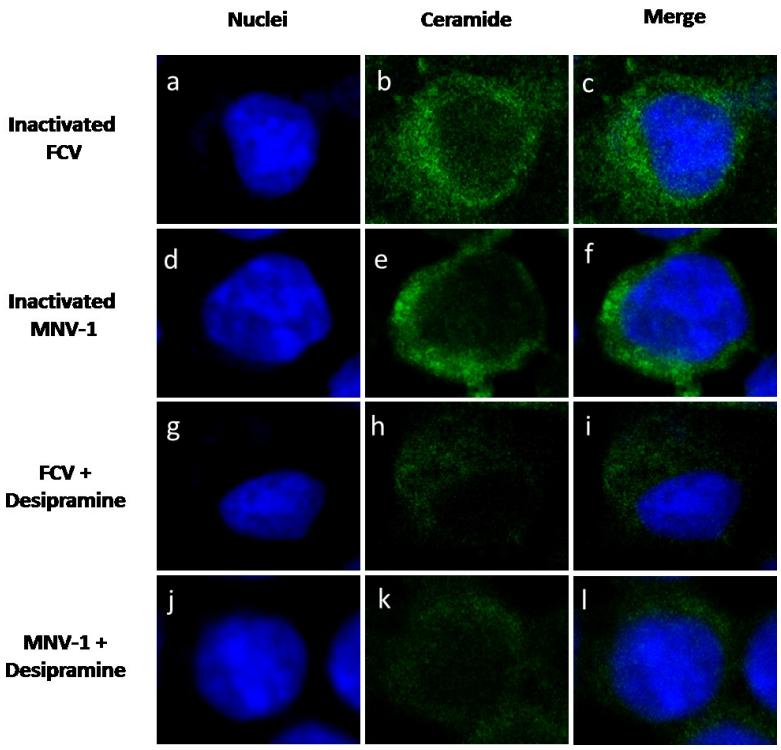
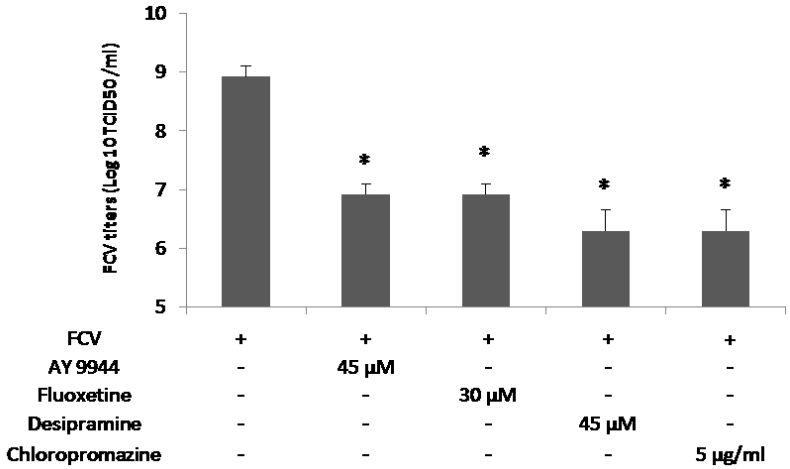

Nuclei
Ceramide
Merge
Inactivated FCV
Inactivated MNV-1
FCV + Desipramine
MNV-1 + Desipramine
a
b
c
d
e
f
g
h
i
j
k
l

Figure 4-6. The effects of ASM inhibitors in the replication of FCV or MNV-1.

Confluent CRFK or RAW264.7 cells were pretreated with various ASM inhibitors at different concentrations as indicated in the Figure for 1 h, and the inoculated with FCV (MOI 50) or MNV-1 (30 MOI) at 37 $^{\circ}$C for 1h. After the inoculation, cells were treated with the same inhibitors for additional 12 h, and viral replication was assessed with titration with $TCID_{50}$ method. An asterisk indicates a significant difference between the groups ($p < 0.05$)

A.



B.

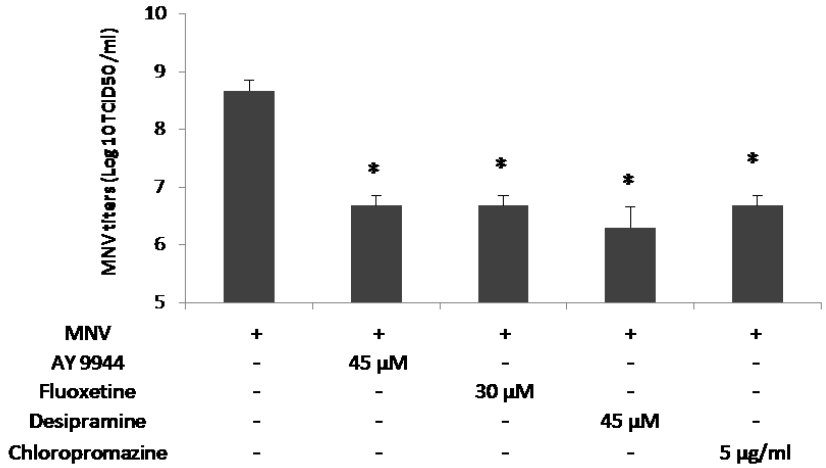
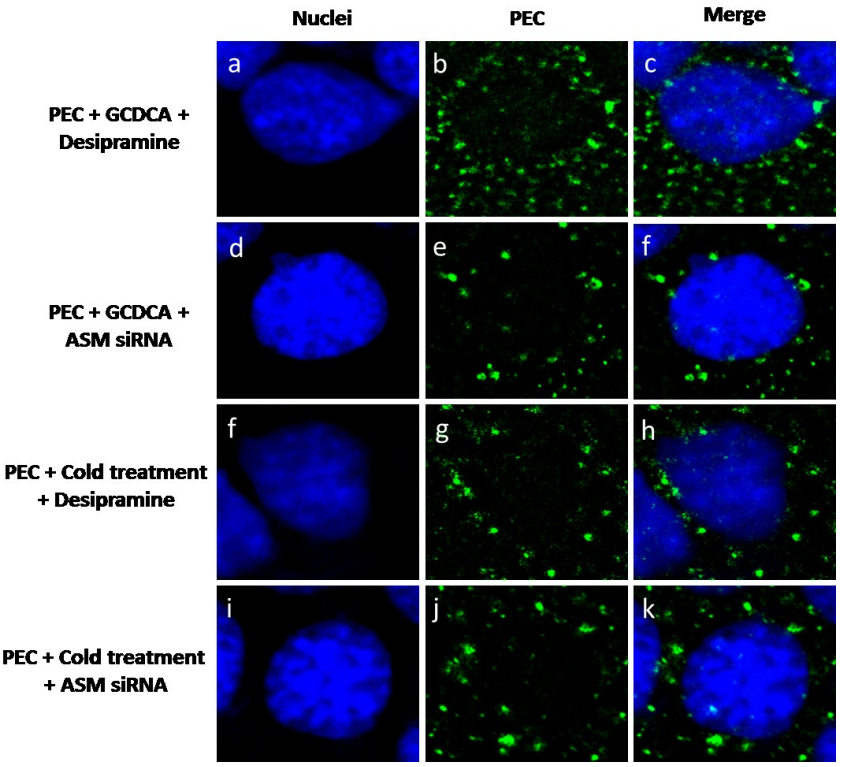


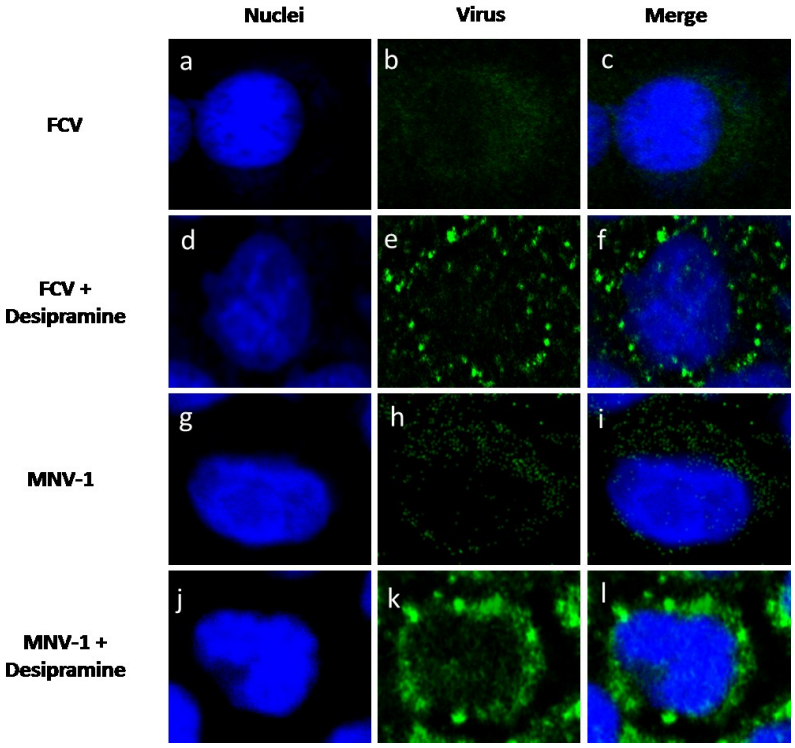
Figure 4-7. Effects of ASM inhibition on the PEC, FCV or MNV-1 escape from endosomes during viral entry.

A). LLC-PK cells were inoculated with PEC (50 MOI) with GCDA (100 µM, 37 °C for 1 h) or cold treatment (4 °C for 1 h) with desipramine (45µM) or ASM siRNA (200 nM) as described above. After additional incubation at 37 °C for 1 h, cells were fixed for confocal microscopy. B). CRFK or RAW264.7 cells were inoculated with either FCV or MNV-1 with or without desipramine (45 µM) as described above. Cells were fixed and probed with PEC, FCV or MNV-1 antibody with the secondary antibody (green) for virus as described above or in text. Merged images of virus (green) with nuclei (blue) were prepared.

A.



B.


Nuclei
Virus
Merge
FCV
FCV + Desipramine
MNV-1
MNV-1 + Desipramine
a
b
c
d
e
f
g
h
i
j
k
l

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Chapter 5 - Conclusions and Future Directions

The family *Caliciviridae* contains viruses affecting humans and animals with infections ranging from acute gastroenteritis in humans and lethal systemic infections in cats to mild oral or non-symptomatic infections in animals. Noroviruses are the major cause of acute gastroenteritis outbreaks in humans and due to their low infectious dose and environmental stability they spread easily and contamination of food and water leads to outbreaks of gastroenteritis. Advances in norovirus research are hindered due to the inability to cultivate noroviruses in cell culture. Other studies have shown that the block for in vitro norovirus replication is at the level of entry or uncoating but the entry events of caliciviruses are not well understood till date. Most of our understanding on the entry events of caliciviruses is made from cultivable caliciviruses namely murine norovirus, feline calicivirus, porcine enteric calicivirus and Tulane virus. Understanding the host factors required and the events during calicivirus entry will help in the development of better models to cultivate human noroviruses and maybe other enteric viruses also.

Early events in virus replication include receptor binding, internalization, trafficking, uncoating and release of viral genome to the sites of replication. Following internalization and during trafficking the capsid undergoes changes that are triggered by receptor binding, exposure to acidic pH, endosomal proteolytic enzymes which lead to events such as capsid destabilization, penetration and uncoating finally releasing its genome. Viruses use the host factors to co-ordinate their movement into the cell and to ensure uncoating and release of their genome occurs at the right time and at the right place. The study presented in this dissertation examines the entry events during calicivirus replication and also identifies the host factors required by caliciviruses for entry, uncoating and final endosomal escape.

In our first study we investigated the role of bile acid in PEC replication. We reported the growth kinetics of PEC in presence and absence of bile acid and showed that bile acid is required only during the early stages of PEC replication. Further we also showed that transfection of PEC viral RNA produces infectious viruses without the requirement of bile acids. Confocal examination of entry events of PEC in LLC-PK cells showed that PEC was able to enter LLC-PK cells and traffic to late endosomes without the requirement of bile acid but viral escape from the late endosomes into cytoplasm required the presence of bile acid. Further bile acid transporters NTCP and ASBT were shown to be involved in bile acid-mediated PEC replication, but bile acid receptors FXR and TGR5 did not have any effect in PEC replication.

The second study identified the importance of endosomal acidification and cathepsin L activity during the entry stages of caliciviruses. We found that inhibition of cathepsin L activity but not cathepsin B and inhibition of endosomal acidification significantly reduced PEC, FCV and MNV-1 replication. Further, incubation of purified virus suspension with recombinant cathepsin L lead to cleavage of VP1 protein of FCV and MNV-1 and VP2 protein of PEC as analyzed by Western blotting. Computational prediction and mutation experiments using reverse genetics for FCV showed F463, L472, and F486 as putative cathepsin L cleavage sites in the VP1 of FCV. Confocal examination showed retention of PEC and MNV-1 in endosomes in the presence of cathepsin L inhibitors and chloroquine during entry stages. Results from this study suggest the crucial role of cathepsin L in calicivirus replication.

The third study further investigated the mechanism of bile acid-mediated PEC replication. Our initial experiments showed that cold-shock treatment induced PEC replication without the requirement of bile acid. Detailed examination using confocal microscopy and specific inhibitors of acid sphingomyelinase (ASM) we showed that cold-shock treatment and

bile acid induced ceramide formation on endosomal membranes through the activity of ASM. Extrapolating these results to FCV and MNV-1, we demonstrated that infection of susceptible cells with FCV and MNV-1 induced ceramide formation and it was also shown that viral replication was not required to induce ceramide formation. Consistent with these results quantification of viral replication showed requirement of ASM activity for productive replication of PEC, FCV and MNV-1. Confocal examination of virus trafficking showed accumulation of viral particles in endosomes following pharmacological inhibition of ASM activity indicating ceramide formation on endosomal membranes was required for endosomal escape of PEC, FCV and MNV-1.

Our studies have revealed important events including endosomal escape of virus, cathepsin L activity and ceramide formation during calicivirus entry. The identified host factors could be potential therapeutic targets for calicivirus infections. Human noroviruses have remained uncultivable to date. Results from our studies will provide new directions for development of better in vitro models for human noroviruses. Further identification of a probable protein receptor/coreceptor and a more detailed study of entry events using human noroviruses will help in understanding the requirements for their productive replication and may also lead to development of better therapeutics for norovirus infections.

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