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Research

# Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection

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## Abstract

**Background:** In order to elucidate how Hepatitis C Virus (HCV) interacts with polarized hepatocytes in vivo and how HCV-induced alterations in cellular function contribute to HCV-associated liver disease, a more physiologically relevant hepatocyte culture model is needed. As such, NASA-engineered three-dimensional (3-D) rotating wall vessel (RWV) bioreactors were used in effort to promote differentiation of HCV-permissive Huh7 hepatoma cells.

**Results:** When cultured in the RWV, Huh7 cells became morphologically and transcriptionally distinct from more standard Huh7 two-dimensional (2-D) monolayers. Specifically, RWV-cultured Huh7 cells formed complex, multilayered 3-D aggregates in which Phase I and Phase II xenobiotic drug metabolism genes, as well as hepatocyte-specific transcripts (HNF4$\alpha$, Albumin, TTR and $\alpha$1AT), were upregulated compared to 2-D cultured Huh7 cells. Immunofluorescence analysis revealed that these HCV-permissive 3-D cultured Huh7 cells were more polarized than their 2D counterparts with the expression of HCV receptors, cell adhesion and tight junction markers (CD81, scavenger receptor class B member 1, claudin-1, occludin, ZO-1, $\beta$-Catenin and E-Cadherin) significantly increased and exhibiting apical, lateral and/or basolateral localization.

**Conclusion:** These findings show that when cultured in 3-D, Huh7 cells acquire a more differentiated hepatocyte-like phenotype. Importantly, we show that these 3D cultures are highly permissive for HCV infection, thus providing an opportunity to study HCV entry and the effects of HCV infection on host cell function in a more physiologically relevant cell culture system.

## Background

Hepatitis C virus (HCV), a liver tropic positive-stranded RNA flavivirus, infects ~170 million people worldwide, causing acute and chronic hepatitis and hepatocellular carcinoma [1]. However, since its discovery in 1989, a major obstacle impeding HCV research has been the lack of robust cell culture and small animal infection models. Notably significant advancement has been made with the identification of a genotype 2a HCV consensus clone (Japanese Fulminant Hepatitis, JFH-1) that can replicate and produce infectious HCV in vitro in the Huh7 human hepatoma-derived cell line [2-4], allowing for the study of the entire viral life cycle. This system, however, is limited in that it makes use of a non-differentiated cell line that does not recapitulate the cellular conditions encountered by HCV in vivo [5,6]. In particular, hepatocyte polarity is likely relevant to HCV entry as growing evidence suggests interplay between HCV and tight junction (TJ) proteins

claudin-1 (CLDN1) [7] and occludin [8,9] is essential for viral uptake. In fact, recent reports surprisingly suggests that hepatocyte polarity may restricts HCV entry [10,11]. While an inverse relationship between viral entry and hepatocyte polarity would potentially represent a unique determinant of HCV entry, to date attempts to dissect this relationship have been difficult and inconclusive due to the inability of cell culture grown hepatocyte-derived cell lines, such as Huh7 cells, to mimic the complex polarized phenotype of hepatocytes in vivo. To circumvent these restriction, studies investigating HCV entry into Caco-2 cells [10] and HepG2 cells [11] have been performed as these cells can polarize to differing degrees in vitro, however, neither Caco-2 or HepG2 cells supports efficient HCV infection limiting their utility. As such, a more physiologically relevant hepatocyte tissue culture model is still needed to assess if cell polarity negatively affects HCV infection.

The NASA-engineered RWV is a horizontally rotating cylindrical culture vessel which reduces shear and turbulence associated with conventional stirred bioreactors; therefore, it simulates aspects of microgravity similar to the environment encountered during fetal development [12-14]. In contrast to conventional static tissue culture systems, cells grown in the RWV are cultured in "suspended animation" where they are continuously free-falling [12,15]. Thus, while the 2-D environment of plastic substrates may alter gene expression and prevent cellular differentiation [12,16-21], the fluid dynamics of the RWV culture system allow cells to co-localize into three-dimensional (3-D) aggregates, promoting in vivo-like exchange of growth factors and efficient cell-to-cell interactions [12-14,20,21]. This in vivo-like environment thus can promote transformed and primary cell lines to become more structurally and functionally similar to their in vivo counterparts [13,15,20-24].

In the current study we demonstrate that RWV-cultured Huh7 cells formed complex, multilayered, 3-D aggregates that exhibited up-regulation of metabolic and hepatocyte-specific transcripts as well as increased expression and re-localization of tight junction, cell adhesion, and polarity markers. Importantly, these aggregates remained highly permissive for HCV infection suggesting that hepatic polarity does not limit HCV entry in 3-D-cultured Huh7 cells. As such, RWV-cultured Huh7 cells may represent a more appropriate physiologically relevant system for further in vitro studies of HCV entry and infection dynamics.

## Methods
### Cell culture and viruses
Huh7 cells (also known as Huh7/scr cells [25,26] and Huh7-1 cells [27]) were obtained from Dr. Chisari (The Scripps Research Institute, La Jolla, CA) [2] and cultured as previously described [2]. 3-D Huh7 cultures were estab-

lished using previously described techniques [13,14], with minor modifications. Briefly, $5 \times 10^6$ Huh7 cells were trypisinized, incubated with 250 mg Cytodex-3 microcarrier beads (Sigma, St. Louis, MO) for 30 minutes at room temperature in a total volume of 30 ml complete DMEM. Cell-bead complexes were introduced into the RWV bioreactor (Synthecon, Inc., Houston, TX) at a ratio of 20 cells/bead, transferred to 37°C, and vessel rotation was initiated at 20 rotations per minute. Medium was replenished every 24 h and rotation speed was increased as aggregates developed to maintain cells in free-falling suspension.

Protocols for JFH-1 in vitro transcription and HCV RNA electroporation have been described elsewhere [28]. JFH-1 cell culture-propagated HCV (HCVcc) viral stocks were obtained by infection of naïve Huh7-1 cells at a multiplicity of infection (MOI) of 0.01 focus forming units (FFU)/cell, using medium collected from Huh7 cells on day 18 post transfection with in vitro transcribed pJFH-1 RNA as previously described [2].

### RNA isolation and RTqPCR
Total cellular RNA was isolated by the guanidine thiocyanate method using standard protocols [29]. One μg of RNA was used for cDNA synthesis using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA), followed by SYBR green real-time quantitative PCR analysis (RTqPCR) using an Applied Biosystems 7300 real-time thermocycler as previously described [30]. Relative expression levels of hepatocyte-specific genes and Phase I and Phase II metabolic genes were assessed using the primers described in [30] and normalized to β-actin levels. HCV JFH-1 and GAPDH transcript levels were determined relative to a standard curve of serially diluted plasmid containing the JFH-1 cDNA or the human GAPDH gene, respectively, using primers described in [28].

### Immunofluorescence
Immunofluorescence analysis of aggregates was performed as previously described [14]. Briefly, Huh7 3-D aggregates were fixed with 4% (v/v) paraformaldehyde (Sigma, St. Louis, MO), free aldehydes quenched with 50 mM $NH_4Cl$ in DPBS for 10 minutes at room temperature and cells permeabilized with 0.1% Triton-X 100 (Fisher). In parallel, Huh7 2-D monolayers were seeded in 8-well chamber slides at 80% confluence and fixed 48 hours post seeding. 3-D aggregates and 2-D monolayer cells were stained with antibodies specific for scavenger receptor class B member 1 (SR-BI) (BD Biosciences, Franklin Lakes, NJ), CD81 (AbD Serotec, Raleigh, NC), CLDN1 (Abnova, Taipei, Taiwan), CD26 (Abcam, Cambridge, UK), β-Catenin (Zymed, San Francisco, CA), E-cadherin (Zymed), zona occludens 1 (ZO-1) (Zymed), Occludin (Zymed) or HCV E2 (C1 [31]) overnight at 4°C, followed by incuba-

tion with a 1:1,000 dilution of an appropriate Alexa555-conjugated secondary antibody (Molecular Probes, Carlsbad, CA) for 1 h at room temperature. Cell nuclei were stained by Hoechst dye. Bound antibodies were visualized via confocal microscopy (630×, Zeiss LSM 510, Germany). Images were analyzed using Zeiss LSM Alpha Imager Browser v4.0 software (Zeiss), and brightness and contrast were adjusted using Adobe® Photoshop® (San Jose, CA). Alternately, 3-D aggregates were embedded in OCT freezing medium (TissueTek, Fisher) or paraffin, sectioned and stained with Hoechst dye or Hematoxylin and Eosin (H&E), respectively.

### HCV infection kinetics
Huh7 3-D aggregates were infected with JFH-1 HCVcc at an MOI of 0.01 FFU/cell at day 1, 7 or 14 post RWV seeding by injection of the viral inoculum directly into the RWV. At indicated times post infection (p.i.), medium was harvested for titration analysis and RNA was isolated from ~0.5 ml of aggregates for reverse transcription followed by RTqPCR as described above.
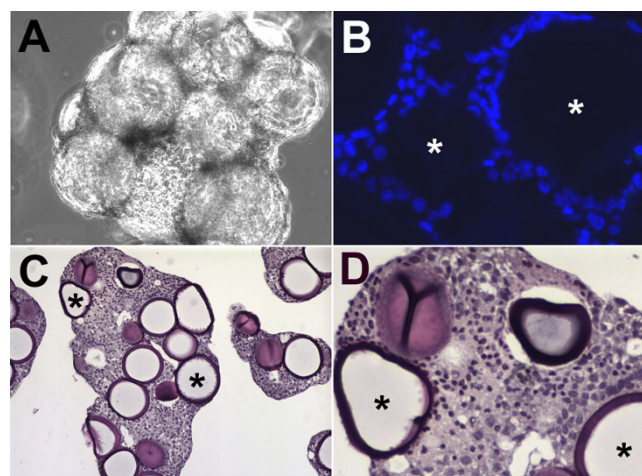
### Infectivity titration assay
Culture supernatants were serially diluted 10-fold and used to infect triplicate Huh7 cultures in 96-well plates. At 24 h p.i., cultures were overlayed with complete DMEM containing 0.4% methylcellulose (Fluka BioChemika, Switzerland) to give a final concentration of 0.25% methylcellulose. Seventy-two hours p.i., cells were fixed in 4% paraformaldehyde (Sigma), and immunohistochemically stained for HCV E2 using the anti-HCV E2 antibody C1 [31]. Viral titers are expressed as FFU/ml, determined by the average E2-positive foci number detected at the highest HCV-positive dilution.

## Results
### Establishment of Huh7 3-D Aggregates
To assess the utility of the RWV as a culture method for Huh7 cells, Huh7 cells were cultured on Cytodex-3 microcarrier beads in the RWV for 26 days. Morphological and cytological examination of cultures demonstrated that Huh7 cells efficiently adhered to the collagen-coated microcarrier beads and that these individual beads then assembled to form 3-D tissue-like aggregates containing ~10–20 beads per aggregate (Fig. 1A). To determine if these aggregates consisted of multilayered cells, aggregates were embedded in OCT freezing medium or paraffin, sectioned, stained with either Hoechst stain (Fig. 1B) or H&E (Fig. 1C–D) and examined by fluorescence or light microscopy, respectively. Panels C and D highlight the multilayered cellular infrastructure of the Huh7 3-D aggregates, while Hoechst's staining in Panel B illustrates similar infrastructure and confirms that the aggregates are devoid of necrotic cores.
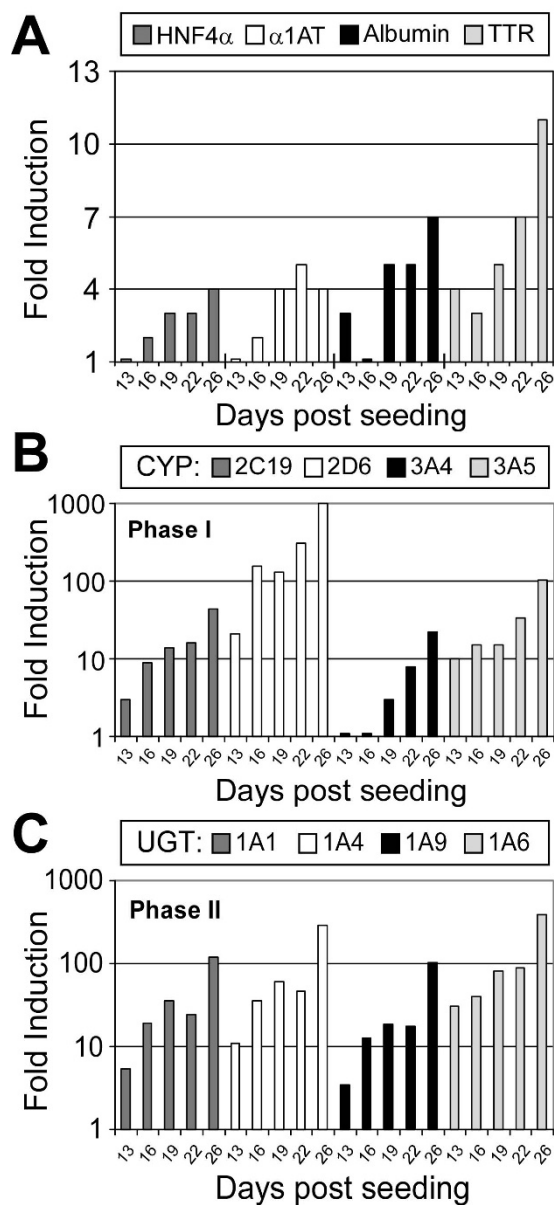


**Figure 1**
**High-fidelity 3-D Huh7 RWV aggregates**. (A) Phase contrast micrograph of Huh7 3-D aggregates cultured in the RWV for 14 days (400×). (B) Fluorescence micrograph of Hoechst-stained OCT sections of 3-D Huh7 aggregates (400×). (C-D) Light micrographs of H&E-stained paraffin sections of 3-D Huh7 aggregates [200× (C), 600× (D)]. (*) = 100 μm microcarrier bead.

### Gene Expression within Huh7 3-D Aggregates
One measure of hepatocyte differentiation is up-regulation of expression of transcription factors such as hepatocyte nuclear factors (HNF) [32,33], which regulate the expression of liver secretory proteins [33] such as albumin [34], alpha-1-antitrypsin (α1AT; [35]), and transthyretin (TTR; [36]). Likewise, induction of enzymes and transporters involved in Phase I and II xenobiotic metabolism [37,38], which include cytochrome P450s (CYPs) and UDP-glucuronosyltransferase (UGTs) enzymes, respectively, is another hallmark of hepatocyte differentiation. Hence, to determine whether culturing Huh7 cells in the RWV allows for cellular differentiation at the transcriptional level, expression of hepatocyte-specific genes, CYPs, and UGTs were analyzed. At indicated times post seeding, total cellular RNA was extracted from 0.5 ml of 3-D Huh7 aggregates or 2-D Huh7 monolayers grown to confluence, and relative gene expression was assessed by RTqPCR analysis. As illustrated in Fig. 2, mRNA levels for the hepatocyte-specific genes and the CYP and UGT enzymes were significantly induced in 3-D Huh7 aggregates (relative to 2-D Huh7 monolayers) and increased in a time-dependent manner while cultured in the RVW.

### Expression and Organization of Cellular Tight Junction and Polarity Markers in 3-D Huh7 Aggregates
While the effect of HCV on cell polarity and TJs (and vice-versa) cannot be accurately studied in 2-D monolayer Huh7 cultures [10], these interactions are of particular
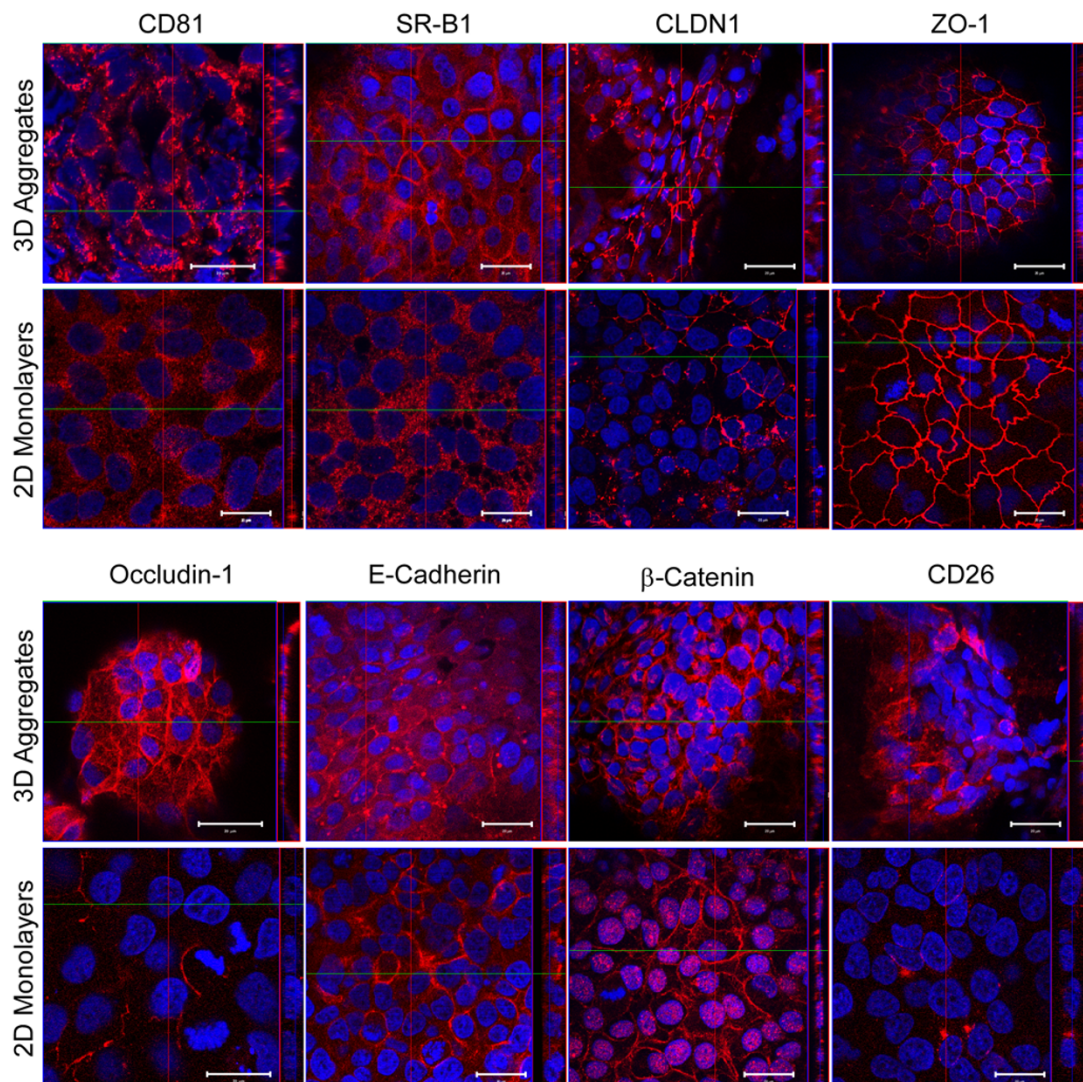
**Figure 2**
**Increased differentiation-specific gene expression in 3-D Huh7 RWV aggregates**. At indicated time points post seeding, 0.5 ml aliquots of 3-D Huh7 aggregates (~5 × 10⁴ cells) were removed from the RWV, pelleted at 1400 RPM for 5 minutes and total RNA extracted. Expression of (A) hepatocyte-specific genes, (B) Phase I (CYP) and (C) Phase II (UGT) metabolic genes in Huh7 3-D aggregates was assessed by RTqPCR. Expression of each transcript, relative to 2-D Huh7 monolayer cultures, was determined using the $2^{-\Delta\Delta C_T}$ method [50], by normalizing to β-actin expression and is graphed as fold induction compared to 2-D monolayers.

interest as TJ proteins are involved in the entry of numerous viruses [39-41] and the TJ proteins CLDN1 [7] and occludin [8,9] have recently been shown to be involved in HCV entry. Therefore, we assessed the expression and organization of the HCV receptors (CD81 and SR-B1), cell adhesion molecules (E-Cadherin and β-Catenin), cellular TJ proteins (CLDN1, ZO-1 and Occludin-1) and the polarity marker (CD26) in 3-D Huh7 aggregates and their 2-D monolayer counterparts (Fig. 3). The expression of known HCV receptors and polarity markers were increased in 3-D Huh7 aggregates as compared 2-D Huh7 monolayers, similar to that observed by Mee et al in polarized Caco-2 cells [10]. This was not a consequence of increased mRNA levels, as normalized transcript levels for all markers examined were similar between 3-D and 2-D Huh7 cultures, as determined by RTqPCR (data not shown).

As expected, the cell adhesion molecules E-Cadherin and β-Catenin were membrane localized both in 2-D and 3-D Huh7 cultures; however, there was a profound decrease in the accumulation of nuclear β-Catenin-containing complexes in the 3-D Huh7 aggregates. Because atypical nuclear localization of β-Catenin in transformed cells has been well documented [42], the loss of this cancer-specific phenotype in the 3-D cultured Huh7 aggregates is consistent with the loss of cancer-specific markers observed in other continuous cell lines when cultured in the RWV [14,23]. Additionally, in contrast to the 2-D Huh7 monolayers, TJ markers localized to apicalateral and/or basolateral planes in the 3-D Huh7 aggregates consistent with localization patterns observed in primary hepatocytes [6,43] and normal liver tissues [44]. Finally, CD26, a cell surface ectopeptidase that localizes to the apical plane of polarized cells [45], was non-detectable in 2-D Huh7 monolayers, while, apical staining of this marker was apparent in distinct areas of 3-D Huh7 aggregates (Fig. 3). Taken together, these data demonstrate that the expression and distribution of cell adhesion and TJ proteins, including known HCV entry receptors, is enhanced and more polarized in 3-D Huh7 cultures than in 2-D monolayers.

### *HCVcc Infection of Huh7 3-D Aggregates*
Because it has been suggested that hepatocyte polarization is inversely related to the permissiveness of the cell for HCVcc infection [10,11], we sought to determine if Huh7 3-D cultures were permissive for HCVcc infection. As such, 3-D Huh7 cultures were inoculated with HCVcc JFH-1 1, 7, or 14 days post RWV-seeding and culture supernatant and cellular RNA were harvested at various time points p.i. for titration of extracellular viral titers and RTqPCR analysis of viral RNA, respectively. Fig. 4A illustrates that HCV not only infected the Huh7 3-D aggregates, but that the kinetics of HCV RNA expansion and

**Figure 3**
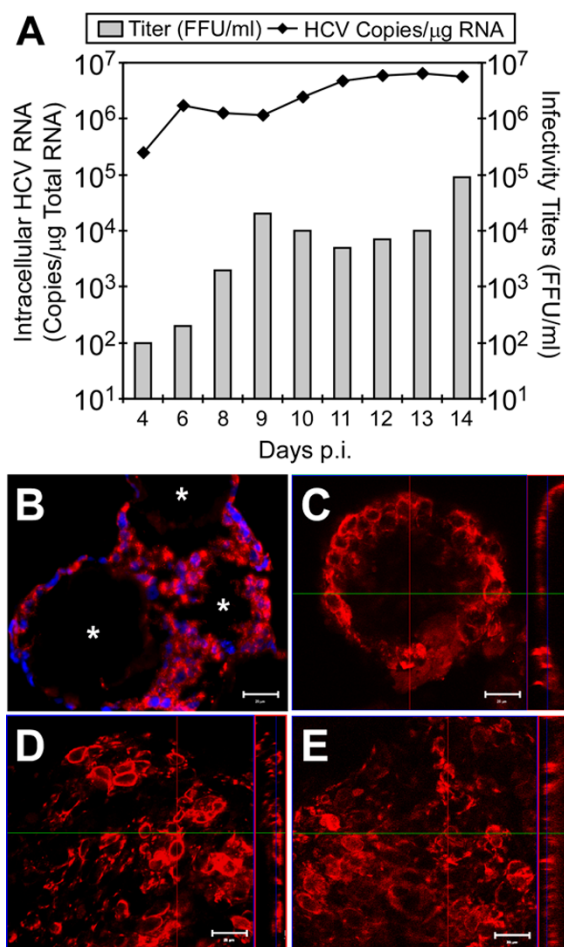**Reorganization of HCV receptor, cell adhesion and tight junction protein localization in 3-D Huh7 aggregates**.
Fourteen days post seeding, Huh7 3-D aggregates and parallel Huh7 2-D confluent monolayers were stained with antibodies specific for SR-BI, CD81, CLDN1, CD26, β-Catenin, E-cadherin, ZO-1 or Occludin and visualized via confocal microscopy (630×, Zeiss LSM 510, Germany). Small vertical panels represent x-z sections (apical = left; basal = right) of larger x-y sections, which were compiled by taking 0.5 μm steps through corresponding x-y sections. Red lines indicate the plane from which the z section was taken. The scale bar equals 20 μm.

infectious virus production increased exponentially to levels comparable to those reported using the robust 2-D Huh7 system [2,46]. To determine the percentage of cells expressing HCV proteins, indirect immunofluorescence analysis of infected 3-D Huh7 aggregates was performed. Fig. 4B shows that the majority of Huh7 cells were positive for the HCV E2 glycoprotein and that the entire aggregate was permissive for HCV infection rather than just the cells at the periphery, demonstrating that HCV can spread throughout the aggregates. Importantly, Fig. 4D and 4E illustrate that aggregates allowed to differentiate in the RWV for 7 or 14 days were as equally permissive for

HCVcc infection as cells infected 1 day post RWV seeding (Fig. 4B-C), suggesting that differentiation and polarization does not negatively affect HCVcc infection in this 3-D cell culture model.

**Discussion**
Here we demonstrate that Huh7 cells cultured in RWV bioreactors form multi-layered tissue-like aggregates that are phenotypically distinct from traditional Huh7 2-D monolayers (Fig. 1 and 2). Specifically, the RWV-environment promoted increases in hepatocyte-specific, as well as Phase I and II metabolic gene transcripts in 3-D Huh7

**Figure 4**
**Robust HCVcc infection in 3-D Huh7 RWV cultures**.
(A) Huh7 3-D aggregates were infected with HCVcc JFH-1 at
an MOI of 0.01 FFU/cell 1 day post seeding in the RWV. Cul-
ture supernatant and intracellular RNA were collected at the
indicated times p.i. Normalized intracellular HCV RNA copy
numbers, displayed as HCV RNA copies/μg total cellular
RNA (line), were determined by RTqPCR. Infectivity titers,
expressed as FFU/ml (bars), were determined by immunohis-
tochemical analysis of 10-fold serially diluted culture superna-
tants on naïve Huh7 cells. (B) Indirect immunofluorescence
analysis of HCV E2 expression in HCV-infected 3-D Huh7
aggregates 14 days p.i. Additional 3-D Huh7 cultures were
infected on day 1 (C), 7 (D) or 14 (E) post seeding in the
RWV. Aggregates were fixed 10 days p.i. and stained with a
human anti-E2 antibody (C1) and anti-human-Alexa 555 sec-
ondary antibody. Images were captured via confocal micros-
copy (630×, Zeiss LSM 510, Germany) and Zeiss LSM Alpha
Imager Browser v4.0 software (Zeiss). Image brightness and
contrast were adjusted using Adobe®Photoshop® (San Jose,
CA). (*) = 100 μm microcarrier bead. Small vertical panels
represent x-z sections of larger x-y sections, which were
compiled by taking 0.5 μm steps through corresponding x-y
sections. Red lines indicate the plane from which the z sec-
tion was taken. The scale bar equals 20 μm.

aggregates relative to Huh7 monolayers (Fig. 2). Addition-
ally, we observed increased expression and organization
of cellular HCV receptors, cell adhesion, tight junction
and polarity-specific proteins, and the loss of cancer-asso-
ciated nuclear localization of β-Catenin, in RWV 3-D
Huh7 aggregates as compared to 2-D monolayers (Fig. 3).
These data therefore suggest that the RWV environment
promotes differentiation of Huh7 cells down a more
hepatocyte-like route. Importantly, since these 3-D Huh7
cultures remain highly permissive for HCVcc infection,
this system represents a new in vitro cell culture system for
the study of HCV infection and antiviral drug studies in
more polarized, xenobiotically-competent cells.

Relevant to the study of HCV, expression of the HCV
receptors CD81 and SR-B1 were both diffuse and poorly
organized in 2-D cultured Huh7s cells, while their expres-
sion was increased and localized to apical TJ regions and
basolateral-sinusoidal surfaces in 3-D aggregates. Like-
wise, TJ proteins, which typically localize to the apical sur-
face in polarized hepatocytes [43], were more
concentrated at the apical surface of 3-D Huh7 aggregates
as compared to monolayer cultures. Notably however, the
TJ protein CLDN1, a recently identified HCV receptor [7],
not only localized to TJs, but was also present at both api-
cal and basolateral surfaces in 3-D aggregates. This locali-
zation pattern is in agreement with other studies [47] and
the model proposed by Reynolds et al., describing tight-
junctional (apical) and nonjunctional (basolateral) forms
of CLDN1 in polarized hepatocytes [44]. As suggested by
Mee et al, it may be that these non-junctional pools of
CLDN1 have a direct role in HCV entry [11]. Interestingly,
Battle et al., have demonstrated a correlation between
HNF4α and cell adhesion and TJ molecules expression
and organization [48]. Whether this is also the case in the
3-D Huh7 aggregates, which have increased HNF4α
expression (Fig. 2A) remains to be determined. Nonethe-
less, the ability of 3-D cultured Huh7 cells to better organ-
ize cell adhesion and TJ proteins is a phenotype consistent
with other RWV-cultured cell types [14,21,23]. As such,
RWV-cultured Huh7 cells provide an appropriate model
for investigating HCV entry, particularly the interaction,
organization, and stoichiometry of HCV receptors and TJ
proteins. Additional analyses to determine the extent of
differentiation and polarization of 3-D Huh7 aggregates is
still warranted and a focus of ongoing studies.

To date, attempts to study HCV in polarized cells have
been limited to colorectal adenocarcinoma Caco-2 cells
[10] or HepG2 cells [11], neither of which support robust
HCVcc infection. Although an inverse relationship
between cell polarization and HCV entry into polarized
Caco-2 [10] and HepG2 [11] cells has been observed no
such phenotype was observed in 3-D Huh7 aggregates.
Specifically, 3-D Huh7 aggregates, infected at various

stages of differentiation (e.g. day 1, 7 or 14 post seeding), were equally permissive for HCVcc infection (Fig. 4B–E). Furthermore, 3-D aggregates treated with PMA, a known disruptor of TJ formation [49], were no more permissive for HCV infection as compared to untreated parallel aggregates (data not shown), suggesting that the TJ barriers formed in 3-D Huh7 aggregates are not inhibitory for HCVcc infection.

## Conclusion

Growing evidence suggests interplay between TJ protein expression, localization and function and HCV infection. Although, the current HCV infectious 2-D Huh7 cell culture system does not amend itself well to elucidating these dynamic relationships, the highly HCV-permissive 3-D Huh7 cell culture system described herein more closely mimics the differentiated and polarized state of hepatocytes. As such the RWV 3-D Huh7 cell culture system should prove useful for understanding the dynamic relationship between HCV and TJ protein expression as well as elucidating how HCV interacts with and disrupts key aspects of hepatocyte physiology.

## Abbreviations

HCV: hepatitis C virus; JFH-1: Japanese Fulminant Hepatitis; RWV: rotating wall vessel; 3-D: three dimensional; 2-D: two-dimensional; HCVcc: hepatitis C virus cell-cultured produced; MOI: multiplicity of infection; FFU: focus forming units; RTqPCR: real-time quantitative PCR; SR-B1: scavenger receptor class B member 1; CLDN1: claudin-1; ZO-1: zona occludens 1; H&E: hematoxylin and eosin; p.i.: post infection; HNF: hepatocyte nuclear factors; α1AT: alpha-1-antitrypisn; TTR: transthyretin; CYP: cytochrome P450s; UGT: UDP-glucuronosyltransferase; TJ: tight junction.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

BS and VT participated in the design of the study, performed the experiments and drafted the manuscript. SLU designed the study and participated in drafting the manuscript. All authors read and approved the final manuscript.

## Acknowledgements

## References

1. Chisari FV: **Unscrambling hepatitis C virus-host interactions.** *Nature* 2005, **436:**930-932.
2. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV: **Robust hepatitis C virus infection in vitro.** *Proc Natl Acad Sci USA* 2005, **102:**9294-9299.
3. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM: **Complete replication of hepatitis C virus in cell culture.** *Science* 2005, **309:**623-626.
4. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ: **Production of infectious hepatitis C virus in tissue culture from a cloned viral genome.** *Nat Med* 2005, **11:**791-796.
5. Moradpour D, Penin F, Rice CM: **Replication of hepatitis C virus.** *Nat Rev Microbiol* 2007, **5:**453-463.
6. Decaens C, Durand M, Grosse B, Cassio D: **Which in vitro models could be best used to study hepatocyte polarity?** *Biol Cell* 2008, **100:**387-398.
7. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, Hatziioannou T, McKeating JA, Bieniasz PD, Rice CM: **Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry.** *Nature* 2007, **446:**801-805.
8. Liu S, Yang W, Shen L, Turner JR, Coyne CB, Wang T: **Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection.** *J Virol* 2009, **83:**2011-2014.
9. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM: **Human occludin is a hepatitis C virus entry factor required for infection of mouse cells.** *Nature* 2009, **457:**882-886.
10. Mee CJ, Grove J, Harris HJ, Hu K, Balfe P, McKeating JA: **Effect of cell polarization on hepatitis C virus entry.** *J Virol* 2008, **82:**461-470.
11. Mee CJ, Harris HJ, Farquhar MJ, Wilson G, Reynolds G, Davis C, van ISC, Balfe P, McKeating JA: **Polarization restricts hepatitis C virus entry into HepG2 hepatoma cells.** *J Virol* 2009, **83:**6211-6221.
12. Schwarz RP, Goodwin TJ, Wolf DA: **Cell culture for three-dimensional modeling in rotating-wall vessels: an application of simulated microgravity.** *J Tissue Cult Methods* 1992, **14:**51-57.
13. 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.
14. Lamarca HL, Ott CM, Honer Zu Bentrup K, Leblanc CL, Pierson DL, Nelson AB, Scandurro AB, Whitley GS, Nickerson CA, Morris CA: **Three-dimensional growth of extravillous cytotrophoblasts promotes differentiation and invasion.** *Placenta* 2005, **26:**709-720.
15. Goodwin TJ, Prewett TL, Wolf DA, Spaulding GF: **Reduced shear stress: a major component in the ability of mammalian tissues to form three-dimensional assemblies in simulated microgravity.** *J Cell Biochem* 1993, **51:**301-311.
16. Gomez-Lechon MJ, Donato MT, Castell JV, Jover R: **Human hepatocytes in primary culture: the choice to investigate drug metabolism in man.** *Curr Drug Metab* 2004, **5:**443-462.
17. Reid LM, Fiorino AS, Sigal SH, Brill S, Holst PA: **Extracellular matrix gradients in the space of Disse: relevance to liver biology.** *Hepatology* 1992, **15:**1198-1203.
18. Zeilinger K, Sauer IM, Pless G, Strobel C, Rudzitis J, Wang A, Nussler AK, Grebe A, Mao L, Auth SH, Unger J, Neuhaus P, Gerlach JC: **Three-dimensional co-culture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions.** *Altern Lab Anim* 2002, **30:**525-538.

19. Freshney RI: *Culture of animal cells: a manual of basic technique* 4th edition. New York, N.Y.: Wiley-Liss; 2000.
20. Unsworth BR, Lelkes PI: **Growing tissues in microgravity.** *Nat Med* 1998, **4**:901-907.
21. Hammond TG, Hammond JM: **Optimized suspension culture: the rotating-wall vessel.** *Am J Physiol Renal Physiol* 2001, **281**:F12-25.
22. Khaoustov VI, Risin D, Pellis NR, Yoffe B: **Microarray analysis of genes differentially expressed in HepG2 cells cultured in simulated microgravity: preliminary report.** In *Vitro Cell Dev Biol Anim* 2001, **37**:84-88.
23. Carterson AJ, Honer zu Bentrup K, Ott CM, Clarke MS, Pierson DL, Vanderburg CR, Buchanan KL, Nickerson CA, Schurr MJ: **A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for Pseudomonas aeruginosa pathogenesis.** *Infect Immun* 2005, **73**:1129-1140.
24. Walther I: **Space bioreactors and their applications.** *Adv Space Biol Med* 2002, **8**:197-213.
25. Gastaminza P, Kapadia SB, Chisari FV: **Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles.** *J Virol* 2006, **80**:11074-11081.
26. Zhong J, Gastaminza P, Chung J, Stamataki Z, Isogawa M, Cheng G, McKeating JA, Chisari FV: **Persistent hepatitis C virus infection in vitro: coevolution of virus and host.** *J Virol* 2006, **80**:11082-11093.
27. Sainz B Jr, Barretto N, Uprichard SL: **Hepatitis C Virus infection in phenotypically distinct Huh7 cell lines.** *PLoS ONE* 2009 in press.
28. Uprichard SL, Chung J, Chisari FV, Wakita T: **Replication of a hepatitis C virus replicon clone in mouse cells.** *Virol J* 2006, **3**:89.
29. Chomczynski P, Sacchi N: **Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.** *Anal Biochem* 1987, **162**:156-159.
30. Choi S, Sainz B Jr, Corcoran P, Uprichard SL, Jeong H: **Characterization of increased drug metabolism activity in dimethyl sulfoxide (DMSO)-treated Huh7 hepatoma cells.** *Xenobiotica* 2009, **39**:205-217.
31. Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamataki Z, Gastaminza P, Chisari FV, Jones IM, Fox RI, Ball JK, McKeating JA, Kneteman NM, Burton DR: **Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge.** *Nat Med* 2008, **14**:25-27.
32. Jochheim A, Hillemann T, Kania G, Scharf J, Attaran M, Manns MP, Wobus AM, Ott M: **Quantitative gene expression profiling reveals a fetal hepatic phenotype of murine ES-derived hepatocytes.** *Int J Dev Biol* 2004, **48**:23-29.
33. Nacer-Cherif H, Bois-Joyeux B, Rousseau GG, Lemaigre FP, Danan JL: **Hepatocyte nuclear factor-6 stimulates transcription of the alpha-fetoprotein gene and synergizes with the retinoic-acid-receptor-related orphan receptor alpha-4.** *Biochem J* 2003, **369**:583-591.
34. Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima A: **Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer.** *Embo J* 1999, **18**:2127-2136.
35. Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J, Whisstock JC: **The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature.** *J Biol Chem* 2001, **276**:33293-33296.
36. Dickson PW, Aldred AR, Menting JG, Marley PD, Sawyer WH, Schreiber G: **Thyroxine transport in choroid plexus.** *J Biol Chem* 1987, **262**:13907-13915.
37. Nakata K, Tanaka Y, Nakano T, Adachi T, Tanaka H, Kaminuma T, Ishikawa T: **Nuclear receptor-mediated transcriptional regulation in Phase I, II, and III xenobiotic metabolizing systems.** *Drug Metab Pharmacokinet* 2006, **21**:437-457.
38. Martinez-Jimenez CP, Jover R, Donato MT, Castell JV, Gomez-Lechon MJ: **Transcriptional regulation and expression of CYP3A4 in hepatocytes.** *Curr Drug Metab* 2007, **8**:185-194.
39. Connolly-Andersen AM, Magnusson KE, Mirazimi A: **Basolateral entry and release of Crimean-Congo hemorrhagic fever virus in polarized MDCK-1 cells.** *J Virol* 2007, **81**:2158-2164.
40. Coyne CB, Bergelson JM: **Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions.** *Cell* 2006, **124**:119-131.
41. Galen B, Cheshenko N, Tuyama A, Ramratnam B, Herold BC: **Access to nectin favors herpes simplex virus infection at the apical surface of polarized human epithelial cells.** *J Virol* 2006, **80**:12209-12218.
42. Levrero M: **Viral hepatitis and liver cancer: the case of hepatitis C.** *Oncogene* 2006, **25**:3834-3847.
43. Stevenson BR, Keon BH: **The tight junction: morphology to molecules.** *Annu Rev Cell Dev Biol* 1998, **14**:89-109.
44. Reynolds GM, Harris HJ, Jennings A, Hu K, Grove J, Lalor PF, Adams DH, Balfe P, Hubscher SG, McKeating JA: **Hepatitis C virus receptor expression in normal and diseased liver tissue.** *Hepatology* 2008, **47**:418-427.
45. Darmoul D, Lacasa M, Baricault L, Marguet D, Sapin C, Trotot P, Barbat A, Trugnan G: **Dipeptidyl peptidase IV (CD 26) gene expression in enterocyte-like colon cancer cell lines HT-29 and Caco-2. Cloning of the complete human coding sequence and changes of dipeptidyl peptidase IV mRNA levels during cell differentiation.** *J Biol Chem* 1992, **267**:4824-4833.
46. Sainz B Jr, Chisari FV: **Production of infectious hepatitis C virus by well-differentiated, growth-arrested human hepatoma-derived cells.** *J Virol* 2006, **80**:10253-10257.
47. Rahner C, Mitic LL, Anderson JM: **Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut.** *Gastroenterology* 2001, **120**:411-422.
48. Battle MA, Konopka G, Parviz F, Gaggl AL, Yang C, Sladek FM, Duncan SA: **Hepatocyte nuclear factor 4alpha orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver.** *Proc Natl Acad Sci USA* 2006, **103**:8419-8424.
49. Schmitt M, Horbach A, Kubitz R, Frilling A, Haussinger D: **Disruption of hepatocellular tight junctions by vascular endothelial growth factor (VEGF): a novel mechanism for tumor invasion.** *J Hepatol* 2004, **41**:274-283.
50. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods* 2001, **25**:402-408.