Efficient hepatitis C virus cell culture system: What a difference the host cell makes

Ralf Bartenschlager* **and Thomas Pietschmann**
Department of Molecular Virology, University of Heidelberg, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany

The availability of a cell culture system is a critical prerequisite to study the replication cycle of a virus and to devise strategies for prophylactic and therapeutic interventions. Such a system depends on two elementary components: an infectable host cell supporting production of infectious virus progeny and a virus that is capable of replicating and assembling infectious particles in these cells. From a technical point of view this is relatively easy to achieve, but what if the virus does not replicate in cultured cells? There are several examples of medically very important viruses that cannot or can only poorly be propagated in cell culture, hepatitis C virus (HCV) being one prominent example. All attempts to culture this pathogen have been faced with major roadblocks that could be overcome only step-by-step. The most recent achievement is a virus production system that is based on the transfection of the human hepatoma cell line Huh-7 with genomic HCV RNA derived from a cloned viral genome (1). Although it was a major step forward, the system was in need of improvement because of limited virus yields and limited spread in cell culture. In a recent issue of PNAS, a study by Zhong *et al.* (2) showed that improvement can be achieved by using particularly permissive cells derived from the human hepatoma cell line Huh-7, yielding virus titers that are ≈50-fold higher and resulting in a more efficient spread of the infection. This is an important observation that broadens the scope of the HCV cell culture system (1) but at the same time raises the important question of what makes this system more efficient.

Host Cell Permissiveness and Cell Culture Adaptations

Numerous examples in the literature document that infection of primary human liver cells and several cell lines with serum-derived HCV is possible and that the viral genome can be kept in some of these cell lines for up to 2 years (reviewed in ref. 3). Nevertheless, in all cases, HCV replication did not exceed copy numbers of 0.01–0.1 RNA genomes per cell, limiting the usefulness of these systems. Moreover, infectivity of serum-derived virus is variable, and the sequences of the genomes used for inoculation usually are unknown. However, only when virus production from cloned genomes is possible, the full power of reverse genetics, in which distinct mutations introduced into the viral genome can be analyzed for their impact on replication and virus production, can be used. For these reasons, much effort was invested to generate HCV by transfection of cultured cells with cloned viral genomes; however, until very recently (1), convincing success had not been reported. A first major obstacle toward an efficient cell culture system was overcome with the invention of the HCV replicon system (4). It is based on the efficient and autonomous replication of viral "minigenomes" into which a selectable marker was inserted. Soon thereafter, it was found that the efficiency of replicon RNA amplification was determined by cell culture adaptive mutations within the viral proteins and by selection for particular cells that are highly permissive (3). The latter conclusion is based on the observation that removal of the replicon from a cell clone by treatment with IFN or a selective drug frequently results in cell clones that support higher levels of HCV RNA replication as compared to naïve Huh-7 cells. The underlying reason for the higher permissiveness is largely unknown, but for one particular cell clone, designated Huh7.5 (5), a single point mutation in the dsRNA sensor retinoic acid-inducible gene-I (RIG-I) was found to be involved in higher permissiveness for HCV RNA replication (6). Activation of RIG-I by dsRNA, such as HCV RNA, results in the phosphorylation and nuclear translocation of IFN regulatory factor-3 (IRF-3), activating innate antiviral defenses. This defect, together with the overall very low expression of the exogenous dsRNA sensor Toll-like receptor 3 in Huh-7 cells (7), could explain why HCV replicates so efficiently in Huh7.5 cells. This is the reason why Zhong *et al.* (2) generated a Huh7.5-derived cell line and used it for their study.

A single point mutation is involved in higher permissiveness for hepatitis C virus RNA replication.

The second observation on which their study is built stems from the recent isolation of an HCV genome designated JFH-1 (for Japan fulminant hepatitis) that replicates to very high levels in Huh-7 cells without requirement for cell culture adaptive mutations (8). This is an important prerequisite for establishment of a complete cell culture system because these mutations were found to interfere with virus production.† Consequently, transfection of the full-length JFH-1 genome into Huh-7 cells leads to the production of virus particles infectious for Huh-7 cells in an E2- and CD81-dependent manner (1).

Virus Production in Huh7.5.1 Cells

Taking advantage of the JFH-1 genome and a highly permissive Huh7.5-derived cell line designated Huh7.5.1, Zhong *et al.* (2) made the perplexing observation that transfected cells released up to 10^5 focus-forming units (ffu) per ml, as determined by the average number of HCV protein (NS5A)-positive foci detected by immunofluorescence analysis of cells infected with the highest virus dilution. This number is ≈50-fold higher as compared to a previous study that also used the same JFH-1 isolate but naïve Huh-7 cells as well as another Huh-7 cell clone (1). The difference appears to be due at least in part to the higher permissiveness of Huh7.5.1 cells, but what renders these cells more permissive, and which stage(s) of the replication cycle is affected? Different scenarios can be envisioned: Huh7.5 cells (from which Huh7.5.1 are derived) have a defect in the RIG-I pathway, making them less responsive to intracellular dsRNA, generated during virus

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*To whom correspondence should be addressed. E-mail: ralf_bartenschlager@med.uni-heidelberg.de.

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replication and inducing an antiviral program (6). The reduced efficiency of the host cell's innate defenses could explain the higher permissiveness. Alternatively, Huh7.5.1 cells may display a higher density of virus (co)receptors allowing attachment and entry to proceed with greatly enhanced efficiency. Also, virus assembly and egress could be more efficient in this particular host cell.

Possible Role for Cell Culture Adaptation of JFH-1

Apart from that, there is an additional possibility emerging from a careful analysis of the kinetics through which infectious virus is released from JFH-1-transfected Huh7.5.1 cells (2). In spite of efficient RNA replication, release of infectious particles was low up to day 11 after electroporation (≈50 ffu/ml). However, a sharp rise in release of infectivity was observed thereafter, first detected ≈14 days after transfection and reaching peak titers in the range of 5×10^4 ffu/ml at 21 days after transfection. This kinetic would be compatible with the emergence of cell-culture-adapted JFH-1 variant(s) carrying mutations that enhance formation and release of infectious virus and thereby accelerate spread of infection in the culture. In agreement with this assumption, infection of Huh7.5.1 cells with virus harvested from cells 19 or 24 days after transfection resulted in high-titer release of infectivity within 3 days after infection, indicating that adapted variants were already present in the inoculum. Although the route of inoculation (RNA transfection versus infection) may have a strong impact on the kinetics of release of infectious HCV particles, the possibility of cell culture adaptation has not been considered, and no sequence analysis of progeny virus was performed. However, this is an important prerequisite to understand the reason for the efficiency of this virus system. Cell culture adaptation has been described for numerous other viruses, and it would well explain the delayed kinetics of high-titer HCV release from transfected cells and the rapid subsequent virus spread.

Characteristics of Cell Culture-Grown HCV

Building on the efficiency of their culture system, Zhong *et al.* (2) demonstrate passage of the virus in Huh7.5.1 cells without loss of virus titer, and they confirmed previous observations that JFH-1 infection can be neutralized by antibodies directed against CD81 (1, †), a molecule that appears to be critically involved in the infection process (9). Moreover, infectivity could be partially neutralized by using a monoclonal antibody directed against envelope protein 2, confirming the studies performed with HCV pseudoparticles and showing that E2 is important for infection (10, 11). Cell culture-grown infectious virus had a surprisingly homogenous density of ≈1.105 g/ml, which is at variance with the heterogeneity of densities described for virus present in patient sera. This finding may be due to the lower association of culture-grown HCV with lipoproteins and antibodies.

Infectivity could be partially neutralized by using a monoclonal antibody against envelope protein 2.

Attempts to infect cell lines other than Huh-7 were not successful. However, as deduced from the low G418-transduction efficiency achieved with selectable replicons and the inability to detect transient HCV RNA replication, these cells have a very limited permissiveness. Therefore, the negative results could be due to a block of infection or merely reflect the limited amplification of viral RNA and protein that is, however, a prerequisite for detection of infected cells. Nevertheless, HCV could also be propagated in naïve Huh-7 cells upon inoculation with virus that was produced in Huh7.5.1 cells. Interestingly, virus release from naïve cells was greatly delayed, and no infectivity could be detected in supernatant of infected Huh-7 cells up to 6 days after inoculation. Thereafter, titers of released infectivity increased ≈500-fold reaching levels comparable to Huh7.5.1 cells that were inoculated in parallel. Whether this is linked to a less efficient infection, for instance, because of lower expression level of (co)receptor molecules or a dsRNA-induced antiviral response operating in naïve Huh-7 cells and delaying spread of virus infection, is not known. If it is an innate immune response that initially controls HCV replication, it appears to be overcome at a later time point, which could be explained by the accumulation of viral proteins interfering with this antiviral program in infected cells. A prime candidate effector is the NS3/4A protease shown to block the dsRNA-induced phosphorylation of IRF-3 (12). Alternatively, HCV genomes replicating in infected naïve Huh-7 cells may have acquired mutations facilitating virus formation and spread in these cells. Unfortunately, passage of the virus using only naïve Huh-7 cells was not done. If adaptation plays a role one would expect a much faster kinetic of virus release and spread upon secondary infection.

Conclusions

In summary, the study by Zhong *et al.* (2) describes a simple and robust HCV cell culture system. Key to the improvement over the previous study was the use of a highly permissive Huh-7 cell clone enhancing titres of (eventually adapted) viruses and spread of infection. Although several questions remain unanswered, this new system illustrates that HCV research has entered an era of classical virology.

Note Added in Proof. While this Commentary was under consideration, Lindenbach *et al.* (13) described the complete replication of chimeric HCV in Huh7.5 cells.

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