OBSERVATION

Stem Cell-Derived Human Intestinal Organoids as an Infection Model for Rotaviruses

Stacy R. Finkbeiner,[a] Xi-Lei Zeng,[a] Budi Utama,[a] Robert L. Atmar,[a,b] Noah F. Shroyer,[c] and Mary K. Estes[a,b]

Department of Molecular Virology, Baylor College of Medicine, Houston, Texas, USA[a]; Department of Medicine, Baylor College of Medicine, Houston, Texas, USA[b]; and Division of Gastroenterology, Hepatology, and Nutrition, Cincinnati Children's Hospital, Cincinnati, Ohio, USA[c]

ABSTRACT **Directed differentiation of stem cell lines into intestine-like tissue called induced human intestinal organoids (iHIOs) is now possible (J. R. Spence, C. N. Mayhew, S. A. Rankin, M. F. Kuhar, J. E. Vallance, K. Tolle, E. E. Hoskins, V. V. Kalinichenko, S. I. Wells, A. M. Zorn, N. F. Shroyer, and J. M. Wells, Nature 470:105-109, 2011). We tested iHIOs as a new model to cultivate and study fecal viruses. Protocols for infection of iHIOs with a laboratory strain of rotavirus, simian SA11, were developed. Proof-of-principle analyses showed that iHIOs support replication of a gastrointestinal virus, rotavirus, on the basis of detection of nonstructural viral proteins (nonstructural protein 4 [NSP4] and NSP2) by immunofluorescence, increased levels of viral RNA by quantitative reverse transcription-PCR (qRT-PCR), and production of infectious progeny virus. iHIOs were also shown to support replication of 12/13 clinical rotavirus isolates directly from stool samples. An unexpected finding was the detection of rotavirus infection not only in the epithelial cells but also in the mesenchymal cell population of the iHIOs. This work demonstrates that iHIOs offer a promising new model to study rotaviruses and other gastrointestinal viruses.**

IMPORTANCE **Gastrointestinal viral infections are a major cause of illness and death in children and adults. The ability to fully understand how viruses interact with human intestinal cells in order to cause disease has been hampered by insufficient methods for growing many gastrointestinal viruses in the laboratory. Induced human intestinal organoids (iHIOs) are a promising new model for generating intestine-like tissue. This is the first report of a study using iHIOs to cultivate any microorganism, in this case, an enteric virus. The evidence that both laboratory and clinical rotavirus isolates can replicate in iHIOs suggests that this model would be useful not only for studies of rotaviruses but also potentially of other infectious agents. Furthermore, detection of rotavirus proteins in unexpected cell types highlights the promise of this system to reveal new questions about pathogenesis that have not been previously recognized or investigated in other intestinal cell culture models.**

Received 24 May 2012 **Accepted** 7 June 2012 **Published** 3 July 2012

Citation: Finkbeiner SR, et al. 2012. Stem cell-derived human intestinal organoids as an infection model for rotaviruses. mBio 3(4):e00159-12. doi:10.1128/mBio.00159-12.

Editor Terence Dermody, Vanderbilt University School of Medicine

Copyright © 2012 Finkbeiner et al. This is an open-access article distributed under the terms of the Creative Commons Attribution-Noncommercial-Share Alike 3.0 Unported License, which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Address correspondence to Mary K. Estes, mestes@bcm.edu.

There are annually an estimated 1.7 billion episodes of acute diarrhea that contribute to the large disease burden of diarrheal diseases (1, 2). Collectively, enteric viruses, predominantly rotaviruses, noroviruses, astroviruses, and enteric adenoviruses, cause the greatest number of annual cases of diarrhea (1, 3, 4). Furthermore, novel viruses could also be responsible for a portion of the ~12% to 40% of sporadic diarrheal episodes and outbreaks that remain of unknown etiology even after extensive testing (5–7). The inability to easily cultivate many enteric viruses presents a major challenge to advancing our understanding of virus-induced diarrhea. While conditions have been established to cultivate, with various degrees of success, some laboratory-adapted strains of human enteric adenoviruses, canonical human astroviruses, and human rotaviruses, efforts to cultivate clinical strains of these viruses have largely been met with only limited success (8–10). Human rotaviruses in clinical samples may be culture adapted if subjected to several passages in primary African green monkey kidney cells, with additional passages in MA104 cells (11). Additionally, there is currently no model that is sufficient for growth of any strain of human norovirus (12). Culturing the increasing number of novel viruses and other microorganisms that are being discovered using sequence-based techniques is also proving to be quite challenging. Therefore, improved intestinal culture systems are needed to enable growth of enteric microorganisms and facilitate further biological investigations of them.

Methods have existed for some time to culture primary epithelial cells from mice as 3-dimensional structures called organoids, and some of these organoid models have been used to study rotaviruses (13, 14). However, these methods were not sufficient for the generation of human intestinal organoids. Recently, a new method was described for directing differentiation of human embryonic or induced pluripotent stem cell lines into intestine-like tissue referred to as induced human intestinal organoids (iHIOs) (15, 16). These organoids contain multiple cell types, including enterocytes, goblet cells, enteroendocrine cells, Paneth cells, and mesenchymal cell populations. The epithelial cells are organized around luminal cavities located within the core of the iHIOs, and mesenchymal cells surround the epithelial cell layer. Furthermore, some functional and physiological properties of the organoids have been demonstrated by the presence of brush borders on en-

terocytes, production of mucin by goblet cells, and functional peptide transport systems as assessed by uptake of a fluorescently labeled dipeptide (15). This breakthrough in the ability to generate human organoids as a model of intestinal tissue prompted the study described here by examining the use of iHIOs as a potential model for the growth of enteric viruses.

Many laboratory strains of rotavirus grow readily in cell culture (8). Rhesus rotavirus (RRV; strain G3P[3]), which has a wide cellular tropism, was chosen to develop methods for carrying out viral infections (IFs) of iHIOs and as proof of principle that iHIOs can support enteric virus replication. Detailed protocols for the development and characterization of iHIOs are described elsewhere (16). Briefly, the NIH-approved embryonic stem cell line WA09 (originating from the WiCell Research Institute and obtained from the Baylor College of Medicine Human Embryonic Stem Cell Core) was cultured using feeder-free conditions. Stem cells were split at a high density, and, once they reached 85 to 90% confluence, cells were treated for 3 days with a series of differentiation media containing activin A to begin differentiation into definitive endoderm. Definitive endoderm was then treated for 2 to 5 days with growth factors Wnt3a and FGF4, leading to formation of hindgut spheroids. Once spheroids spontaneously detached from monolayers, they were collected, embedded into matrigel (BD Biosciences), and supplied with media supplemented with intestinal growth factors (Wnt3a, R-Spondin1, Noggin, and epidermal growth factor [EGF]; all supplied from R&D Systems). Spheroids matured into intestinal organoids over the course of ~1 to 2 months before they were used for experiments. We examined the expression of a number of cellular markers by immunofluorescence and confirmed that our iHIOs showed staining patterns similar to those of the iHIOs published in the original paper (15). These included villin (Fig. S1) as well as Cdx2, Muc2, dipeptidyl peptidase IV (DPPIV), chromogranin A, E-cadherin (E-cad), cystic fibrosis transmembrane conductance regulator (CFTR), and alkaline phosphatase (data not shown). For all experiments, similar numbers of organoids, ranging from 7 to 15 organoids per set of conditions, were used under all conditions within a given experiment.

The luminal cavities and surrounding layer of epithelial cells of the iHIOs are encased by mesenchymal cells (15), so iHIOs were physically cut open using tungsten needles to allow viral particles access to the epithelial cells. In doing this, iHIOs were also removed from the matrigel in which they were embedded and collected into 1.5-ml Eppendorf tubes. To remove excess matrigel and accumulated dead cells and mucus in the luminal cavities, iHIOs were then washed twice with phosphate-buffered saline (PBS). Infections were carried out by suspending the cut organoids in 200 μl of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM-F12) (Invitrogen) containing 10 μM of the anti-apoptotic rho-associated coiled-coil-containing protein kinase (ROCK) inhibitor compound Y-27632 (StemCell Technologies) and adding 10 μl of RRV inoculum containing 10^7 PFU (multiplicity of infection [MOI], ~1) or DMEM-F12 for mock controls. iHIOs were inoculated with virus for 6 h and were manually agitated by inverting and flicking the tubes approximately every hour. At 6 h postinoculation (hpi), the virus inoculum was removed and iHIOs were washed twice with 1 ml of PBS before they were re-embedded into matrigel and covered with the normal growth media containing intestinal growth factors, after which iHIOs resealed to form a continuous epithelium surrounding the luminal cavity. At 24 hpi, iHIOs were fixed with 4% paraformaldehyde–PBS for 20 min at 4°C, stained with methylene blue for tracking purposes, embedded into optimal-cutting-temperature (OCT) freezing compound, and frozen at −80°C to make frozen blocks for sectioning. Sections (5 μm) of iHIOs were then cut using a cryostat and stained for immunofluorescence analysis using antibodies against rotavirus nonstructural protein 4 (NSP4) and the adherens junction protein E-cadherin (BD Biosciences; catalog no. 610182) to identify epithelial cells. NSP4 staining was evident in RRV-infected iHIOs but not in mock-infected organoids (Fig. 1A). Confocal microscopy showed typical viroplasms (virus replication factories), as demonstrated by NSP2 staining, surrounded by the rotavirus NSP4 protein (Fig. 1B) (17).

Proteolytic cleavage of VP4 rotavirus surface proteins enhances infectivity of the virus (18, 19), so further efforts were undertaken to optimize conditions of infection of iHIOs with rotavirus. Infections were carried out as described above, with the addition of either trypsin or pancreatin to both the inoculation media and the normal growth media used once the organoids were re-embedded into matrigel. Bovine trypsin (Worthington) was tested at ≥180 U per mg of protein (10 μg/ml), as this is the concentration routinely used for rotavirus studies in other cell lines (8). A range of pancreatin (Sigma) concentrations (from 1 μg/ml to 5 mg/ml) was tested based on concentrations that had previously been used to generate *in vitro* digestion models and to test for growth of other enteric viruses (12, 20). Preliminary analysis by qualitative assessment of immunofluorescence images indicated that use of trypsin at 10 μg/ml and pancreatin treatments of 2.5 μg/ml or higher yielded a greater number of rotavirus-positive cells compared to no proteolytic treatment (data not shown). The optimal condition for growth of RRV in iHIOs was addition of pancreatin at 2.5 mg/ml to the medium.

To quantitatively measure the effects of adding proteases to the iHIO infections, a TaqMan quantitative reverse transcription-PCR (qRT-PCR) assay targeting the gene for rotavirus structural protein 7 (VP7) was used to measure the increase in rotavirus RNA levels over time (21). RNA was extracted from mock-infected and infected organoids 1 hpi (as a baseline to account for any input virus that could be bound to the cells) and then at 24 and 48 hpi. The VP7 assay was carried out as previously described (21), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA was amplified in parallel under the same conditions using a commercially available assay (Applied Biosystems; catalog no. Hs99999905). Fold increases in VP7 RNA levels were calculated using the delta-delta threshold cycle (C_T) formula [fold increase = $2^{(-\Delta\Delta Ct)}$], normalizing to GAPDH, and using the 1 hpi time points as the calibrators for each set of conditions. For baseline samples in which there was no amplification of VP7, a C_T value of 46 was assigned in order to calculate fold increases. While the addition of pancreatin at 2.5 mg/ml to the media represented the optimal concentration to use for rotavirus infections of iHIOs based on the preliminary immunofluorescence experiments, the high concentration caused some proteolytic degradation of the organoids during the 24-h infection period (data not shown). This presumably affected only the outer layers of mesenchymal cells, as the epithelial cells in the cores could still be visualized and were still surrounded by mesenchymal cells. Based on this observation, an additional condition was included in the qRT-PCR experiments to determine if the high concentration of pancreatin is needed throughout the course of infection or if it is dispensable after ini-

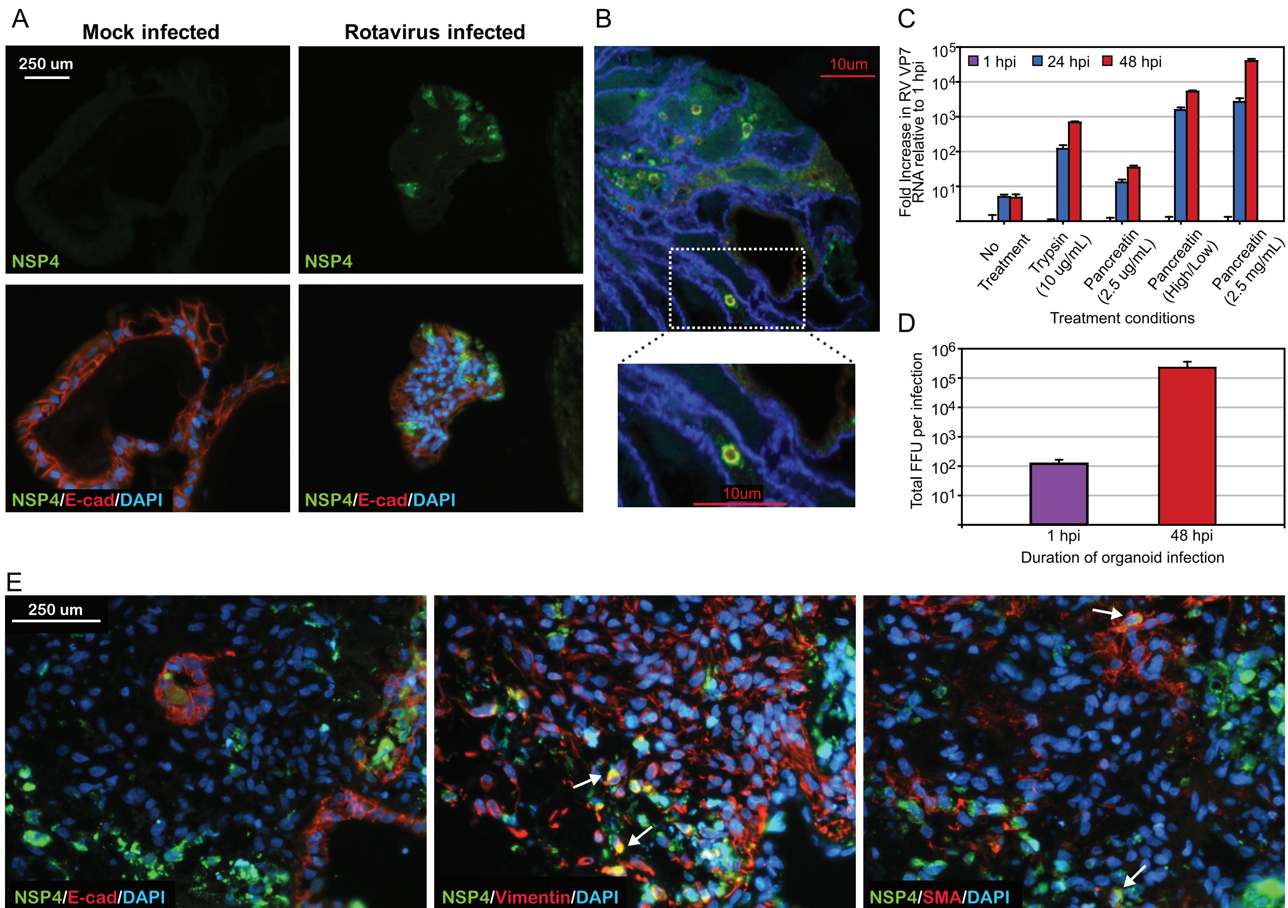

FIG 1 Rhesus rotavirus (RRV) replicates in induced human intestinal organoids (iHIOs). (A) Replication of virus in iHIOs inoculated with 10^7 PFU of RRV was evaluated by immunofluorescence analysis for nonstructural protein NSP4 (nonstructural protein 4) (green), E-cadherin (red), and DAPI (4′,6-diamidino-2-phenylindole) nuclear staining (blue). (B) Confocal microscopy was used to visualize the formation of viral replication structures called viroplasms, which contain the nonstructural protein NSP2 (red) surrounded by NSP4 (green). The epithelial cells are marked by E-cadherin (blue). (C) Trypsin and various concentrations of pancreatin ("High/Low" = 2.5 mg/ml during the inoculation period followed by 2.5 μg/ml for the remainder of the infection period) were tested to determine optimal conditions for growth of rotaviruses in iHIOs. GAPDH-normalized rotavirus VP7 RNA levels are shown as fold increases relative to those seen at 1 h postinfection. (D) Levels of infectious virus produced during iHIO infections were determined by fluorescent focus assays using MA104 cells. (E) Sequential sections from the same infected organoid showed NSP4 staining in E-cadherin-negative cells (left panel), which were positive for the mesenchymal cell markers vimentin and smooth-muscle actin (middle and right panels, respectively). White arrows indicate RV-infected mesenchymal cells.

tial activation of the virus. For this condition (labeled the "High/Low" condition), pancreatin was added at 2.5 mg/ml to the rotavirus infection of iHIOs during the 6-h inoculation period and then the medium was supplemented with the lower concentration of pancreatin at 2.5 μg/ml once the organoids were re-embedded into matrigel. Three replicate experiments were carried out using different batches of iHIOs. The fold increases in RNA levels were highly variable, resulting in ~1 to 2 $\log_{10}$ differences between different experiments. However, the trends were the same for each experiment in that the highest increase of RNA levels resulted from virus cultivation in the presence of pancreatin at 2.5 mg/ml (the results of a representative experiment are shown in Fig. 1C). However, the High/Low condition enhanced RNA levels in only 1 of 3 replicates compared to continuous treatment with 2.5 mg/ml (as shown in Fig. 1C). One drawback of the iHIOs is that they are heterogeneous and can vary considerably in size and cellular content, and this likely explains the degree of variation observed. In all experiments, the "High/Low" conditions resulted in a greater increase in RNA levels than the trypsin, low pancreatin, or no treatment conditions; however, the fold changes were less than those observed when pancreatin was present at 2.5 mg/ml throughout the course of the infection. This suggests that a high concentration of pancreatin is required throughout the course of infection for optimal virus replication, perhaps facilitating continual viral spread throughout the organoids. Therefore, pancreatin at 2.5 mg/ml was included under all sets of infection conditions for subsequent experiments.

To determine whether full replication of RRV occurred and infectious viral particles were produced in iHIOs, the yield of virus was measured with a rotavirus fluorescent focus assay (FFA) (8). iHIOs were infected with RRV as described for previous experiments, and at 1 and 48 hpi, the tubes containing 400 μl of media and organoids were frozen at −80°C. After thawing, the organoids were sonicated for 1 min on ice and then subjected to two rounds of freeze/thawing before a final sonication step was performed for 1 min. The lysates were then treated with trypsin at 10 μg/ml for 30 min at 37°C to ensure complete activation of virus. This is the standard treatment used for FFAs and was used instead of pancre-

atin treatment because MA104 cells are highly sensitive to the level of pancreatin used for infection of organoids, as they quickly detached from the plates in initial experiments (data not shown). Trypsin-treated lysates were then spun down to pellet cellular debris, and then 100 μl of each lysate supernatant was added to 3 wells of a 96-well plate and was also used as the starting material for 10-fold serial dilutions in DMEM. Virus levels in the supernatant dilutions were then determined by measuring titers using MA104 cells and a fluorescent focus assay. Figure 1D shows the average levels of fluorescent focus units (FFU) produced from 4 experiments. iHIOs infected with RRV for 48 h produced an average of 2.2×10^5 FFUs. In contrast, iHIOs infected for 1 h averaged only 100 FFUs, indicating *de novo* synthesis of infectious virus produced during the course of the 48-h infection. Unlike the RV RNA levels, the levels of infectious virus produced during the course of infections in replicates were surprisingly similar.

Examination of the cells within the iHIOs infected with RRV showed infection of epithelial cells, as expected. Surprisingly, rotavirus NSP4 protein was also detected in cells lacking E-cadherin (E-cad) expression. Figure 1E shows staining of three sections focused on the same region of a single infected organoid. The E-cad-negative cells were confirmed to be part of the mesenchymal cell population based on the location and organization of the cells and their expression of two mesenchymal cell markers, vimentin (AnaSpec; catalog no. 53945) and smooth-muscle actin (SMA) (Abcam; catalog no. ab5494) (Fig. 1E). Vimentin, an intermediate filament protein and the major cytoskeletal component of mesenchymal cells, serves as a general marker of mesenchymal cells. The cytoskeletal protein SMA is a marker of intestinal myofibroblasts and smooth-muscle cells. To our knowledge, there have been no previous reports of rotavirus infection of intestinal mesenchymal cells. However, this finding is consistent with previous reports describing the ability of rotavirus strains to bind to the $\alpha 4\beta 1$ integrin subunit, which is expressed on intestinal mesenchymal cells (22). It remains unclear whether this finding suggests a true role for mesenchymal cells in rotavirus infection or whether it is an artifact of exposure to a population of cells that the virus does not normally encounter in a natural infection. Furthermore, it is important to determine whether mesenchymal cells support production of infectious virus, since incomplete replication in mesenchymal cells could be an explanation for the high variation in viral RNA levels compared to the similar levels of infectious particles observed in the experiments. These matters require further investigation and further characterization of which specific mesenchymal cell populations are susceptible to rotavirus infection.

Growth of clinical isolates of rotavirus from stool is typically challenging, as is the case for many enteric viruses. Often, clinical isolates of rotavirus must be subjected to multiple rounds of blind passaging in primary cells before they can then be grown in continuous cell lines (11). Even then, this approach is not always successful. Since iHIOs seemingly recapitulate intestinal tissue, we hypothesized that they may provide a useful means of studying clinical isolates of rotavirus in addition to laboratory strains. Thirteen stool samples were therefore selected from an archive of pediatric stools submitted to Texas Children's Hospital between 2002 and 2010 for viral enteropathogen testing. Samples that were positive for rotavirus either by electron microscopic analysis or by enzyme-linked immunoassay (VIDAS rotavirus [RTV] assay; bioMérieux, South Africa) were selected for cultivation. Suspensions (10% [wt/vol]) of stool were prepared in PBS and subjected to low-speed centrifugation to pellet debris, and the supernatants were then sequentially filtered through 0.8-μm- and 0.45-μm-pore-size filters. RNA was extracted from filtrates in order to determine the genotype of each isolate by the use of standard assays (23, 24). Genotyping showed that the panel of stools contained a variety of strains, including 5 G9P[8], 4 G1P[8], 2 G3P[8], 1 G2P[4], and 1 G3P[untypeable] (Fig. 2A; Fig. S2).

Infections of iHIOs were carried out as described above using pancreatin (2.5 mg/ml). Since the virus titers in the clinical specimens were unknown and presumed to be variable among the samples, 100 μl of filtrate was added to 100 μl of DMEM and supplemented with 10 μM ROCK inhibitor Y-27632 for each infection. Rotavirus infection (NSP4-positive staining) was observed in organoids infected with 12 of the clinical isolates. Sample TCH-03-319 was the only sample for which no positive cells were detected. Images from a subset of these infections are shown in Fig. 2A, and images from the remaining infections are shown in Fig. S2. Infections with some isolates resulted in many very brightly stained cells, while others were less robust.

To further analyze the ability of iHIOs to support replication of rotavirus clinical isolates, the RV VP7 qRT-PCR assay was used to assess whether there was an increase in RNA levels over time for each isolate. Results of experiments conducted with a subset of isolates in which three independent infections were carried out for each isolate are shown in Fig. 2B. Infections with the remaining isolates were carried out only once for qRT-PCR analysis (Fig. S3). Use of the VP7 qRT-PCR assay with RNA extracted from the original filtrates revealed that one isolate (TCH-04-35) was not amplified by the VP7 qRT-PCR primer/probe set (data not shown), so RNA levels could not be determined for that isolate. That virus was perhaps more divergent from the others, as it was the only G2P[4] strain in the panel. An increase in RNA levels at 24 and 48 hpi was detected for all the other isolates (11/11) that had been IF positive; no VP7 RNA was detected at any time point for TCH-03-319, which had also been IF negative (Fig. 2B; Fig. S3). As seen with the RRV infections, the RNA levels were highly variable in the cases where multiple replicates were carried out for a given isolate. However, at 24 hpi, the majority of samples had a $>$10-fold increase in VP7 RNA levels, and by 48 hpi, all samples had a $>$10-fold increase, with several samples (9/12 in at least one replicate) having a $>$100-fold increase. Despite the variability in the levels of RNA produced, successful infection of iHIOs with clinical isolates of rotavirus directly from stool was achieved for the majority of samples tested, making this a promising system for studying clinically important strains of rotavirus. The results of experiments performed to grow 12 of the clinical isolates (the isolate not detected by the VP7 qRT-PCR assay was excluded) in MA104 cells suggest a distinct growth advantage for most of the isolates in the organoids compared to MA104 cells. The fold increases of VP7 RNA levels at 24 hpi were at least ~10 times greater in the organoids than in MA104 cells for the majority (9/12) of samples (data not shown). Thus, the ability to use iHIOs as an infection model for clinical RV isolates represents an advantage over other culture systems that involve using immortalized or cancer-derived cell lines.

This was an original and inaugural study designed to demonstrate the functional relevance of stem cell-derived organoids in relation to an infectious agent. The demonstration that iHIOs support replication of rotaviruses suggests that iHIOs are an in-

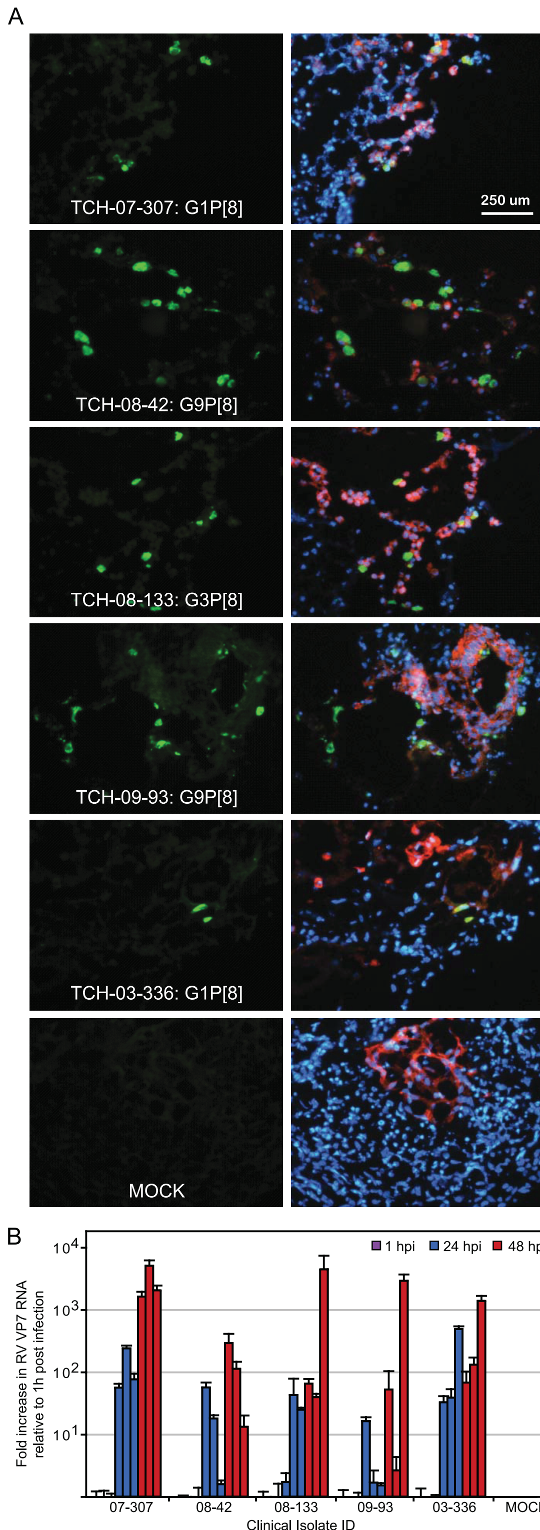

FIG 2 Clinical isolates of human rotavirus replicate in iHIOs. (A) A total of 13 stool filtrates (10% [wt/vol] in PBS) were used to infect iHIOs in the presence of pancreatin at 2.5 mg/ml. NSP4 was detected in 12 of 13 iHIO infections with isolates at 24 hpi (additional samples are shown in Fig. S2). (B) Increases in rotavirus VP7 RNA levels over time, as determined by normalizing to GAPDH and plotting the fold increases relative to 1 hpi, are shown. Due to high variation between experiments, the data for 3 replicates are shown and error bars are for 3 amplifications per sample. Results for additional samples are shown in Fig. S3.

valuable tool to improve understanding of aspects of rotavirus biology. Furthermore, the proof of principle that iHIOs support growth of enteric viral pathogens directly from stool provides promise that this system can support replication of other enteric agents and perhaps even those that have traditionally been difficult or impossible to grow. Another protocol for the generation of human intestine-like tissue has been described since this study began in which epithelial organoids are generated from human intestinal biopsy samples (25). The organoids generated by that protocol have not yet been evaluated for their ability to support rotavirus replication, but comparative studies in those organoids, which do not contain mesenchymal cells, since they are exclusively derived from epithelium, would be of interest for the study of infectious agents.

iHIOs were maintained up to 3 months, although they can be maintained for even longer periods. Once established, iHIOs can be frozen and expanded as needed and they may negate the need for euthanizing African green monkeys in order to prepare primary cells for the cultivation of rotavirus clinical isolates. The iHIOs described in this report were used at 1 to 2 months of age, but we also used 3-month-old iHIOs successfully in other experiments. In our hands, the age of the iHIOs did not affect their ability to be infected with rotaviruses. This may not be true for other infectious agents, as they may have specific requirements with respect to the differentiation state or composition of the iHIOs. Therefore, in future studies using organoid models of intestinal tissue, it will be important to better characterize the cellular composition, architecture, and differentiation state of the iHIOs as they age and also the expression profiles of proteins located along the different segments of the intestine. Such a biologically relevant model of intestinal tissue offers promise to open up new avenues of research into host-pathogen interactions in the gut and could reveal new insights into a variety of topics, including epithelial innate responses, activation of death pathways, and physiological responses to enteric infections.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00159-12/-/DCSupplemental.

Figure S1, TIF file, 2.3 MB.
Figure S2, EPS file, 9.8 MB.
Figure S3, EPS file, 2.7 MB.

ACKNOWLEDGMENTS

We thank Jason Spence, Jeff Vallance, Kyle McCrackin, and James Wells at Cincinnati Children's Hospital for training in how to establish and maintain the human intestinal organoid model. We further thank Jason Spence for helpful discussions and Khalil Ettayebi for performing the confocal microscopy for Fig. S1. We thank Robert Milczarek and Ping Zhang of the Baylor College of Medicine Human Embryonic Stem Cell Core for training in stem cell-culturing methods and for providing us stocks of stem cell lines.

This study was supported in part by Public Health Service Awards from the National Institutes of Health (PO1 AI57788, RO1 AI080656, and P30 DK56338). S.R.F. was also supported in part by T32 AI007471-14.

REFERENCES

1. **Klein EJ, et al.** 2006. Diarrhea etiology in a Children's Hospital Emergency Department: a prospective cohort study. Clin. Infect. Dis. **43**: 807–813.
2. **O'Ryan M, Prado V, Pickering LK.** 2005. A millennium update on

pediatric diarrheal illness in the developing world. Semin. Pediatr. Infect. Dis. **16**:125–136.
3. **Kirkwood CD, Clark R, Bogdanovic-Sakran N, Bishop RF.** 2005. A 5-year study of the prevalence and genetic diversity of human caliciviruses associated with sporadic cases of acute gastroenteritis in young children admitted to hospital in Melbourne, Australia (1998–2002). J. Med. Virol. **77**:96–101.
4. **Wilhelmi I, Roman E, Sánchez-Fauquier A.** 2003. Viruses causing gastroenteritis. Clin. Microbiol. Infect. **9**:247–262.
5. **Kapikian AZ.** 1993. Viral gastroenteritis. JAMA **269**:627–630.
6. **Svraka S, et al.** 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J. Clin. Microbiol. **45**:1389–1394.
7. **Simpson R, Aliyu S, Iturriza-Gómara M, Desselberger U, Gray J.** 2003. Infantile viral gastroenteritis: on the way to closing the diagnostic gap. J. Med. Virol. **70**:258–262.
8. **Arnold M, Patton JT, McDonald SM.** 2009. Chapter 15: Culturing, storage, and quantification of rotaviruses. Curr. Protoc. Microbiol. **15**: 15C.3.1–15C.3.24.
9. **Brinker JP, Blacklow NR, Herrmann JE.** 2000. Human astrovirus isolation and propagation in multiple cell lines. Arch. Virol. **145**:1847–1856.
10. **Tiemessen CT, Kidd AH.** 1995. The subgroup F adenoviruses. J. Gen. Virol. **76**(Pt. 3):481–497.
11. **Ward RL, et al.** 1991. Culture adaptation and characterization of group A rotaviruses causing diarrheal illnesses in Bangladesh from 1985 to 1986. J. Clin. Microbiol. **29**:1915–1923.
12. **Duizer E, et al.** 2004. Laboratory efforts to cultivate noroviruses. J. Gen. Virol. **85**:79–87.
13. **Macartney KK, Baumgart DC, Carding SR, Brubaker JO, Offit PA.** 2000. Primary murine small intestinal epithelial cells, maintained in long-term culture, are susceptible to rotavirus infection. J. Virol. **74**:5597–5603.
14. **Sato T, et al.** 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature **459**:262–265.
15. **Spence JR, et al.** 2011. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature **470**:105–109.
16. **McCracken KW, Howell JC, Wells JM, Spence JR.** 2011. Generating human intestinal tissue from pluripotent stem cells in vitro. Nat. Protoc. **6**:1920–1928.
17. **Berkova Z, et al.** 2006. Rotavirus NSP4 induces a novel vesicular compartment regulated by calcium and associated with viroplasms. J. Virol. **80**:6061–6071.
18. **Estes MK, Graham DY, Mason BB.** 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J. Virol. **39**:879–888.
19. **Graham DY, Estes MK.** 1980. Proteolytic enhancement of rotavirus infectivity: biology mechanism. Virology **101**:432–439.
20. **Oomen AG, et al.** 2003. Development of an in vitro digestion model for estimating the bioaccessibility of soil contaminants. Arch. Environ. Contam. Toxicol. **44**:281–287.
21. **Feeney SA, et al.** 2011. Development and clinical validation of multiplex TaqMan® assays for rapid diagnosis of viral gastroenteritis. J. Med. Virol. **83**:1650–1656.
22. **Graham KL, et al.** 2005. Rotaviruses interact with alpha4beta7 and alpha4beta1 integrins by binding the same integrin domains as natural ligands. J. Gen. Virol. **86**:3397–3408.
23. **Iturriza-Gómara M, Kang G, Gray J.** 2004. Rotavirus genotyping: keeping up with an evolving population of human rotaviruses. J. Clin. Virol. **31**:259–265.
24. **Maes P, Matthijnssens J, Rahman M, Van Ranst M.** 2009. RotaC: a web-based tool for the complete genome classification of group A rotaviruses. BMC Microbiol. **9**:238.
25. **Sato T, et al.** 2011. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology **141**:1762–1772.