Chapter 2 Cell Culture Systems for Hepatitis C Virus

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Abstract Due to the obligatory intracellular life style of viruses, cell culture systems for efficient viral propagation are crucial to obtain a detailed understanding of the virus host cell interaction. For hepatitis C virus (HCV) the development of permissive and authentic culture models has been and continues to be a challenging task. The first efforts to culture HCV had limited success and range back to before the virus was molecularly cloned in 1989. Since then several major breakthroughs have gradually overcome limitations in culturing the virus and sequentially permitted analysis of viral RNA replication, cell entry and ultimately the complete replication cycle in cultured cells in 2005. Until today, basic and applied HCV research greatly benefit from these tremendous efforts which spurred multiple complementary cell based model systems for distinct steps of the HCV replication cycle. When used in combination they now permit deep insights into the fascinating biology of HCV and its interplay with the host cell. In fact drug development has been much facilitated and our understanding of the molecular determinants of HCV replication has grown in parallel to these advances. Building on this groundwork and further refining our cellular models to better mimic the architecture, polarization and differentiation of natural hepatocytes should reveal novel unique aspects of HCV replication. Ultimately, models to culture primary HCV isolates across all genotypes may teach us important new lessons about viral functional adaptations that have evolved in exchange with its human host and that may explain the variable natural course of hepatitis C.

Abbreviations

BMEC	Brain microvascular endothelial cells
CNS	central nervous system
Con1	Consensus genome 1
DAA	Direct acting antiviral
DC-SIGN	dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
EGFR	Epidermal growth factor receptor
EMCV	Encephalomyocarditis virus
EphA2	Ephrin receptor A2
GFP	Green fluorescent protein
HCV	Hepatitis C virus
HCV_{TCP}	Hepatitis C virus trans-complemented particles
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
iPSC	induced pluripotent stem cells
IRES	Internal ribosomal entry site
JFH1	Japanese fulminant hepatitis
LDL-R	Low density lipoprotein receptor
MEF	Mouse embryonic fibroblasts
MPCC	Micropattern co-cultures
mL	Milliliter
MLV	Murine leukemia virus
NPC1L1	Niemann-Pick C1-like cholesterol adsorption receptor
PBMC	Peripheral blood mononuclear cells
PHH	Primary human hepatocytes
REM	Replication enhancing mutations
RIG-I	Retinoic acid-inducible gene I
SEAP	Secreted embryonic alkaline phosphatase
siRNA	small interfering RNAs
$TCID_{50}$	Tissue culture infectious dose 50
VSV	Vesicular stomatitis virus

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2.1 Introduction

When HCV, the causative agent of hepatitis C, was first cloned in 1989 many attempts to culture the elusive infectious agent *in vitro* had already failed. These efforts were a prelude of the hurdles ahead to push the doors open for model systems fully permissive to cell culture replication of HCV. Some of these early limitations, like for instance the inefficient replication of primary HCV isolates, prevail until today. However, during the past decades several breakthrough developments have much improved our repertoire to study this virus *in vitro*. In fact, our increasing knowledge of molecular replication mechanisms may help to overcome the remaining roadblocks that prevent us from analysing the complex interplay of HCV with its host cells in a yet further refined fashion.
The development of HCV-permissive cell culture models was a step-wise process. The establishment of subgenomic replicons that autonomously amplify in cultured human hepatoma cells was a first major breakthrough. Another important achievement was the generation of infectious retroviral pseudotypes displaying functional HCV glycoproteins for the study of HCV entry. Finally, the identification of a novel HCV isolate, termed JFH1, paved the way for the production of infectious virions to investigate all steps of the viral life cycle. Recently, remarkable advances were also made with regard to measuring HCV infection and replication in primary cell cultures. In this chapter, we will highlight essential components of HCV cell culture models and provide an overview of viral adaptation to replication in cell culture. In addition, we attempt to provide a perspective on future developments that may help to unravel new features of the HCV host cell interaction.
Historically, three key achievements build the foundation of the most widely used HCV tissue culture systems. Besides these models described below in greater detail, during the past years a variety of additional cell based systems to monitor HCV cell entry and receptor interactions have been reported. The interested reader is referred to a recent review for a detailed description of these systems . Details on the HCV entry pathway are reviewed in Chapter 4.

2.2 HCV replicon system

During the 1990ies, numerous attempts were made to initiate robust HCV infection and replication in cultured cells after inoculation with patient sera or transfection with cloned viral RNA. Although in part long term productive HCV replication was reported, these experimental systems suffered from low replication efficiency . Highly sensitive, but also error-prone techniques, like RT-PCR were necessary to document HCV replication and only small amounts of viral proteins or infectious virus were produced precluding molecular dissection of HCV replication mechanisms. In fact, until today replication of the vast majority of cloned HCV genomes is poor in cultured cells (see also below section 2.4).
Encouraged by reports that subgenomic RNA molecules of other plus strand RNA viruses readily replicate in transfected cells (Khromykh and Westaway 1997; Mittelholzer, Moser et al. 1997), similar approaches were attempted for HCV. By trimming the HCV genome to those components essential for RNA-replication (see Chapter 7), so called replicons were created . Due to deletion of viral structural genes (core, envelope 1 and envelope 2), p7 and NS2 these RNA molecules were much smaller than the authentic viral genome. This provided the freedom to insert a heterologous dominant selectable marker (e.g. neomycin phosphotransferase, neo) without exceeding the natural length of the HCV genome. The prototype replicon was a bicistronic RNA of genotype 1b (Con1 isolate) encoding a neomycin resistance gene under the control of the HCV internal ribosomal entry site (IRES), followed by a second IRES from Encephalomyocarditis virus (EMCV) that controlled expression of the genes for NS3-NS5B. Upon transfection of synthetic RNAs derived from such a construct into the human hepatoma cell line Huh-7 and G418 selection, cell lines containing high amounts of self-replicating HCV RNAs could be obtained . Based on quantification by Northern hybridization, an average copy number of 1000 to 5000 positive strand RNA molecules per cell was determined. Minus-strand RNA was present in about 10-fold lower amounts in comparison with plus strand RNA and HCV protein expression was readily detected by metabolic radiolabeling and immunopreciptaion . Replicon cell clones continuously passaged under selective pressure maintain the

viral RNA for many years. After the introduction of the replicon system in 1999 this cell culture system has been widely applied in HCV research. During the following years an increasing number of replicon constructs with varying reporter genes including luciferases and fluorescent proteins was developed to tailor the system to the needs of the researcher and to facilitate exploration of the mechanisms of HCV RNA replication. A detailed summary of replicon constructs currently in use was compiled by Bartenschlager et al. . While initially replicons were developed for the genotype 1b (GT1b) consensus genome Con1 (Lohmann, Korner et al. 1999), meanwhile replicons are available for GT1a, various GT1b isolates, GT2a and GT4a strains (Table 1), thus increasing the versatility of this important model tremendously.

2.2.1 Replication enhancing mutations

The HCV RNA dependent RNA polymerase NS5B lacks a proofreading activity and as observed for many other viruses HCV replicates with a high mutation rate (see Chapter 7). Initially, genotype 1b replicons showed a low G418 transduction efficiency despite of high level of RNA replication within the surviving cell clones. It turned out that the reason for this was twofold. First, during the selection procedure replicons acquired so-called replication enhancing mutations (REMs) permitting more efficient RNA replication in transfected Huh-7 cells. Second, the selection process enriched for those few host cells in the total population of transfected cells that were more permissive to HCV replication than "standard" Huh-7 cells. Evidence for this second mechanism was elegantly provided by transfection of replicons into individual Huh-7 clones that had been obtained after transfection and selection with selectable replicons and subsequent purging of the replicon by inhibitor or IFN-treatment . In fact, the most widely used host cells for HCV research -Huh-7.5, Huh-7.5.1 and Huh7-Lunet- all were obtained by this strategy .
While for most of these highly permissive cells it remains elusive why they are so amenable for HCV replication, viral adaptation permitting increased replication has been linked to distinct mutations within individual non-structural proteins. These mutations have ori-

ginally been designated 'cell culture adaptive mutations', but should be renamed as 'replication enhancing mutations' (REMs) in order to discriminate them from cell culture adaptive mutations that increase virus titers without affecting replication . Sequence analysis of replicons within selected cell clones identified numerous conserved changes within the coding region of the viral non-structural proteins. Introduction of these mutations back into the parental genome and transfection of *in vitro* transcribed RNA revealed an enhancement of RNA replication to various degrees as determined by the number of G418-resistant colonies .

Replication enhancing mutations were mainly located in the N-terminus of NS3, at two distinct amino acids in NS4B and in the central domain of NS5A. Several of the most potent replication enhancing mutations in NS5A change phosphorylation sites within the protein suggesting that replication efficiency may be regulated via phosphorylation. Interestingly, Evans et al. observed an interaction between HCV NS5A and human vesicle-associated membrane protein-associated protein A (hVAP-A) which is modulated by NS5A phosphorylation . Their findings support a model where NS5A hyperphosphorylation disrupts the interaction with h-VAP-A which negatively regulates viral RNA replication . With the generation of replicons from other HCV isolates it could be shown that Con1 adaptive mutations also enhanced replication efficiency of other genotype 1b strains, including HCV-O , HCV-BK , J4 and AH1 . In case of the HCV N-isolate adaptive mutations were not required for efficient replication due to a unique four amino acid insertion naturally present in NS5A .

The establishment of genotype 1a replicons turned out to be more difficult as even the introduction of genotype 1b-specific mutations did not result in high levels of RNA replication . However, with passage of genotype 1a replicon RNA in highly permissive cell lines replication-enhancing mutations could be identified in NS3, NS4B or NS5A . Generation of non-genotype 1 replicons have not been described so far except for a genotype 4a (B. Peng 2012) and genotype 2a isolate that replicates with high efficiency without the requirement of adaptive mutations . The latter genome was cloned from a Japanese patient suffering a fulminant course of hepatitis and thus designated "Japanese fulminant hepatitis 1" (JFH1). This genome has become the basis of the most widely used HCV cell culture system which will be described in more detail below (see section 2.4). With the identifications of REMs further improvements of the original replicon system could be developed. These include alternative drug resistance genes , monocistronic replicons and transient replication assays that are based on the detection of reporter genes like luciferase, ß-lactamase, green fluorescent protein (GFP) and secreted alkaline phospatase . The exact mode of action of cell culture replication enhancing mutations is still not fully understood. Generally, these mutations have not been observed in natural HCV isolates suggesting that the stimulatory effect on HCV RNA replication *in vitro* does not increase viral fitness *in vivo*. In fact, at least for Con1 evidence has been provided that REMs interfere with production of infection virus und viral spread *in vitro* and *in vivo* and see also below section 2.4).

2.3 Retroviral pseudoparticles

In the absence of an efficient cell culture system encompassing the entire life cycle of the virus, surrogate models were developed that were useful to study the role of HCV glycoproteins in virus entry (see Chapter 4). The most successful among the models to investigate early steps of HCV infection was the establishment of retroviral pseudotypes bearing unmodified HCV glycoproteins (HCVpp) . This system is based on the co-transfection of 293T cells with expression vectors encoding HCV E1 and E2, the gag-pol proteins of either murine leukemia virus (MLV) or human immunodeficiency virus (HIV) and a retroviral genome encoding a reporter gene. Entry of these particles leads to the delivery of the retroviral capsid into the cytoplasm of the target cell following reverse transcription and integration of the viral genome into the host cell genome. The reporter gene is expressed by the integrated provirus to detect productive entry events in a rapid manner. Importantly, attachment and receptor interaction of these retroviral pseudotypes is governed by the functional HCV E1-E2 protein complex incorporated into the envelope of these particles. Therefore, HCVpp's can be neutralized with antibodies targeting the viral glycoproteins E1, E2 and with sera of infected patients . The incorporation of patient-derived glycoproteins has also been described for HCVpp and can be used to study cross-neutralizing antibodies . Moreover, utilisation of HCVpp is an elegant way of analysing HCV cell entry independent of the other parts of the viral life cycle. Consequently, this system offers the freedom to investigate cell entry into cells that are not permissive to HCV RNA replication. Various viral entry attachment factors and receptors have been identified or verified using this system including glycosaminoglycans, low-density lipoprotein receptor (LDL-R), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), claudin-1, claudin-6, claudin-9, occludin and recently also epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) and the Niemann-Pick C1-like cholesterol adsorption receptor (NPC1L1) (for review see (Ploss and Evans 2012)). A limitation of the HCVpp system is that these particles are produced in a non-liver cell line (293T) and that they assemble in post-Golgi compartments and/or the plasma membrane as retroviruses do. Therefore, the close association of HCV particles with lipoproteins cannot be reproduced which may affect studies including antibody neutralization assays and entry studies with lipid receptors LDL-R, SR-BI and NPC1L1. However, this "limitation" offers the exciting opportunity to learn about the relevance of these host derived modifications of HCV particles by directly comparing cell entry properties of HCVpp with natural HCV particles.

In summary, although not covered with lipoproteins, HCVpps can be used to study viral entry events of HCV independent of RNA replication and assembly and have been a valuable tool to identify cellular entry molecules.

2.3.1 Other models to study individual steps of HCV cell entry

In addition to HCVpp, additional model systems have been developed to study HCV entry. These include the most widely used cell culture model systems HCVcc and HCV_{TCP} that are covered in separate sections. For the identification of cellular receptors C-terminally truncated secreted forms or cell surface expressed versions of the glycoprotein E2 have been described . One of the first tools

used to study HCV cell entry and to discover receptors involved in this pathway was a soluble and truncated form of the E2 glycoprotein ($sE2_{661}$), in which the last 85 amino acids, encompassing the hydrophobic transmembrane domain, are deleted . It is not clear to which degree this truncated form reflects the proper folding of E2 in the context of the E1/E2 complexes within the HCV envelope (see also Chapter 5). However, the discovery of CD81 and SR-BI as part of the HCV receptor complex was achieved using $sE2_{661}$. Current approaches to solve the structure of the E2 glycoprotein are based on differently truncated forms of the E2 protein (GreyKrey, d'Alayer et al. 2010; McCaffrey, Gouklani et al. 2011). Further surrogate models to study glycoprotein and receptor interaction as well as early entry events include E1-E2 liposomes , virus-like particles generated in insect cells and vesicular stomatitis virus (VSV) pseudotyped with chimeric glycoproteins consisting of the ectodomains of HCV E1 and E2 fused to the transmembrane domain of the VSV-G glycoprotein . More recently, a soluble form of E2 was reported that blocks HCVcc entry and is produced in mammalian or insect cells .
Virus binding to the cellular surface can be measured by quantification of cell-bound RNA copy numbers (Vieyres, Angus et al. 2009; Calland, Albecka et al. 2012) or by radioactive labelling of HCV virions (Ciesek, von Hahn et al. 2011). After cell binding, receptor interactions and conformational changes in the glycoproteins the virus is taken up by endocytosis (Ploss and Evans 2012). Molecular inhibitors like small interfering RNAs (siRNAs) and dominant-negative constructs have been applied in addition to the use of chemical inhibitors in specifically blocking distinct stages of endocytosis . With respect to HCV, trafficking, acidification and clathrin-mediated endocytosis has been studied with different inhibitors . Recently, Coller et al developed infectious fluorescent particles to visualize the association of HCV virions with the endocytosis maschinery . They labeled particles with membrane-permeable lipophilic dyes, called DiD (1,1´dioctadecyl-3,3,3´,3´tetramethyl-indodicarbocyanine 4-chlorobenzenesulfonate salt) and DiI (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate), that can be used to study HCV-cell interactions in the HCV entry process . Uptake of HCV particles can also be analyzed by protease treatment.

Internalized viral particles are resistant to proteolysis whereas viruses remaining on the cell surface are inactivated .
As structural information about the HCV glycoproteins are lacking, the fusion process is not fully understood at a molecular level. Several different fusion assays have been designed that rely on cell to cell fusion or fusion between HCVpp/HCVcc and liposomes or target cells. The 'cell to cell' fusion assay is based on 293T cells that ectopically express the HCV glycoproteins and that encode a T7-polymerase dependent GFP gene. These cells are co-cultured with Huh7 cells expressing the T7-polymerase and successful fusion results in multinucleated cells expressing the GFP reporter gene . Modifications of this system for example with luciferase as reporter gene have been developed . Productive fusion *in vitro* can be also monitored with fluorescent probes that are incorporated into either the virus particles or liposomes and upon fusion membrane mixing results in fluorescence dequenching and emission . Recently, another fluorescence-based fusion assay was developed in which HCVcc viruses were labeled with the hydrophobic DiD fluorophore that inserts into the membrane at self-quenching doses. After fusion of viral and target membranes the DiD fluorophores diffuse away causing dequenching which can be monitored in real time (Sainz, Barretto et al. 2012).
It has been reported that HCV can also be transmitted via cell-to-cell spread. This mode of transmission may be particularly relevant *in vivo* in the context of infected liver tissue. It was reported that infection via cell to cell spread was refractory to neutralization by E2 monoclonal antibodies and that it may occur in a CD81-independent manner . Cell-to-cell spread of HCV can be studied by co-culturing HCV-positive donor cells with target cells that can be monitored by fluorescent markers. Cell-free spread can be blocked by the presence of neutralizing antibodies or an agarose overlay (Timpe, Stamataki et al. 2008; Witteveldt, Evans et al. 2009; Brimacombe, Grove et al. 2011). A recently described cell-based reporter system that is based on NS3/4A-mediated cleavage of a fluorescent substrate can also be applied to investigate this route of transmission at a single cell level (Jones, Catanese et al. 2010). Combination of this system with an agarose overlay facilitates this assay setup (Ciesek, von Hahn et al. 2011; Ciesek, Westhaus et al. 2011). Instead of using two distinct

cell populations, quantification of the number of infected cells per infection focus under an agarose overlay similarly allows to study cell-to-cell spread (Baldick, Wichroski et al. 2010; Calland, Albecka et al. 2012)

2.4 Cell culture infectious HCV genomes and host cells

Initial attempts of transfecting genomic *in vitro* transcripts of HCV derived from cloned viral genomes into human liver cells were unsuccessful, due to non-functional sequences or mutations introduced by RT-PCR. To circumvent these problems, consensus genomes were constructed which were based on a master sequence that is representative of the dominant nucleotide sequences at each position of the genome. The consensus sequence was established by sequencing of multiple clones of a single isolate which then guided construction a consensus genome based on this sequence information. The first constructs generated by this procedure were derived from a patient designated “H77” who had been infected with a genotype 1a virus . Importantly, intrahepatic inoculation of these consensus RNA genomes into chimpanzees initiated a productive infection of the animals (for detailed description of HCV animal models see Chapter 3). This evidence provided formal proof that indeed these H77 consensus genomes are functional and infectious *in vivo* . However, despite of the availability of these and a few other consensus genomes with proven infectivity *in vivo*, attempts to initiate robust replication and production of infectious progeny with these genomes were initially not fruitful.
After the successful construction of autonomously replicating selectable subgenomic Con1-derived replicons, we added the viral structural genes and thus created selectable full-length genomes expressing the complete HCV open reading frame of this isolate. Adaptive mutations initially identified with the subgenomic replicons were added to increase RNA replication and virus protein expression. While these selectable genomes as well as authentic genomes with only the adaptive mutations but no selectable marker replicated relatively efficiently, neither stable cell lines harbouring selectable full length replicons of Con1, nor the transiently replicating Con1 full length

RNAs gave rise to infectious HCV progeny . Likewise full length RNA of the HCV N strain (GT1b) did not support the production of infectious particles . It was suggested that either the host cells lack factors important for particle formation or that REMs interfere with the production of infectious particles. In line with the second hypothesis, replication-promoting mutations selected for in tissue culture experiments are rarely found in HCV sequences from human or chimpanzee . Pietschmann et al. recently reported that those very same replication enhancing mutations which were used to increase the replication capacity of Con1 genomes actually interfered with production of infectious virus . In fact most of the Con1-replication enhancing changes within the viral non-structural proteins stimulated replication at the expense of production of infectious particles. This observation was a first hint suggesting that the non-structural proteins contribute to production of infectious HCV, an observation which was much refined and extended using the infectious JFH1 system (Jones, Murray et al. 2007; Steinmann, Penin et al. 2007; Yi, Ma et al. 2007; Appel, Zayas et al. 2008; Ma, Yates et al. 2008; Jones, Patel et al. 2009; Phan, Beran et al. 2009). Besides this, the observation that REMs can interfere with virus production in Con1 genomes provided a simple explanation why the adapted Con1 genomes unlike the wild type were non-infectious in Chimpanzee or reverted back to the wild type sequence . It is, however, important to stress here that REMs not necessarily lead to inactivation of virus production. This is exemplified by one of the most potent adaptive changes within the Con1 replicase (i.e. the K1846T exchange within the NS4B protein of Con1) that does not interfere with production of infectious particles (Pietschmann, Zayas et al. 2009). Even more striking, an adapted H77 genome designated H77S has been generated that carries multiple mutations which substantially enhance virus production and at the same time permit production of infectious virus . Thus, there are apparently different modes of increasing replication fitness of HCV consensus genomes in tissue culture, some of which interfere with production of virus particles. Certainly, gaining a deeper understanding of how REMs enhance replication in tissue culture is an important challenge for future research.

When Kato and colleagues in 2001 reported construction of a subgenomic replicon termed JFH1 which replicated with very high efficiency and without the requirement of adaptive mutations , this observation was a major finding itself. However, it turned out to be the prelude of a yet more important major breakthrough in HCV research: A few years later, three groups reported that the complete wild type JFH1 genome or chimeras consisting of the JFH1 replicase genes NS3-NS5B and Core to NS2 regions of alternative HCV genomes replicated efficiently in Huh-7 cells and produced infectious viral progeny both in tissue culture and in animal models . These particles were designated cell culture derived HCV (HCVcc) and they are now routinely used in many laboratories. Each step of the viral life cycle can be studied with this system including viral entry, replication and also the late events like genome packaging, virion assembly, maturation and release. Immunoelectron microscopy with E2-specific antibodies demonstrated the presence of spherical particles with 50 to 65 diameter and cell culture viruses had a density profile comparable to serum-derived viruses (for detailed description of HCV particles see Chapter 8). The authenticity of recombinant virus particles was confirmed by demonstrating HCVcc infectivity in chimpanzees and in mice containing human liver xenografts (Chapter 3). Interestingly, the specific infectivity of HCVcc recovered after passage *in vivo* was increased in comparison to cell culture produced viruses . The highly infectious nature of the animal-derived viruses correlated with a lower buoyant density compared to cell culture derived HCV. Interestingly, these features (lower density and higher infectivity) were lost after a single round of passaging in cell culture, suggesting that modifications that were not fixed within the viral genome were responsible for these alterations. Infection could be neutralized with patient sera or E2 and CD81 specific antibodies. Importantly, HCVcc were sensitive to inhibitors targeting the viral protease and polymerase as well as to interferon-α .

As the JFH1-based infection system belongs to genotype 2a, a major challenge is still the generation of molecular clones from other genotype supporting production of infectious virus in cell culture. As alluded to above, Yi et al. reported in 2006 an infectious clone of the genotype 1a designated H77S that contains five REMs . These muta-

tions were selected through a tedious and iterative process of adaptation of subgenomic H77 replicons and are located therefore in the non-structural genes . However, virus titers that were recovered from transfected Huh7.5 cells were about 100 to 1,000-fold lower compared to the JFH1 system. Passaging of the full-length H77S genome did not result in increased viral titers as observed for JFH1, probably due to a lower replication efficiency of H77S .
As mentioned above, transfection of a full-length wildtype Con1 clone resulted in a transient release of virus particles which was blocked by cell culture adaptive mutations in NS5A or NS3 . Recently, another genotype 1b isolate (NC1) from a patient with acute severe hepatitis was described that shares 91% nucleotide and 94% amino acid sequence homology with the Con1 isolate (Date, Morikawa et al. 2012). The replication efficiency of a NC1 subgenomic replicon was lower compared to JFH1 but could be enhanced with the introduction of REMs. After transfection of full-length RNA only cells harbouring genomes with NS5A mutations S2197 or S2204G showed significant amounts of core protein secreted into the cell culture supernatant. Next, the authors combined the identified REMs with previously described mutations in NS3 and NS4B to increase replication and virus production. Enhanced production of infectious particles was observed, however, the efficiency was not sufficient for autonomous virus propagation in cell culture and for infectivity studies *in vivo* (Date, Morikawa et al. 2012).

2.4.1 JFH1 and chimeric genomes

Although these infection models described so far are an important achievement permitting studies of the complete HCV replication cycle in cell culture, these systems are restricted to specific isolates and limit comparable studies of all HCV genotypes. To overcome this restraint, a comprehensive panel of chimeric genomes was constructed by combining the JFH1 isolate with heterologous strains of all major HCV genotypes (Table 1). In most cases, the replicase proteins necessary for generating the membrane-bound replicase complex and non-translated regions are derived from the highly efficient JFH1 strain. The proteins Core to NS2 which are required for viral morphogenesis (Chapter 8) are derived from another genotype. With this strategy the yield of HCVcc particles was subsequently enhanced by creating an intragenotypic chimera using the C-NS2 part of a different genotype 2a isolate, J6 . To analyse whether it is also possible to generate intergenotypic chimeras, an analogous chimeric genome that carried the Core to NS2 part of the GT 1b Con1 isolate was constructed . However, although this genome produced infectious HCV, virus titers were very low probably due to incompatibilities between the Con1 (GT1b) and JFH1 (GT2a) proteins. Therefore, a series of intergenotypic reporter chimeras were generated with different cross-over sites varying from the C-terminus of E2 to the NS2-NS3 cleavage site. The results of this mapping analysis identified a cross-over site located right after the first trans-membrane domain of NS2 as the best choice for construction of infectious JFH1-Con1 chimeras JFH-J6 and JFH1-H77 chimeras . Similar studies describing the generation of chimeric genomes of genotype 3a, 4a, 5a and finally all major seven genotypes have been reported (Pietschmann, Kaul et al. 2006; Gottwein, Scheel et al. 2007; McMullan, Grakoui et al. 2007; Yi, Ma et al. 2007; Jensen, Gottwein et al. 2008; Scheel, Gottwein et al. 2008; Gottwein, Scheel et al. 2009; Gottwein, Jensen et al. 2011; Scheel, Gottwein et al. 2011). These chimeric genomes were shown to be highly useful to study entry, neutralization and virus assembly of all seven known HCV genotypes. They have been further validated to be infectious *in vivo* as human liver-chimeric mice developed high-titer infections after inoculation with HCV of genotypes 1-6 (Bukh, Meuleman et al. 2010). Highest virus titers could be achieved with a J6-JFH1 chimera designated Jc1 that allows the production of virus particles of about 10^6 infectious units per ml .

Further improvements were the construction of reporter genomes of different HCV chimeric genomes (luciferase or GFP) for rapid and sensitive detection of replication or infection (Koutsoudakis, Kaul et al. 2006; Tscherne, Jones et al. 2006; Schaller, Appel et al. 2007; Gottwein, Jensen et al. 2011; Reiss, Rebhan et al. 2011) (recently summarized in .

The idea of chimeric genomes was further expanded to also include non-structural proteins. As mentioned above, HCV replicons have only been described for genotype 1 and 2 and efficiency or resistance of direct acting antiviral agents (DAAs) targeting non-structural proteins could not be tested for all genotypes. Recently, the construction of viable JFH1-based chimeras in which sequences encoding NS3/4A or NS5A were replaced with homologous sequences of other genotypes was described (Gottwein, Scheel et al. 2011; Scheel, Gottwein et al. 2011). These technical developments that are based on adaptation approaches allow analysing effects of antiviral compounds against NS3/4A and NS5A and antiviral resistance for all HCV genotypes in the context of infectious full length HCV RNAs.

2.4.2 Adaptation of infectious HCV genomes to cell culture

The generation of chimeric genomes as discussed in the previous section is one way to increase viral yields in cell culture. However, genetic incompatibility between JFH1 and the alternative HCV genome segment fused to it often limits production of infectious virus This restriction can be overcome by serial passage of the chimeras in cell culture which over time results in the accumulation of adaptive changes compensating the genetic differences between the fused genomes and thus increases virus yields (Abe, Ikeda et al. 2007; Gottwein, Scheel et al. 2007; McMullan, Grakoui et al. 2007; Yi, Ma et al. 2007; Jensen, Gottwein et al. 2008; Scheel, Gottwein et al. 2008; Bungyoku, Shoji et al. 2009; Gottwein, Scheel et al. 2009; Gottwein, Jensen et al. 2011; Koutsoudakis, Perez-del-Pulgar et al. 2011; Chan, Cheng et al. 2012). For instance, Yi and colleagues demonstrated that mutations in E1, p7, NS2 and NS3 contribute to the ability of a H77/JFH1 chimeric genome to assemble and release high amounts of virus particles . These mutations act independent of any detectable effect on viral RNA-replication or polyprotein processing indicating a crucial role of these proteins in virus assembly and relase .

It is important to realize that the process of adapting inter- or intra-genotypic chimeric genomes is fundamentally different from the selection process which yields REM in replicons. In the former, functional incompatibility between JFH1 and the fused non-JFH1 derived proteins is overcome. In other words, the selection process "shapes" these specific partners to better cooperate in the viral replication cycle. In the latter, however, the monogenetic replicon is modified to better fit to the host cell environment (e.g. Huh-7). Likely as a consequence, most of the changes adapting a given non-JFH1 strain to the JFH1-derived NS3 to NS5B replicase in full length chimeras are strictly chimera-specific and cannot be transferred to other chimeras. In contrast, there is a certain degree of flexibility with REMs which can be successfully transferred from Con1 to H77 and even to genotype 2a replicons (Grobler, Markel et al. 2003; Kato, Sugiyama et al. 2003; Maekawa, Enomoto et al. 2004; Ikeda, Abe et al. 2005; Abe, Ikeda et al. 2007; Mori, Abe et al. 2008).

Cell culture adaptations of JFH1-chimeras were mostly conducted in the highly permissive cell line Huh7.5 and are based on the passage of JFH1 infected cells or by serial passages of viral supernatants. Due to this strategy, the selection process optimizes viral fitness of the chimera across the entire replication cycle and not only the processes of RNA translation and RNA-replication as in the selection scheme of subgenomic replicons. Over time, viral variants emerge that harbour adaptive mutations leading to increased viral titers up to 100 to 1,000-fold over the parental genome.

Interestingly, several groups found that also the JFH1 wildtype genome can be efficiently adapted in cell culture with an increase in viral titers from 10^3 tissue culture-infectious doses ($TCID_{50}$) per millilitrer (mL) to 10^5-10^6 $TCID_{50}$/mL . This indicates that also JFH1 *per se* is not optimally suited for replication and propagation in Huh-7 cells. However, compared to all other known HCV isolates the degree of replication competence of this particular isolate in these cells is certainly unprecedented.

Titer-enhancing mutations were identified throughout the HCV genome (Core, E2, p7, NS2, NS3, NS5A, NS5B) and interestingly, repetition of an adaptation process showed that none of the mutations identified in the first experiment reappeared in the second selection . Thus, there are likely varying independent options to adapt JFH1 to cell culture replication in Huh-7-derived cells. Notably, adaptation of JFH1 itself yielded one of the few adaptive changes (V2440L within domain III of NS5A) which boosts virus production not only of the construct it was selected with but also of other JFH1-based chimeras including GT1a, 1b and 3a . Therefore, this adaptive change likely optimize JFH1-based virus assembly in a manner that is compatible with divergent viral structural proteins – possibly by acting on properties of the viral replicase that generally favour assembly of infectious progeny. Notably, this mutation which is located at the P3 position of the NS5A-NS5B cleavage site was shown to delay polyprotein processing at this junction . Although this alteration did not measurably change RNA-replication, a subtle change in polyprotein processing could modulate interaction of the replicase with viral structural proteins and in turn efficiency of virus production. Interestingly, the very same mutation was later shown to confer partial resistance against drugs inhibiting cyclophilin A, a crucial replication co-factor of HCV . Therefore, it is possible that not only RNA replication but also the efficiency of HCV assembly is modulated by cyclophilin A, possibly via modulation of polyprotein processing and folding. In many cases, however, the underlying mechanisms by which adaptive mutations facilitate production of infectious JFH1 or JFH1-chimeras are only beginning to emerge. First evidence suggests that enhanced physical interactions between the structural and non-structural proteins as well as within non-structural proteins during virus morphogenesis may be in part responsible for increased virus yields (Murray, Jones et al. 2008; Jiang and Luo 2012). Alternatively mutations were described which reduced cytotoxicity JFH1 or increased specific infectivity of released particles likely by altered recognition of CD81 . Notably, the G451R mutation within E2 of JFH1 falls into the latter category of mutations: First described as adaptive mutation for JFH1 by Zhong et al. (Zhong, Gastaminza et al. 2006) additional work by Grove and Bitzegeio revealed that this mutation reduces dependence on SR-BI and increases exposure of the CD81 binding site on the virus particle (Grove, Nielsen et al. 2008; Bitzegeio, Bankwitz et al. 2010). While these changes optimize cell entry in cell culture they are unlikely to confer a gain of fitness *in vivo* as these modification increase virus neutralization through a number of neutralizing antibodies (Grove, Nielsen et al. 2008; Bitzegeio, Bankwitz et al. 2010). This example illustrates how cell culture adapative changes increase viral fitness in tissue culture but at the same time skew viability in vivo. While such subtle changes facilitate *in vitro* experimentation and can give important and unique clues to the function and interplay of viral factors among themselves as well as with host determinants, this example also highlights the urgent need for better replication models based on primary HCV isolates.

2.4.5 HCV trans-complemented particles (HCV$_{TCP}$)

It is known from other plus-strand RNA viruses that assembly of progeny virus can be achieved when structural proteins are expressed in *trans* and independently from the RNA molecule that encodes the replicase proteins. This flexibility has been widely used to generate viral vectors for gene delivery and immunization approaches . Subgenomic replicons of HCV contain all genetic elements needed for replication in human liver cells, while lacking the coding region of the viral structural proteins, p7 and NS2 . Consequently, these RNAs replicate in cells but are unable to produce infectious progeny virus. It was reported by several groups that this defect can be rescued by expression of the HCV structural proteins in *trans* via helper viruses or varying DNA-based expression systems in so called in packaging cell lines . With the production of these *trans*-complemented JFH1 particles (referred to as HCV$_{TCP}$) virus entry and replication can be studied independently from the late steps of the viral life cycle and thus from secondary rounds of infection. Moreover, due to the inability of HCV$_{TCP}$ to spread, this model has improved biosafety . Naturally occurring HCV subgenomic RNAs with different deletions in the structural proteins have been found in several patients and could also be *trans*-complemented *in vitro* highlighting that HCV$_{TCP}$ can circulate in vivo and may modulate disease progression and outcome.

2.4.6 Permissive host cells

HCV replicates primarily in human hepatocytes, but multiple reports suggest that also extrahepatic reservoirs exist which may include the lymphatic system, gut and the brain . Besides mutations that affect viral fitness in cell culture, also the host cell plays a crucial role in HCV replication. The most permissive cell line for efficient RNA replication *in vitro* is the human hepatoma cell line Huh-7 and its clonal descendants. Studies with HCV replicons demonstrated that only a subpopulation of Huh-7 cells allowed high levels of replication and efficiency was dependent on the cell passage number and cell density . In fact the observation that during the replicon selection process a cell population which sustains elevated replication fitness emerges was utilized to create the most HCV permissive cell lines currently available. Using IFN-α or a selective HCV inhibitor numerous highly permissive Huh-7-desendent cell clones were established including Huh7.5 and Huh7-Lunet cells as well as other derivatives . . The often dramatic differences between the permissiveness of individual Huh-7-derived cell clones including individual cell passages of the same polyclonal Huh-7 cell population highlights the strong host factor dependence of HCV the high variability of these cultured cells. As mentioned above, the molecular mechanisms that govern differential permissiveness of these cells are poorly defined. However, it is assumed that the abundance of crucial host cell factors critical for replication plays an important role . Interestingly, in case of Huh7.5 cells a lesion in the innate antiviral defence signalling pathway caused by a mutation in RIG-I has been implicated in the phenotype of high permissiveness of these cells . Although another group has not been able confirm a tight correlation between HCV permissiveness and RIG-I status in these cells , this observation nevertheless illustrates that also lack of antiviral restrictions may substantially increase permissiveness for HCV (for further details see Chapter 9).

Initial approaches to establish robust HCV replication in cells other than Huh-7 were difficult. A first report that HCV replication is possible in human non-liver cells and even in non-human, mouse liver cells was published by Zhu et al. who described moderate replication of subgenomic replicons of the HCV-N isolate in Hela and and the murine hepatoma cells Hepa1-6 cells . Interestingly, these cell lines were transfected with RNA isolated from stable Huh-7 cells instead of *in vitro* transcribed RNA to have a higher genetic variability . However, unlike with selection of replicons in Huh-7 cells, in the mouse context no conserved mutations were identified that increase viral replication fitness in these non-human cells. Although the reason for this remains unclear, it is possible that the genetic barrier to adapt HCV proteins to murine replication co-factors was too high to permit selection of adaptive changes. Importantly, these findings provided formal proof that the essential host factors needed for HCV RNA replication can be found outside of human liver cells. Meanwhile numerous authors have described various alternative human liver-derived HCV-permissive cells including HuH6 , HepG2, IMY-N9 and LH86 . Moreover, several human cells of non-liver origin are well established . Finally, a spectrum of non-human cells have been reported that sustain HCV replication (Zhu, Guo et al. 2003; Chang, Cai et al. 2006; Uprichard, Chung et al. 2006; Long, Hiet et al. 2011) (Figure 2).

However, it is important to note that RNA-replication is in general lower in these cells, particularly in transient replication assays, compared to Huh7-derived cell clones. HCV RNA replication has also been demonstrated in mouse embryonic fibroblast (MEFs) using JFH1 replicons . This study could be confirmed and extended by Lin and colleagues who showed that expression of the liver-specific miR-122 in MEFs stimulated the synthesis of HCV replicons in the rodent fibroblasts and that the combined effects of miR-122 expression and deletion of IRF-3 lead to cooperative stimulation of HCV subgenome replication (Lin, Noyce et al. 2010). Therefore, MEFs now provide an important opportunity to utilize the powerful mouse genetic systems and the available mouse strains to unravel host factors that determine or preclude efficient HCV replication in these animals.

Similar to what has been observed in the replicon system, the identification of highly permissive Huh-7 cell lines is a prerequisite of a robust infection system. Huh7.5 and Huh7-Lunet/CD81 cells are two examples that support high levels of RNA replication and infection . It was shown that some Huh-7 cell clones express low levels of the important HCV entry factor CD81 and that ectopic expression of this tetraspanin leads to much higher viral spread and infection events . Conversely, selection of Huh-Lunet cell clones essentially lacking CD81 expression permitted receptor complementation studies for this important entry factor and the analysis of HCV cell-to-cell spread in the presence or absence of CD81. In a similar fashion, we have now HCV permissive cell lines available permitting receptor complementation assays for SR-BI (Dreux, Dao Thi et al. 2009; Catanese, Ansuini et al. 2010), CLDN1 (Haid, Windisch et al. 2010), and OCLN (Ciesek, Westhaus et al. 2011), thus greatly facilitating HCV cell entry studies with HCVcc. In parallel, a novel human hepatoma cell line, named LH86, was demonstrated to be permissive and susceptible to HCVcc and overexpression of CD81 and miR122 rendered HepG2 cells which were initially refractory to HCVcc infection fully permissive to HCV propagation (Narbus, Israelow et al. 2011). Since the latter cells are known to polarize in cell culture HepG2-CD81-miR122 cells provide a unique opportunity to assess HCV infection and replication in polarized cells.

Instead of construction of reporter viruses, host cells can be modified for rapid and sensitive scoring of HCV infection events especially in a high-through put format. One reported assay is based on a reporter cell line stably expressing the enhanced green fluorescent protein (EGFP) fused in-frame to the secreted alkaline phosphatise (SEAP) via a recognition sequence of the viral NS3/4A serine protease . Upon HCV infection and cleavage of the NS3/4A protease SEAP is released into the cell culture supernatant. Cell lines were also engineered to express the pro-apoptotic factor n4mBid, where NS3-dependent cleavage and activation led to an easily measurable cytopathic effect (Chockalingam, Simeon et al. 2010).

Although HCV infects mainly hepatocytes, there is evidence for the existence of non-hepatic reservoirs suggesting that the virus might have a broader cell tropism. Genomic viral RNA could be detected in peripheral blood mononuclear cells (PBMCs) and negative-strand RNA and HCV were reported in brain autopsies of HCV-infected patients with neuropathological abnormalities (reviewed in). Additionally, microscope techniques and strand-specific detection of HCV showed that microglia and macrophages are the dominant brain cell population positive for HCV . Interestingly, there seemed to be differences between HCV sequences in the brain and those circulating in plasma strengthening the possibility of HCV replication in cells of CNS origin. Direct detection of HCV antigens in the brain

remains technically challenging as reported for the liver probably due to low HCV replication. However, it could recently be shown by *in vitro* studies that two neuroepithelioma cell lines express all HCV receptors essential for viral entry (Fletcher, Yang et al. 2010; Burgel, Friesland et al. 2011) and support RNA replication (Fletcher, Yang et al. 2010). These cell lines were the first extra-hepatic cells that sustain HCV infection without ectopic expression of cellular factor required for viral entry (Lindenbach 2010). HCV tropism for the brain is further supported by a recent study by Fletcher et al. demonstrating productive HCV infection of brain microvascular endothelial cells (BMEC), a major component of the blood/brain barrier (Fletcher, Wilson et al. 2012). In this study, two independent derived brain microvascular endothelial cell lines were described to express all HCV receptor molecules and could be infected with HCVpp and HCVcc.
Collectively, the cell tropism of HCV could be expanded to several other human liver cell lines plus murine hepatoma cells and non-liver cell lines. With the discovery of novel HCV-specific dependency and restriction factors and genetic modifications of host cells further *in vitro* systems that sustain the entire HCV life cycle in cell culture are in development.

2.5 HCV replication models in primary cells and patient isolates

Studies on virus-host interactions have been hampered by limited *in vivo* and *ex vivo* models that mimic the natural environment of the liver. Due to the narrow host tropism of HCV small animal models are challenging and primary HCV isolates show a poor ability to replicate in tissue culture.
One drawback of human hepatoma cell lines like Huh-7 and its derivatives is that they do not polarize or express markers of mature hepatocytes and therefore may not fully recapitulate the polarized status of human hepatocytes *in vivo* . However, chemical treatment or ectopic expression of host factors required for viral propagation may overcome some of these hurdles. Interestingly, treatment of human hepatoma cells with 1% dimethyl sulfoxide was shown to differentiate cells in culture, induce the expression of hepatocyte-spe-

cific genes and arrest cell growth . These more hepatic-like cell cultures were still highly permissive for HCV infection and represent a more physiological relevant system compared to dividing Huh7 cells and allow studies e.g. of HCV persistence (Bauhofer, Ruggieri et al. 2012) HepG2 cells can also grow in a polarized manner that mimics the bile canalicular configuration of hepatocytes. After ectopic expression of CD81 these cells have been used as a model of polarized culture to study HCV, however, these cells weakly support HCV RNA replication . Overcoming this bottleneck, it was recently reported that the ectopic expression of miR122 in HepG2 cells permitted efficient RNA replication and support the entire HCV life cycle (Narbus, Israelow et al. 2011). Furthermore, the growth of hepatoma cells in 3D cultures can also resemble the natural host cells of HCV *in vivo* and can be used to study HCV infections (Sainz, TenCate et al. 2009; Molina-Jimenez, Benedicto et al. 2012). A human liver progenitor cell line, named HepaRG, can be differentiated into hepatocytes with specific cell culture conditions and has been widely used for HBV infection experiments. It could now be shown that these cells are also susceptible to serum-derived HCV particles and support long-term production of viral particles, albeit at very low levels (Ndongo-Thiam, Berthillon et al. 2011).

Primary human hepatocytes (PHH) provide the closest *in vitro* model for the natural host cell of HCV. However, their use in HCV research is limited as PHH are difficult to obtain. In addition to limited availability, these cells have high donor variability, and the rapid loss of their differentiation status complicates tissue culture experiments. Nevertheless, several groups reported infection of cultured PHH using sera from HCV-infected patients and could demonstrate CD81- and LDL receptor-dependent entry of serum-derived particles or inhibition of HCV replication by interferon. However, in general low-level replication was observed and results were difficult to reproduce . Recently, a study by Podevin et al. described a method of culturing PHH with hepatocyte-specific gene expression for up to two weeks (Podevin, Carpentier et al. 2010). Importantly, under these conditions PHH supported the complete infectious cycle of HCV, including production of new progeny virus, termed primary culture-derived HCV (HCVpc). The authors further could show that HCVpc had a lower average buoyant density and a higher specific infectivity than HCVcc particles produced in Huh7 cells (Podevin, Carpentier et al. 2010). A limitation of this study is still that this model is restricted to cell culture-derived viruses and it is unclear if also patient-derived viruses can be propagated in this system. A very recent study which is based on *ex vivo* human adult liver slices demonstrated a productive infection using human primary isolates of genotype 1b as well as JFH1 viruses and genotype 1 JFH1 chimeric genomes (Lagaye, Shen et al. 2012). This new experimental model system in which viral titers above 10^5 ffu/ml were achieved allows in addition the validation of antiviral drugs.

Improvements of PHH cultivation were also made with the addition of non-parenchymal feeder cells to hepatocytes in micopatterned co-cultures (MPCC) (Ploss, Khetani et al. 2010). MPCCs displayed hepatic function for several weeks, could be adapted to high-throughput format and were susceptible to HCV infection with limited virus spread for cell culture- and patient-derived viruses (Ploss, Khetani et al. 2010). HCV detection techniques in PHH such as RNA quantification turned out to be sensitive but at the same time limited due to high background of input RNA. Detecting HCV infection events can be facilitated with a novel cell-based reporter system, in which the NS3/4A protease cleaves a fluorescent substrate that then relocated the reporter signal from a mitochondrial localization to the nucleus (Jones, Catanese et al. 2010). This method permits visualizing HCV infection events in Huh7 cells as well as PHH and could be extended to several other cell culture systems (Jones, Catanese et al. 2010; Ploss, Khetani et al. 2010).

Robust experimental model systems to study the role of host genetics like *IL-28B* polymorphism are restricted to needle biopsies, surgical resections and organ donation. Two recent studies by Schwartz et al. and Wu et al. reported that hepatocyte-like cells derived from induced pluripotent stem cells (iPSC) allowed the productive infection with HCV, including inflammatory responses to infection (Schwartz, Trehan et al. 2012; Wu, Robotham et al. 2012). This novel development of an iPSC model can have the potential to study the impact of host genetics on hepatitis viral pathogenesis (Schwartz, Trehan et al. 2012; Wu, Robotham et al. 2012). Furthermore, these pluripotent stem cells can be genetically modified prior to differentiation and used to generate e.g. HCV-resistant hepato-

cytes. Importantly, these hepatic-like cells also permitted direct infection by patient sera. Similar findings were also recently reported with human embryonic stem cells (hESC)-derived hepatocytes demonstrating that these cells could be infected with JFH1 viruses and supported the complete HCV replication cycle (Roelandt, Obeid et al. 2012).
In conclusion, recent advances in the development of more physiologically relevant infection systems will advance our understanding of host-pathogen interactions in the liver.

2.6 Future perspectives and conclusions

After the molecular cloning of the HCV genome, it took more than a decade to establish functional cell culture systems for this human pathogen. Since then, step by step improvements were achieved that finally led to an infection system covering every step of the viral life cycle. Further improvements allow now to apply all virological techniques to study viral replication facilitating drug discovery. Chimeric genomes were created in which the structural proteins of all genotypes were fused to the JFH1 replicase, however, the challenge remains to propagate additional genotypes *in vitro*. Moreover, development of cell based models to culture primary HCV isolates across different genotypes would open novel and unique perspectives to investigate viral determinants responsible for the different natural course and treatment outcome of hepatitis C. As Huh-7-derived cell lines do not recapitulate the functional liver tissue including differentiation and polarization more relevant host cell system are needed. Along these lines, a robust supply of primary human cells with differentiated hepatocyte function and morphology would greatly facilitate our ability to study the relevance of host factors for replication and pathogenesis of HCV. The recent exciting reports about HCV replication in human stem cell-derived cells raise hopes that these models are in reach and will permit robust molecular studies of pathogenesis and replication mechanisms. These achievements will further close the gap between *in vitro* studies and the clinical situation of HCV infections.

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Figure legends:

Figure 1: Key HCV cell culture systems to investigate different steps of the viral replication cycle

For dissection of the of entry process HCV pseudoparticles (HCVpp) can be utilized. HCVpp are produced by transfection of 293T cells with three plasmids encoding for (I) retroviral gag and pol genes, (II) a retroviral vector harboring a reporter gene, and (III) the HCV glycoproteins E1, E2. These retroviral particles contain a vector that encodes the reporter gene and display the HCV glycoproteins in their envelope and thus, enter cells in a HCV-dependent manner. (B) HCV RNA replication can be quantified using subgenomic replicons. Those self-replicating HCV RNAs are based on a selectable marker or reporter gene replacing the coding region from core to NS2 upstream of a second IRES from EMCV that allows translation of the non-structural proteins NS3 to NS5B. After transfection, the viral RNA is directly translated and replication can be monitored for example by reporter gene expression. (C) HCV trans-complemented particles (HCV_{TCP}) are authentic viral particles that contain a replicon RNA instead of the full-length genome. They are produced by transfection of replicon RNA into so-called packaging cell lines that stably express the lacking structural proteins and thus, provide them in *trans*. Infection of naïve cells with HCV_{TCP} results in a single-round infection with only viral entry and RNA replication taking place since the structural proteins necessary for virus production are missing. (D) Production of cell culture-derived HCV particles (HCVcc) are based on the genotype 2a isolate JFH1 and derivatives thereof. Full-length viral genomes are transfected into permissive human hepatocytes which leads to translation and RNA replication giving rise to the production of viral particles that are able to infect new target cells, thereby completing the whole viral life cycle of HCV.

Figure 2: Primary cells and cell lines supporting HCV replication. The origin of human and murine cell lines permissive for HCV replication is given. Cells that reported to support the complete HCV replication cycle including cell entry, RNA replication and de novo production of infectious viral progeny are marked by an asterisk. Note that permissiveness between cells varies greately and is generally highest in Huh-7-derived cell clones. References to the individual reported cell lines are given in the text.

Table I: Molecular HCV clones

Financial & competing interest disclosure

The authors declare no conflict of interest. T.P. has received consulting fees from Biotest AG and from Janssen Global Servies , LLC.