Published in final edited form as:

Arch Virol. 2014 February ; 159(2): 257–266. doi:10.1007/s00705-013-1806-4.

Failure of propagation of human norovirus in intestinal epithelial cells with microvilli grown in three-dimensional cultures

Sayaka Takanashi^{1,2,3}, Linda J. Saif^{1,2}, John H. Hughes⁴, Tea Meulia⁵, Kwonil Jung^{1,2}, Kelly A. Scheuer^{1,2}, and Qihong Wang^{1,2,*}

¹Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, Ohio, USA

²Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, 1900 Coffey Road, Columbus, Ohio, USA

³Department of Developmental Medical Sciences, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

⁴Department of Molecular Virology, Immunology and Medical Genetics, College of Medicine, The Ohio State University, 700 Children's Drive, Columbus, Ohio, USA

⁵Molecular and Cellular Imaging Center, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue Wooster, Ohio, USA

Abstract

Human noroviruses (HuNoVs) are a leading cause of acute gastroenteritis. Establishment of a cell culture system for *in vitro* HuNoV growth remains challenging. Replication of HuNoVs in human intestinal cell lines (INT-407 and Caco-2) that differentiate to produce microvilli in rotation wall vessel (RWV) three-dimensional cultures has been reported (Straub et al., *Emerg Infect Dis* 13:396–403 2007, *J Water Health* 9:225–240 2011, and *Water Sci Technol* 67:863–868 2013). We used a similar RWV system, intestinal cell lines, and the same (Genogroup [G] I.1) plus additional (GII.4 and GII.12) HuNoV strains to test the system's reproducibility and to expand the earlier findings. Apical microvilli were observed on the surface of both cell lines by light and electron microscopy. However, none of the cell types tested resulted in productive viral replication of any of the HuNoV strains, as confirmed by plateau or declining viral RNA titers in the supernatants and cell lysates of HuNoV-infected cells, determined by real-time reverse transcription PCR. These trends were the same when culture supplements were added that have been reported to be effective for replication of other fastidious enteric viruses *in vitro*. Additionally, by confocal microscopy and orthoslice analysis, viral capsid proteins were mainly observed above the actin filament signals, which suggested that the majority of viral antigens were on the cell surface. We conclude that even intestinal cells displaying microvilli were not sufficient to support HuNoV replication under the conditions tested.

Introduction

Acute gastroenteritis remains a major disease burden worldwide, with an estimated 1.7 billion episodes annually [22, 25]. Human noroviruses (HuNoVs) are the leading cause of

*Corresponding author. Mailing address: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, Ohio 44691, USA. Phone: (330) 263-3744. Fax: (330) 263-3677. wang.655@osu.edu.

Conflict of interest

The authors declare that they have no conflict of interest.

food-borne gastroenteritis in the US, accounting for 58% of all cases [38]. It has been estimated that HuNoVs cause up to 200,000 deaths of children <5 years of age in developing countries annually [33]. HuNoVs belong to the family *Caliciviridae* and have a single-stranded, positive-sense RNA genome. Noroviruses are genetically diverse and are classified into five genogroups (GI–GV). Viruses within each genogroup are further divided into genotypes based on sequence similarity. GI, GII and GIV are associated with human infections [50]. There are more than 30 genotypes, with the GI.1/Norwalk strain being the prototype and GII.4 being the dominant genotype worldwide, responsible for 70–80% of HuNoV outbreaks [14]. The lack of an efficient cell culture system for HuNoVs has greatly hampered an understanding of their life cycle and the development of antiviral drugs and vaccines [10, 13].

Human volunteer studies have provided information on HuNoV pathogenesis, including the following observations: (1) The primary site for viral replication was the small intestine, with blunting and shortening of villi, which was observed by intestinal biopsy from individuals inoculated with GI.1/Norwalk virus [9, 13]. (2) Individuals who have inactive α (1, 2) fucosyltransferase (*FUT2*) genes were resistant to Norwalk virus infection [19, 24, 45]. Those who have active *FUT2* are called secretors (Se^+), expressing histo-blood group antigens (HBGAs) not only on red blood cells but also on the surface of mucosal epithelial cells and in body fluids such as saliva [45]. Thus, HBGAs are considered receptors/co-receptors for Norwalk virus. Although HBGAs are present in the human colorectal adenocarcinoma cell line Caco-2 [1, 16, 17], the use of this cell line in conventional cultures has failed to support HuNoV replication [10]. Because human volunteer studies are difficult and expensive, continuous efforts are being made to establish an *in vitro* cell culture system for HuNoVs. A novel approach was reported recently by Lay et al. [23] by using primary macrophages and dendritic cells from peripheral blood mononuclear cells of Se^+ individuals. However, Norwalk virus failed to replicate in these cells and showed a different cell tropism from that of murine NoV, which replicates in mouse macrophages and dendritic cells [23, 47].

Most publications reporting attempts to establish a cell culture system for HuNoVs have shown negative results [10, 23, 28, 30, 31, 44], except for sequential papers from a single group who reported successful HuNoV replication in a three-dimensional (3-D) culture model using human intestinal epithelial cell lines, INT-407 and Caco-2 cells [39–41]. The authors reported that the critical factor for HuNoV replication in these cells was having apical microvillus structures [41]. In the present study, we report contradictory results using a similar 3-D culture system with the same GI.1/Norwalk virus strain as well as two additional HuNoV strains, GII.4/HS194, belonging to the dominant genotype [12], and GII.12/HS206, an emerging genotype [38].

Materials and methods

3-D culture of human intestinal cell lines

The human epithelial cell line INT-407 (ATCC #CCL-6, HeLa contaminant) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units of penicillin, 100 μ g of streptomycin, and 0.25 μ g of amphotericin B per ml). The human colorectal adenocarcinoma cell line Caco-2 (ATCC #HTB-37) was cultured in minimal essential medium (MEM) supplemented with 20% FBS, 1% non-essential amino acids, 1 mM sodium pyruvate, and antibiotics. Fetal bovine serum was from Thermo Scientific (Waltham, MA, USA), and the other reagents were from Invitrogen (Carlsbad, CA, USA). Three-D cultures were established according to the techniques described previously [27, 39, 40]. Briefly, a total of $\sim 2 \times 10^6$ cells were loaded into a rotating wall vessel (RWV) bioreactor (Synthecon, Inc., Houston, TX, USA).

containing 250 mg of Cytodex 2-trimethyl-2-hydroxyaminopropyl-coated microcarrier beads or Cytodex 3 porous collagen I-coated microcarrier beads (Sigma, St. Louis, MO, USA) and incubated at 37°C in 5% CO₂. Vessel rotation was initiated at 15 rotations per minute, and the rotation speed was increased as aggregate size increased to maintain cell aggregates in free-fall suspension. Partial growth medium was replaced every 48–72 hours. Cell aggregates were harvested at 21–28 days after seeding using a wide-bore pipette and rinsed three times with 0.01 M phosphate-buffered saline (PBS, pH 7.2). After confirming a viability of greater than 95% by trypan blue exclusion, cell aggregates were used for HuNoV replication trials in 24-well tissue culture plates (50 µl, with 2–3 aggregates/well, ~5,000 cells/well). Basically, we aimed to follow the same approach and set-up described by Straub and colleagues [39, 40] except that we attempted using additional materials such as Cytodex 2 microcarrier beads, additional HuNoV genotype strains as inocula, culture medium supplements and an additional inoculation method described in the following section.

HuNoV stocks and infection trials

Three strains of HuNoVs were used in infection trials: (1) Human-volunteer-passaged Hu/NoV/GI.1/Norwalk/1968/US (GenBank accession number M87661), which was generously provided by Dr. Mary Estes of Baylor College of Medicine (Houston, Texas), (2) Hu/NoV/GII.4/HS194/2010/US (GenBank accession number GU325839) [43], and (3) Hu/NoV/GII.12/HS206/2010/US (GenBank accession number HQ664990) [43]. The original stool samples were suspended in sterile MEM at 10% (w/v) and were processed by vigorous vortexing, centrifugation at 1,800 × g for 30 min, and filtration through 0.2-µm-pore-size filters. These samples were screened for common diarrheal viruses, including NoV GI and GII, human rotavirus groups A, B and C, sapovirus, astrovirus, and adenovirus by RT-PCR or PCR assays as described previously [21, 48, 49]. The suspensions were aliquoted to individual tubes, stored at –70°C, and thawed only once for infection trials. The viral RNA titer of each sample was determined using real-time reverse transcription PCR (RT-qPCR). For virus inoculation, approximately 4 log₁₀ genomic equivalents (GE)/cell (~ 5 × 10⁷ GE/well) were applied in 100 µl of sample for a total volume of 150 µl/well, as used by Straub et al. [40] and indicated to be important to allow maximal viral contact with the cell suspension. Serum-free media were used as mock controls. Plates were gently rocked every 15 minutes during the 1-hour absorption phase. Subsequently, all wells were overlaid with an additional 1 ml of fresh serum-free medium. Plates were incubated at 37°C in 5% CO₂. At 0, 24, 48, 72, and 96 hours post-inoculation (hpi), culture supernatants were harvested for viral RNA assays. At 24 hpi, plates were fixed and examined for viral antigens by immunofluorescence assays. Three wells were allocated for each condition per experiment, and experiments were repeated at least twice. For some trials, direct inoculation to the RWV was performed, as has been shown to be effective for rhinovirus [26] and hepatitis C virus replication [37]. In addition, culture supplements that have been effective for the growth of fastidious enteric viruses *in vitro* were also added at the time of inoculation to determine their influence on supporting HuNoV replication. These supplements included 2% intestinal content preparations (IC) from gnotobiotic pigs, 100 µM glycochenodeoxycholic acid (GCDCA, Sigma, St. Louis, MO, USA) [6, 12], and 1 µM simvastatin (Zocor; Merck and Co, Inc., Whitehouse station, NJ) [7, 20, 35].

Viral RNA extraction and TaqMan RT-qPCR

RNA was extracted from samples by using a MagMAX Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) or an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. All of the NoV-positive samples were titrated in duplicate by RT-qPCR [21] with a standard curve generated from a serial dilution of an RNA transcript, which was generously provided by Dr. Estes (Prep1397FN, GI.1/Norwalk

virus ORF1/2) for NoV GI and plasmid DNA carrying the GII.4/HS66-specific COG2F/2R amplicon for NoV GII.

Production of guinea pig hyperimmune serum

Hyperimmune serum to virus-like particles (VLPs) of GII.12/HS206 strain [43] was prepared according to an approved IACUC protocol as described previously [8].

Microscopic analysis

The cellular histology of mature 3-D aggregates was monitored using an Olympus inverted microscope system (Olympus, Tokyo, Japan) before and after the inoculation trials. To assess the subcellular pathology, cell aggregates harvested from a vessel were prepared and examined as described previously [36, 39, 40]. Samples were fixed in 3% glutaraldehyde-1% paraformaldehyde in 0.1 M potassium phosphate buffer (PPB; pH 7.2) for 2 to 3 hours at room temperature. Samples were washed three times with excess PPB and then post-fixed with 1% osmium tetroxide and 1% uranyl acetate in distilled water for 1 h at room temperature. After dehydration in a graded ethanol-propylene oxide series, samples were embedded in EM Bed-812 resin (Electron Microscopy Sciences, Hartfield PA, Cat. #14120). Two- μ m sections were collected, stained with 0.5% toluidine blue in 25% ethanol and 0.1% sodium carbonate solution, viewed with a Leica DM IRB light microscope (Leica, Solms, Germany), and imaged with a Retiga 2000R digital camera (Scientifica, East Sussex, UK). On 200-nm mesh copper grids, 70-nm thin sections were collected, stained with 2% aqueous uranyl acetate and Reynolds' lead citrate stain, and viewed using a Hitachi H-7500 (Hitachi, Tokyo, Japan) transmission electron microscope. Images were captured with an Optronics QuantiFire model S99835 digital camera [8]. For analysis by confocal microscopy, cell aggregates harvested from a cell culture plate at 24 hpi were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M PPB for 2 h at room temperature, washed four times with PPB, and quenched with PPB containing 50 mM glycine for 1 h at room temperature or kept overnight at 4°C. After permeabilization with 0.1% Triton X-100 in PBS for 1 h at room temperature, cells were washed with PBS, blocked with PBS containing 2% bovine serum albumin and 5% normal goat serum for 30 min at room temperature, and incubated with genotype-specific primary antibodies overnight at 4°C in incubation buffer (10 mM PPB containing 150 mM NaCl, 10 mM sodium azide, and 0.2% bovine serum albumin). The primary antibodies used were anti-GI.1/Norwalk polyclonal antibody produced in a rabbit, anti-GII.4/Houston monoclonal antibody produced in a mouse (both kindly provided by Dr. Estes), anti-GII.4/HS194 polyclonal antibody produced in a guinea pig [20] and anti-GII.12/HS206 polyclonal antibody produced in a guinea pig at dilutions of 1:800, 1:500, 1:800, and 1:800, respectively. After six washes with PBS, incubation with a 1:600 dilution of a secondary antibody (goat anti-rabbit IgG labeled with Alexa Fluor 488, goat anti-mouse IgG labeled with Alexa Fluor 488, or goat anti-guinea-pig IgG labeled with Alexa Fluor 543) was performed overnight at 4°C. All of the secondary antibodies were from Invitrogen. Samples were counterstained with the nuclear stain diamidino-2-phenylindole (DAPI), giving a blue color, and the cytoskeleton stain Alexa Fluor 633-labeled phalloidin, producing a cyan color (both from Invitrogen). Pre-immunization sera from the same guinea pig that produced the polyclonal antibody against HS194 or HS206 were included as a negative control. Samples were examined using a Leica model TCS SP5 laser scanning confocal microscope (Leica, Wetzlar, Germany).

Results

Morphology of cells grown in the 3-D culture system

To investigate the morphological characteristics of mature intestinal cell aggregates, we observed them microscopically after being cultured for 3–4 weeks and harvested from a

RWV. Both INT-407 cells and Caco-2 cells adhered efficiently to the microcarrier beads and formed 3-D tissue-like aggregates, ranging from 2 to 4 mm in diameter (Fig. 1A and E). Each aggregate possessed ~10 microcarrier beads and multilayers of cells (Fig. 1B and F). The surface of each cell aggregate had apical microvilli that were clearly seen with both light ($\times 1,000$) (Fig. 1C and G) and electron microscopes ($\times 10,000$) (Fig. 1D and H). Compared to the INT-407 cells, the microvilli of the Caco-2 cells were more numerous and slightly longer, and morphologically, they more closely resembled the brush border of human intestine epithelial cells [34].

Immunofluorescence assay

To determine if HuNoV proteins were present in the inoculated cell aggregates, immunofluorescence analysis was performed at 24 hpi for both Caco-2 and INT-407 cell lines inoculated with GI.1/Norwalk, GII.4/HS194, or GII.12/HS206. All of the stool samples were negative for other common diarrheal viruses (human rotavirus groups A, B and C, sapovirus, astrovirus, and adenovirus) as confirmed by RT-PCR or PCR. We performed comprehensive analysis in combinations of two cell lines inoculated with one of three genotypes and observed that ~1% of the cells stained positive for viral capsid protein (VP1) in INT-407 cells inoculated with GII.4/HS194 (Fig. 2, left) and GII.12/HS206 (Fig. 2, middle), and in Caco-2 cells inoculated with GI.1/Norwalk (Fig. 2, right). By co-localization analysis with DAPI staining, the locations of these positive signals were found to be outside of the nucleus. To further investigate the location of the viral antigen-positive signals, we performed an orthoslice analysis by confocal microscopy. Although a few signals appeared to be in the cells (under the cytoskeleton-actin filaments), the majority of the signals were outside of the cells. A typical case was captured in INT-407 cells inoculated with GII.12/HS206 strain (Fig. 3), showing the VP1 signal (red) above the cytoskeleton signal (cyan). These results suggested that the viral capsid protein VP1 was mainly located on the surface of the cells.

Monitoring of viral RNA by TaqMan RT-qPCR

Next, we examined whether viral RNA replication could be observed by titrating viral RNA in the supernatants (released and input virus) and cell lysates (cell-associated and intracellular virus) at multiple time points after inoculation with $\sim 10^7$ GE/ml of HuNoV. Typical results from multiple trials for each of the three tested genotypes of HuNoVs in the Caco-2 and INT-407 cell lines are shown in Figure 4. We consistently detected HuNoV RNA both in supernatants ($\sim 10^5$ – 10^7 GE/ml) and cell lysate samples ($\sim 10^1$ – 10^3 GE/ μ g of total RNA from cell lysates), but never in the mock control samples (medium only; data not shown). However, the viral RNA levels did not increase at any time points assayed, remaining at a plateau or declining, and the viral RNA in the supernatants did not surpass the input HuNoV RNA level. Additional trials with the direct inoculation method and culture supplements in the medium are summarized in Table 1. There was no significant increase in viral RNA at 24 hpi for any combination tested. These data suggest that although some HuNoVs might have attached or entered the cells after inoculation, they did not productively replicate to show dynamically increasing viral RNA titers.

Discussion

For more than four decades after the discovery of HuNoVs, an understanding of their life cycle and the development of antiviral drugs have been hampered due to lack of an efficient cell culture system to propagate these viruses. Significant efforts have been made to overcome this obstacle. Representative studies were summarized by Duizer *et al.* [10], who tested HuNoV cultivation with more than 25 human and animal cell lines in various *in vitro* culture conditions but failed to replicate the virus. In 2007, Straub *et al.* [39] reported, for

the first time, that HuNoV replicated in INT-407 cells using a 3-D RWV cell culture system. Although several researchers had questioned the validity of this study [5, 30], we were motivated to re-evaluate the significance of the 3-D RWV system for two reasons; First, the 3-D RWV system was confirmed to be a more physiologically relevant cell culture system compared to conventional 2-D cultures, and it has been successfully used to study the pathogenesis of other enteric bacterial and viral pathogens, including salmonella [18, 29], hepatitis C virus [37], and rhinovirus [26]. Second, a confirmatory paper published by Straub *et al.* [40] reported that Caco-2 cells cultured in a 3-D RWV also supported HuNoV replication.

In our study, we clearly showed the organoid structure of INT-407 and Caco-2 cell aggregates grown in the 3-D RWV for 21–28 days and confirmed the presence of the apical microvilli, especially on Caco-2 cells. These were described as critical features for the success of HuNoV propagation in a brush-border-producing Caco-2 cell line [40, 41]. However, in spite of the presence of microvilli on the cell aggregates, we did not observe measurable increases in viral RNA after inoculation with three different genotypes of HuNoVs. We attempted to monitor dynamic changes of viral RNA levels, which would be expected in the case of productive viral infection, by sampling additional time points (2-, 4-, 6-, 8-, 120-, 144-, 168-hpi) in some trials, but the viral titers resulted in a plateau or declined and were similar to that illustrated in Figure 4. These results provide clear evidence that in spite of the microvilli structures produced by intestinal cells grown in 3-D RWV, they failed to support the replication of the HuNoVs tested. This is in concordance with a recent paper [28] showing no increase of the viral RNA titer in Norwalk-virus-inoculated INT-407 cells up to 96 hpi.

The GI.1/Norwalk-positive stool samples provided by Dr. Estes enabled us to compare the same HuNoV strain as inoculum in our HuNoV cell culture project with that conducted in different laboratories. The infectivity of these samples was well described in human volunteer studies [2–4]. Similarly, the infectivity of the other two genotype samples, namely GII.4/HS194 and GII.12/HS206, was confirmed by the studies using the gnotobiotic (Gn) pig model for HuNoV replication [20, 43]. The selection of well-characterized samples enabled us to use strain-specific antibodies, which greatly improved the sensitivity of the immunological assays. We detected ~1% of the cells staining positive at 24 hpi in both Caco-2 and INT-407 cell lines (Fig. 2). The pattern of the signals, especially in Caco-2 cells inoculated with GI.1 shown in Figure 2, was unexpected, as we observed a whole cell surface labeled with antibody. There are two possible explanations for this configuration of signals. The first is localized viral replication, which is theoretically possible but was not supported by the real-time RT-PCR results. The second possibility is that the beating of microvilli on these polarized cells has caused the localized accumulation of inoculum virus without infection. This possibility was partially supported by the result of our pilot experiment using heat-inactivated (56°C, 1 hour) virus as inoculum, which occasionally showed accumulated signals in a single cell (data not shown). This possibility could be further tested by specific labeling of microvilli to investigate the locational relationship between viral antigen and microvilli. By orthoslice analysis, we showed that the positive signals remained on the surface of the inoculated cells, suggesting that the entry of the virus into the cells may have been blocked (Fig. 3). Strong expression of HBGA H1 and H2 antigens and weak expression of H1 antigen was observed on the surface of Caco-2 and INT-407 cells, respectively (data not shown), and this is in concordance with previous studies [1, 15, 28]. Guix *et al.* [15] reported that overexpression of the *FUT2* gene, which determines the secretor phenotype of HBGA, in human hepatoma Huh7 cells enhanced Norwalk virus attachment, but entry and replication were not observed. Their report and our observations suggest that some steps other than viral attachment may have been hampered, precluding productive viral replication in the cell lines tested.

By light and electron microscopic analysis, extensive attempts were made to document the presence of HuNoV particles and cytopathic effects (CPE) described by Straub *et al.* [39, 40], such as vacuolization in the cytoplasm and shortening of microvilli. However, our observations were contradictory to their reports, i.e., neither HuNoV particles nor CPE were observed in the inoculated cells at 24 hpi (data not shown). The contrary findings could be due to different experimental conditions, such as different cell passages of INT-407 and Caco-2 cells, a different clone of Caco-2 cells, and different passages of GI.1/Norwalk virus used as inoculum. Nevertheless, the propagation of HuNoVs is still extremely difficult, even in well-established labs.

We also explored other possibility of using the 3-D RWV system to promote HuNoV replication, which was not examined by Straub's group. These attempts included the following: (1) direct virus inoculation onto cells and cell aggregates inside RWV, which was effective with rhinovirus [26] and HCV replication [37]; (2) including culture supplements that were effective for the growth of fastidious enteric viruses *in vitro* (IC from non-infected Gn pigs [12, 32], bile acid GCDCA [6], and simvastatin [7, 20, 35]); and (3) blind passage by retaining one fifth of the inoculated cells from the previous RWV to the following one. None of the trials showed evidence of HuNoV replication (Table 1).

Collectively, the INT-407 and Caco-2 cells grew and differentiated to produce apical microvilli in 3-D RWV, but these cells did not support the replication of any of the three HuNoV strains. These results are contradictory to the previous reports [39, 40]. Future efforts could target the use of human primary intestinal cells and human small-intestinal organs to attempt to grow HuNoVs *in vitro*. The intrinsic ethical issues in these methods could be overcome by using an intestinal organoid model derived from human pluripotent stem cells, as has been done successfully in a rotavirus infection study [11], and an induced pluripotent stem cell technology that might enable the production of a small-intestinal counterpart [42].

Acknowledgments

We thank Juliette Hanson and Kingsly Berlin for assistance with animal care, Andrea Kaszas for assistance with microscopic examination, and Tomoichiro Oka for helpful discussions. This work was supported by a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (R21 AI081009-2). Salaries and research support were provided by state and federal funds provided to the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University.

References

1. Amano J, Oshima M. Expression of the H type 1 blood group antigen during enterocytic differentiation of Caco-2 cells. *J Biol Chem.* 1999; 274:21209–21216. [PubMed: 10409676]
2. Atmar RL, Opekun AR, Gilger MA, Estes MK, Crawford SE, Neill FH, Graham DY. Norwalk virus shedding after experimental human infection. *Emerg Infect Dis.* 2008; 14:1553–1557. [PubMed: 18826818]
3. Atmar RL. Noroviruses - State of the Art. *Food Environ Virol.* 2010; 2:117–126. [PubMed: 20814448]
4. Atmar RL, Estes MK. Norovirus vaccine development: next steps. *Expert Rev Vaccines.* 2012; 11:1023–1025. [PubMed: 23151158]
5. Chan MC, Wong YP, Leung WK. Cell culture assay for human noroviruses. *Emerg Infect Dis.* 2007; 13:1117. author reply 1117–1118. [PubMed: 18214197]
6. Chang KO, Sosnovtsev SV, Belliot G, Kim Y, Saif LJ, Green KY. Bile acids are essential for porcine enteric calicivirus replication in association with down-regulation of signal transducer and activator of transcription 1. *Proc Natl Acad Sci U S A.* 2004; 101:8733–8738. [PubMed: 15161971]

7. Chang KO. Role of cholesterol pathways in norovirus replication. *J Virol.* 2009; 83:8587–8595. [PubMed: 19515767]
8. Cheetham S, Souza M, Meulia T, Grimes S, Han MG, Saif LJ. Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. *J Virol.* 2006; 80:10372–10381. [PubMed: 17041218]
9. Dolin R, Blacklow NR, Malmgren RA, Chanock RM. Establishment of human fetal intestinal organ cultures for growth of viruses. *J Infect Dis.* 1970; 122:227–231. [PubMed: 4317758]
10. Duizer E, Schwab KJ, Neill FH, Atmar RL, Koopmans MP, Estes MK. Laboratory efforts to cultivate noroviruses. *J Gen Virol.* 2004; 85:79–87. [PubMed: 14718622]
11. Finkbeiner SR, Zeng XL, Utama B, Atmar RL, Shroyer NF, Estes MK. Stem cell-derived human intestinal organoids as an infection model for rotaviruses. *MBio.* 2012; 3:e00159–e00112. [PubMed: 22761392]
12. Flynn WT, Saif LJ. Serial propagation of porcine enteric calicivirus-like virus in primary porcine kidney cell cultures. *J Clin Microbiol.* 1988; 26:206–212. [PubMed: 2830305]
13. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. *N Engl J Med.* 2009; 361:1776–1785. [PubMed: 19864676]
14. Green K. The noroviruses. 2007
15. Guix S, Asanaka M, Katayama K, Crawford SE, Neill FH, Atmar RL, Estes MK. Norwalk virus RNA is infectious in mammalian cells. *J Virol.* 2007; 81:12238–12248. [PubMed: 17855551]
16. Harrington PR, Lindesmith L, Yount B, Moe CL, Baric RS. Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice. *J Virol.* 2002; 76:12335–12343. [PubMed: 12414974]
17. Harrington PR, Vinje J, Moe CL, Baric RS. Norovirus capture with histo-blood group antigens reveals novel virus-ligand interactions. *J Virol.* 2004; 78:3035–3045. [PubMed: 14990722]
18. Honer zu Bentrop K, Ramamurthy R, Ott CM, Emami K, Nelman-Gonzalez M, Wilson JW, Richter EG, Goodwin TJ, Alexander JS, Pierson DL, Pellis N, Buchanan KL, Nickerson CA. Three-dimensional organotypic models of human colonic epithelium to study the early stages of enteric salmonellosis. *Microbes Infect.* 2006; 8:1813–1825. [PubMed: 16730210]
19. Hutson AM, Atmar RL, Graham DY, Estes MK. Norwalk virus infection and disease is associated with ABO histo-blood group type. *J Infect Dis.* 2002; 185:1335–1337. [PubMed: 12001052]
20. Jung K, Wang Q, Kim Y, Scheuer K, Zhang Z, Shen Q, Chang KO, Saif LJ. The effects of simvastatin or interferon-alpha on infectivity of human norovirus using a gnotobiotic pig model for the study of antivirals. *PLoS One.* 2012; 7:e41619. [PubMed: 22911825]
21. Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J Clin Microbiol.* 2003; 41:1548–1557. [PubMed: 12682144]
22. Klein EJ, Boster DR, Stapp JR, Wells JG, Qin X, Clausen CR, Sverdlow DL, Braden CR, Tarr PI. Diarrhea etiology in a Children's Hospital Emergency Department: a prospective cohort study. *Clin Infect Dis.* 2006; 43:807–813. [PubMed: 16941358]
23. Lay MK, Atmar RL, Guix S, Bharadwaj U, He H, Neill FH, Sastry KJ, Yao Q, Estes MK. Norwalk virus does not replicate in human macrophages or dendritic cells derived from the peripheral blood of susceptible humans. *Virology.* 2010; 406:1–11. [PubMed: 20667573]
24. Lindesmith L, Moe C, Marionneau S, Ruvoen N, Jiang X, Lindblad L, Stewart P, LePendou J, Baric R. Human susceptibility and resistance to Norwalk virus infection. *Nat Med.* 2003; 9:548–553. [PubMed: 12692541]
25. Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, Rudan I, Campbell H, Cibulskis R, Li M, Mathers C, Black RE. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet.* 2012; 379:2151–2161. [PubMed: 22579125]
26. Long JP, Pierson S, Hughes JH. Rhinovirus replication in HeLa cells cultured under conditions of simulated microgravity. *Aviat Space Environ Med.* 1998; 69:851–856. [PubMed: 9737755]
27. Long JP, Hughes JH. Epstein-Barr virus latently infected cells are selectively deleted in simulated-microgravity cultures. *In Vitro Cell Dev Biol Anim.* 2001; 37:223–230. [PubMed: 11409687]

28. Melissa M, Herbst-Kralovetz ALR, Margarita K Lay, Brooke E Hjelm, Alice N Bolick, Shameema S Sarker, Robert L Atmar, David H Kingsley, Charles J Arntzen, Mary K Estes, Cheryl A Nickerson. Lack of Norovirus Replication and Histo-Blood Group Antigen Expression in 3-Dimensional Intestinal Epithelial Cells. *Emerg Infect Dis.* 2013; Volume 19
29. Nickerson CA, Goodwin TJ, Terlonge J, Ott CM, Buchanan KL, Uicker WC, Emami K, LeBlanc CL, Ramamurthy R, Clarke MS, Vanderburg CR, Hammond T, Pierson DL. Three-dimensional tissue assemblies: novel models for the study of *Salmonella enterica* serovar Typhimurium pathogenesis. *Infect Immun.* 2001; 69:7106–7120. [PubMed: 11598087]
30. Papafragkou E, Hewitt J, Park G, Straub T, Greening G, Vinje J. Challenges of Culturing Human Norovirus in a 3-D Organoid Cell Culture Model. In *Proceedings of the ASM 109th General Meeting*: 09-GM-A-2871. 2009
31. Papafragkou E, Hewitt J, Park GW, Greening G, Vinje J. Challenges of culturing human norovirus in three-dimensional organoid intestinal cell culture models. *PLoS One.* 2013; 8:e63485. [PubMed: 23755105]
32. Parwani AV, Flynn WT, Gadfield KL, Saif LJ. Serial propagation of porcine enteric calicivirus in a continuous cell line. Effect of medium supplementation with intestinal contents or enzymes. *Arch Virol.* 1991; 120:115–122. [PubMed: 1929875]
33. Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinje J, Parashar UD. Systematic literature review of role of noroviruses in sporadic gastroenteritis. *Emerg Infect Dis.* 2008; 14:1224–1231. [PubMed: 18680645]
34. Peterson MD, Mooseker MS. Characterization of the enterocyte-like brush border cytoskeleton of the C2BBE clones of the human intestinal cell line, Caco-2. *J Cell Sci.* 1992; 102(Pt 3):581–600. [PubMed: 1506435]
35. Rondy M, Koopmans M, Rotsaert C, Van Loon T, Beljaars B, Van Dijk G, Siebenga J, Svraka S, Rossen JW, Teunis P, Van Pelt W, Verhoef L. Norovirus disease associated with excess mortality and use of statins: a retrospective cohort study of an outbreak following a pilgrimage to Lourdes. *Epidemiol Infect.* 2011; 139:453–463. [PubMed: 20492742]
36. Sainz B Jr, Barretto N, Uprichard SL. Hepatitis C virus infection in phenotypically distinct Huh7 cell lines. *PLoS One.* 2009; 4:e6561. [PubMed: 19668344]
37. Sainz B Jr, TenCate V, Uprichard SL. Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. *Virol J.* 2009; 6:103. [PubMed: 19604376]
38. Scallan E, Griffin PM, Angulo FJ, Tauxe RV, Hoekstra RM. Foodborne illness acquired in the United States--unspecified agents. *Emerg Infect Dis.* 2011; 17:16–22. [PubMed: 21192849]
39. Straub TM, Honer zu Bentrup K, Orosz-Coghlan P, Dohnalkova A, Mayer BK, Bartholomew RA, Valdez CO, Bruckner-Lea CJ, Gerba CP, Abbaszadegan M, Nickerson CA. In vitro cell culture infectivity assay for human noroviruses. *Emerg Infect Dis.* 2007; 13:396–403. [PubMed: 17552092]
40. Straub TM, Bartholomew RA, Valdez CO, Valentine NB, Dohnalkova A, Ozanich RM, Bruckner-Lea CJ, Call DR. Human norovirus infection of caco-2 cells grown as a three-dimensional tissue structure. *J Water Health.* 2011; 9:225–240. [PubMed: 21942189]
41. Straub TM, Hutchison JR, Bartholomew RA, Valdez CO, Valentine NB, Dohnalkova A, Ozanich RM, Bruckner-Lea CJ. Defining cell culture conditions to improve human norovirus infectivity assays. *Water Sci Technol.* 2013; 67:863–868. [PubMed: 23306266]
42. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006; 126:663–676. [PubMed: 16904174]
43. Takanashi S, Wang Q, Chen N, Shen Q, Jung K, Zhang Z, Yokoyama M, Lindesmith LC, Baric RS, Saif LJ. Characterization of emerging GII.g/GII.12 noroviruses from a gastroenteritis outbreak in the United States in 2010. *J Clin Microbiol.* 2011; 49:3234–3244. [PubMed: 21752978]
44. Tan M, Jiang X. Norovirus gastroenteritis, carbohydrate receptors, and animal models. *PLoS Pathog.* 2010; 6:e1000983. [PubMed: 20865168]
45. Thorven M, Grahn A, Hedlund KO, Johansson H, Wahlfrid C, Larson G, Svensson L. A homozygous nonsense mutation (428G-->A) in the human secretor (FUT2) gene provides resistance to symptomatic norovirus (GGII) infections. *J Virol.* 2005; 79:15351–15355. [PubMed: 16306606]

46. Wang Q, Zhang Z, Saif LJ. Stability of and attachment to lettuce by a culturable porcine sapovirus surrogate for human caliciviruses. *Appl Environ Microbiol.* 2012; 78:3932–3940. [PubMed: 22447610]
47. Wobus CE, Thackray LB, Virgin HWt. Murine norovirus: a model system to study norovirus biology and pathogenesis. *J Virol.* 2006; 80:5104–5112. [PubMed: 16698991]
48. Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H. Detection of norovirus (GI, GII), Sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. *J Virol Methods.* 2003; 114:37–44. [PubMed: 14599677]
49. Yan H NT, Phan TG, Okitsu S, Li Y, Ushijima H. Development of RT-multiplex PCR assay for detection of adenovirus and group A and C rotaviruses in diarrheal fecal specimens from children in China. *Kansenshogaku Zasshi.* 2004; 78:699–709. [PubMed: 15478645]
50. Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS. Norovirus classification and proposed strain nomenclature. *Virology.* 2006; 346:312–323. [PubMed: 16343580]

Figure 1. Morphology of INT-407 and Caco-2 cell aggregates cultured in a rotating wall vessel for 21 and 28 days, respectively. A-D, INT-407; E-H, Caco-2. A and E, phase contrast micrograph, 200 $\times$. B, C, F, and G, light micrographs of toluidine-blue-stained cell aggregates [B and F, 100 $\times$; *, ~175 μ m microcarrier bead. C and G, 1000 $\times$]. D and H, transmission electron micrograph of uranyl-acetate-stained cell aggregates, 10,000 $\times$. Arrows in C, D, G, and H indicate microvilli. The bold arrow in D indicates a tight junction. Bold arrowheads in D and H indicate mitochondria. "N" indicates the nucleus.

Figure 2.

Detection of HuNoV antigens at 24 hpi in INT-407 and Caco-2 cells by confocal microscopy. Green or red, HuNoV capsid proteins detected using mouse anti-GII.4 monoclonal antibody and rabbit anti-GI.1 polyclonal antibody for green, and guinea pig anti-GII.12 polyclonal antibody for red; cyan, cytoskeleton actin filaments using Alexa Fluor 633-labeled phalloidin; blue, nuclei stained with DAPI. Arrows indicate viral antigens.

Figure 3.

Orthoslice analysis of HuNoV GII.12/HS206-inoculated INT-407 cells by confocal microscopy. Typical Z-axis images of INT-407 cells incubated with GII.12/HS206 were constructed digitally in the XZ and YZ planes, shown as horizontal and vertical lines in the XY plane. Red, HuNoV capsid proteins detected using guinea pig anti-GII.12 polyclonal antibody; cyan, cytoskeleton actin filaments detected using Alexa Fluor633-labeled phalloidin; blue, nuclei stained using DAPI.

Figure 4.

HuNoV RNA levels at different times after inoculation in culture supernatants (lines expressed as GE/ml), and in cell lysates (bars expressed as GE/ug of total RNA). INT407 and Caco-2 cells were inoculated with three different HuNoV strains (GI.1/Norwalk, GII.4/HS194 and GII.12/HS206). RNA was titrated using RT-qPCR. Error bars indicate standard deviation (n=3).

Table 1

Summary of 3D cultures using Wall Bioreactors to propagate HuNoVs

Cell line	Type of microcarrier beads	HuNoV strain	Inoculation method ^a	Supplements ^b	Fold change in viral titer in the supernatant at 24 hpi compared to 0 hpi ^c
INT-407	Cytodex 3	GI.1/Norwalk	Plate	None	1.19±0.36
INT-407	Cytodex 3	GI.4/HS194	RWV	None	0.91±0.11
INT-407	Cytodex 3	GI.4/HS194	Plate	IC 2%	0.91±0.32
				simvastatin 1uM	0.8±0.15
				None	0.71±0.13
INT-407	Cytodex 3	GI.12/HS206	Plate	IC 2%	0.83±0.05
				simvastatin 1uM	0.87±0.08
				None	0.92±0.02
Caco-2	Cytodex 3	GI.1/Norwalk	Plate	None	1.14±0.02
Caco-2	Cytodex 3	GI.4/HS194	RWV	IC 2%	0.97±0.06
Caco-2	Cytodex 3	GI.4/HS194	Plate	IC 2%	0.61±0.05
				simvastatin 1uM	0.91±0.25
				None	0.77±0.16
Caco-2	Cytodex	GI.4/HS194	Plate	IC 2%	ND ^d
				GCDCA 100uM	ND
				None	0.18±0.21
Caco-2	Cytodex 3	GI.12/HS206	Plate	IC 2%	ND
				GCDCA 100uM	ND
				None	0.68±0.28
Caco-2	Cytodex 2	GI.12/HS206	RWV	None	0.83±0.26
Caco-2	Cytodex 3	GI.12/HS206	RWV	None	1.14±0.62
Caco-2 ^e	Cytodex 3	GI.12/HS206	RWV	None	0.54±0.27
Caco-2	Cytodex	GI.12/HS206	RWV	IC 2%	0.62±0.22
Caco-2	Cytodex 3	GI.12/HS206	Plate	IC 2%	0.86±0.21
				simvastatin 1uM	0.91±0.35

Cell line	Type of microcarrier beads	HuNoV strain	Inoculation method ^a	Supplements ^b	Fold change in viral titer in the supernatant at 24 hpi compared to 0 hpi ^c
				None	0.74±0.18

^a RWB: The inoculum was directly injected into the Rotating Wall-Bioreactor (RWB) (Sainz et al. 2009); Plate: The aggregated cells were transferred to the wells of a Plate for virus inoculation (Straub
^b IC: intestinal content preparations from gnotobiotic pigs.
^c Mean fold change±SEM
^d ND: not determined because either or both 0 hpi and 24 hpi were negative.
^e This passage was composed of cells from prior passage (1/5) and new cells(4/5).