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Cellular models for the screening and development of anti-hepatitis C virus agents

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Abstract

Investigations on the biology of hepatitis C virus (HCV) have been hampered by the lack of small animal models. Efforts have therefore been directed to designing practical and robust cellular models of human origin able to support HCV replication and production in a reproducible, reliable and consistent manner. Many different models based on different forms of virions and hepatoma or other cell types have been described including virus-like particles, pseudotyped particles, subgenomic and full length replicons, virion productive replicons, immortalised hepatocytes, fetal and adult primary human hepatocytes. This review focuses on these different cellular models, their advantages and disadvantages at the biological and experimental levels, and their respective use for evaluating the effect of antiviral molecules on different steps of HCV biology including virus entry, replication, particles generation and excretion, as well as on the modulation by the virus of the host cell response to infection.

Abbreviations

HCV, hepatitis c virus; HCV-LP, HCV virus-like particles; HCVpp, HCV pseudoparticles; HCVcc, HCV virions produced in cell culture; HCVser, serum-derived HCV natural virions ; LVP, lipovirions; IRES, internal ribosomal entry site; IFN, interferon; ISG, interferon stimulated gene; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDLR, low density lipoprotein receptor; SR-BI, scavenger receptor BI; CD81, tetraspanin CD81; CLDN, claudin; OCLN, occludin; TJ, tight junction; nHRP, heterologous ribonucleoprotein; PBP, poly-C binding protein; PTBP, pyrimidine track-binding protein ; RNAi, RNA interference; shRNA, small herpin RNA; siRNA, small interfering RNA; miR, microRNA; 5' or 3'-NTR, 5' or 3'-non translated region; dsRNA, double stranded RNA; ER, endoplasmic reticulum; GFP, green fluorescence protein; VSV, vesicular stomatitis virus; HTS, high throughput screening; IHH, immortalized human hepatocyte; PHH, primary human hepatocyte ; MTP, microsomal triglyceride transporter protein ; TLR, Toll-like receptor ; RIG, retinoic acid inducible gene.

Keywords: hepatoma cell line, replicon, hepatocyte, virion, antiviral drugs, RNA interference, pharmaceutical screening

Contents

1. Introduction	2
2. HCV biology	2
3. Mechanisms of host cell response to infection and modulation by HCV	7

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4. Cellular models for HCV investigations	11
5. Comparative analysis of HCV cellular models	17
6. Cellular models for the development of anti-HCV agents	20
7. Cellular models for the development of anti-HCV vaccine and the characterization of neutralizing antibody activity	27
8. Conclusion	28
References	29

1. Introduction

Hepatitis C virus (HCV) is an enveloped RNA virus which belongs to the *Flaviviridae* family and is the unique member of the *Hepacivirus* genus (Choo et al., 1989; Houghton et al., 1991). Within the last twenty years, HCV has emerged as a common cause of liver disease with an estimate of 170 million people infected annually on a worldwide basis. HCV infection is characterized by viral persistence and chronic liver disease in approximately 80% of cases. The complications of chronic hepatitis C include cirrhosis in 20% of cases and hepatocellular carcinoma, which has an incidence of up to 4 to 5% per year in patients with HCV-related cirrhosis. HCV-related end stage liver disease is now the principal indication for liver transplantation in industrialized countries (Pawlotsky, 2004). Efficient vaccine against HCV does not exist and the only available treatment which associates pegylated interferon α (IFN-Peg) and ribavirine is efficient in only 50 % of patients chronically infected with a virus of genotype 1 (the most frequent in Europe, Japan and North America) or 4, and in approximately 80 % of the patients infected with a virus of genotype 2 and 3 (Chevaliez & Pawlotsky, 2007a). It is therefore of major importance to develop new and efficient therapeutic strategies (Wakita, 2007).

2. HCV biology

2.1. HCV virions

HCV genome consists of an approximately 9.6 kb-long single strand RNA coding for a polyprotein of approximately 3000 amino acids (Choo et al., 1989). The polyprotein is translated from an IRES (internal ribosomal entry site), a highly conserved region of the genome, and is cleaved by host and viral proteases. Host proteases generate the capsid, envelope viral glycoproteins E1 and E2 and protein p7, while the viral proteases NS2/NS3 (cysteine protease) and NS3/NS4A (serine protease) generate the non-structural proteins (NS2 to NS5B). Non-structural proteins are involved in the replication of HCV genome and the building up of virions (Suzuki et al., 2007). There are six major genotypes. The sequence similarity within two different genotypes is in the range of 70% at both nucleotide and amino acid levels (Martell et al., 1992; Pawlotsky, 2006). The HCV virion consists of a complex (nucleocapsid) between genomic RNA and the core protein, embedded within a membrane derived from the host cell and containing E1/E2 protein heterodimers (Lavie et al., 2007). Both E1 and E2 ectodomains are multi-glycosylated and these proteins play a major part in the virus entry in host cells. In the plasma, HCV virions exhibit a wide range of density (from <1.06 to 1.30 g/ml). The low density virions appear to be the most infectious in chimpanzee. They have been shown to form complex particles with low and very low density lipoproteins (LDL, VLDL, apoB100, apoE, apoCII and apoCIII) that are enriched in triglycerides (Andre et al., 2002; Andre et al., 2005; Diaz et al., 2006). These complexes are named lipovirions (LVP) although the actual organisation of virion and lipoprotein within the

LVPs is not yet clearly established. In infected patients, on average, 40% of the plasma-derived HCV RNA appears to be associated with triglyceride-rich LVP.

2.2. HCV cellular entry

HCV virion enter host cells through a multistep process involving sequential attachment to several entry factors (Dubuisson et al., 2008) (**Fig 1**). It is likely that each step is necessary for the next one and for full coordination of the entry. The participation of glycosaminoglycans (Barth et al., 2006; Morikawa et al., 2007) and LDL receptor (LDLR) (Agnello et al., 1999; Molina et al., 2007) as attachment factors to concentrate HCV at the host cell membrane has been documented and is consistent with the fact that both E1 and E2 are highly glycosylated and with the nature of the HCV LVPs (Andre et al., 2002) (**Fig 1, step 1**). After initial recruitment at the membrane, HCV particles appear to bind to high affinity entry factors in a sequential manner. These factors involve the scavenger receptor BI (SR-BI), tetraspanin CD81 and proteins of tight junctions (TJ). SR-BI belongs to the CD36 family of lipid transporters and regulators of lipid homeostasis and is considered as the major high density lipoprotein (HDL) receptor (M. Krieger, 2001). However, it is a promiscuous receptor of other lipoproteins including LDL, VLDL, acetylated and oxidized LDL (Acton et al., 1994; Connelly & Williams, 2004). SR-BI has been shown to interact with the soluble E2 protein with high affinity and its implication as an HCV entry factor is well documented (Bartosch et al., 2003; Dreux et al., 2009; Maillard et al., 2006; Scarselli et al., 2002; Voisset et al., 2005; von Hahn et al., 2006). Interestingly however, HDLs do not appear to compete with HCV entry but rather favor it (Voisset et al., 2005; Zeisel et al., 2007). Recently SR-BII, a form generated by alternative splicing of SR-BI gene, has been shown to bind E2 and to be involved in HCV cell entry as well. Apparently, SR-BI and SR-BII may trigger the entry of different HCV variants (Grove et al., 2007). Finally, Dreux et al. (Dreux et al., 2009) recently demonstrated that SR-BI is an essential HCV entry factor by investigating the impact of ectopic expression of human SR-BI in the entry of HCVpp and HCVcc in rat hepatoma cells that do not express SR-BI (**Fig 1, step 2**).

Tetraspanin CD81 has been well characterized as a major factor for HCV cellular entry (Bartosch et al., 2003; Buck, 2008; Cormier et al., 2004; Hsu et al., 2003; Kapadia et al., 2007; Koutsoudakis et al., 2007; Molina et al., 2008; Petracca et al., 2000; Pileri et al., 1998; Wunschmann et al., 2000; Zhang et al., 2004) (**Fig 1, step 3**). E2 protein has been shown to exhibit a very high affinity ($K_d \sim 10^{-9}$ M) for the large extracellular loop of CD81 (Petracca et al., 2000; Pileri et al., 1998). CD81 expression and HCV entry are dependent on the plasma membrane sphingomyelin/ceramide ratio and cholesterol content, suggesting that CD81 must be located in tetraspanin-enriched microdomains to function as a viral entry mediator (Voisset et al., 2008). Recent investigations suggest that both CD81 and SR-BI may function cooperatively to mediate HCV entry in target cells (Kapadia et al., 2007; Zeisel et al., 2007), while CD81 expression is absolutely required to confer Huh-7 cell permissiveness to HCV infection (Akazawa et al., 2007). Interestingly, a soluble form of E2 has been suggested to link both CD81 and SR-BI (Heo et al., 2006). A recent work confirms these finding at the *in vivo* level (Meuleman et al., 2008).

Recent investigations suggest that two proteins entering the constitution of tight junctions (TJ) claudin (CLDN) (Evans et al., 2007) and occludin (OCLN) (Ploss et al., 2009) play a part in HCV entry. CLDNs constitute a family of four transmembrane domain proteins with two extracellular loops that are essential for the formation of TJs by forming homo or heterodimers on opposing cells, and for cell polarisation (Furuse et al., 1998). OCLN is a four transmembrane domain protein as well, present in the TJ complex of polarized epithelial cells. It appears to be associated with the CLDN-based strands and seems to play a role by

regulating TJ permeability (Forster, 2008). CLDN1 was discovered as a positive candidate in a genetic screen aimed at identifying genes that render a nonpermissive cell line (293T) infectable by HCVpp (HCV pseudoparticles, see section 4) and HCVcc (HCV particles produced in cell culture, see section 4) (Evans et al., 2007) (**Fig 1, step 4**). The residues involved in the CLDN-mediated entry of HCV particles have been mapped to the first N-terminal half extracellular loop of the protein although no direct interaction between HCV E1/E2 and CLDN1 has been demonstrated. Comparative analyses of the kinetics of the inhibitory effect of antibodies against CD81 and CLDN1 on HCV entry suggested that CLDN1 plays a role in a step of the process that occurs *after* interaction with CD81 (Evans et al., 2007). CLDN6 and CLDN9 which only differ by one amino acid, have been both reported to mediate HCVpp and HCVcc entry in Huh-7.5 cells (Meertens et al., 2008), while other members of the CLDN family do not (Zheng et al., 2007). There is apparently no obvious preference in CLDN1/6/9 usage by the various HCV genotypes. Using the intestinal Caco-2 cell line which displays increased polarisation in culture, Mee et al. observed that the role of CLDNs in HCV entry does not correlate with their function as TJ components, and that disruption of TJ increases viral entry suggesting that HCV uses nonjunctional forms of CLDN1 (Mee et al., 2008). This would imply that HCV preferentially targets hepatocytes in which cell-cell contacts are damaged. Using fluorescence resonance energy transfer, Harris et al. observed that CD81 and CLDN1 colocalize and form coreceptor complexes in liver tissue (Harris et al., 2008) (**Fig 1, step 4**). SRBI, CD81 and CLDN1 appear to be expressed in the basolateral-sinusoidal membrane of hepatocytes in man, consistent with the fact that HCV enters the liver *via* the sinusoidal blood (Reynolds et al., 2008). In addition, these authors demonstrated that HCV infection positively regulates CLDN1 expression in Huh-7.5 cells and in human liver. It is suspected that CLDNs favor cell to cell virus spread in the liver. However, there are several cell lines which although being resistant to HCV infection express SRBI, CD81 and CLDN1. Evidence has been recently provided that binding of the virions to CD81, triggers Rho GTPase-dependent actin rearrangement that allows lateral movement of the CD81-virion complexes to the TJ linking hepatocytes (Brazzoli et al., 2008). Besides these observations, it was demonstrated that human OCLN plays a critical part in the process of HCV entry into human and rodent cells (Ploss et al., 2009) (**Fig 1, step 4**). Silencing of OCLN, specifically inhibited both HCVpp and HCVcc infection of Huh-7.5 cells. The second extracellular loop of OCLN was identified as the specific site of this action. Using different cell lines as well as mouse and human proteins and combinations thereof, these authors demonstrated that CD81 and OCLN represent the minimal human-specific entry factors for mouse and hamster cell infection, while both SR-BI and CLDN1 exhibited minor species-specificity, if at all. While recent indications suggested that polarisation restricts HCV entry into hepatocytes (Jane McKeating, personal communication) and (Farquhar & McKeating, 2008; Mee et al., 2008), Benedicto et al. provided direct evidence that infection affects TJs (Benedicto et al., 2008). Using the full genomic replicon system (Huh-7/JFH1) these authors demonstrated that HCV infection alters the distribution of TJ proteins including CLDN1, OCLN and ZO-1 by downregulating their accumulation, in disagreement with data reported by others (Reynolds et al., 2008). Notably and in contrast, OCLN exhibited a marked accumulation in the endoplasmic reticulum (ER) of infected cells. E2 protein colocalized and coimmunoprecipitated with OCLN suggesting that both proteins establish a direct virus-host protein interaction. Both normal OCLN localization and TJ organization were rescued by antiviral treatments of cells. Most of these results were confirmed in a recent paper (Liu et al., 2009). In sum, these data provide new arguments allowing to establish a link between HCV-mediated liver pathogenesis (notably cholestatic disorders) and the quality of hepatocyte-hepatocyte TJs.

Finally, it has been recently shown that EWI-2wint, a cleavage product of EWI-2 a partner of CD81, is a potent inhibitor of the E2-CD81 interaction (Rocha-Perugini et al., 2008). Although being expressed in different cell lines, EWI-2wint is not expressed in hepatocytes. It is therefore possible that the hepatotropism of HCV might result either from the presence of other specific entry factor(s) or from the absence of specific entry inhibitor(s).

2.3. HCV endocytosis and genome replication

After binding to entry factors, HCV appears to enter the cells by clathrin-mediated endocytosis (Blanchard et al., 2006; Meertens et al., 2006) (**Fig 1, step 5**). Indeed, siRNA (small interfering RNA) directed to clathrin heavy chain and molecules that neutralise the acidic pH of endosomes produce a potent inhibition of the entry of HCVpp and HCVcc in various cell lines. ApoC-I, an exchangeable apolipoprotein that predominantly resides in HDL, was shown to increase HCV infectivity and the rate of fusion between viral and target membranes *via* a direct interaction with the virion surface (Dreux et al., 2007). Consistent with this, recent studies demonstrated that the C-terminal region of ApoC-1 associates with surface components of virions during morphogenesis (Meunier et al., 2008). The combination between interactions with receptors, low pH encountered in endosomes (**Fig 1, step 6**), and mutual chaperoning or cooperation activity of E1 and E2 is likely to trigger a conformational change on the envelope glycoproteins that leads to the fusion of the virus membrane with the endosome membrane (Bartosch et al., 2003a; Hsu et al., 2003; Tscherne et al., 2006) (**Fig 1, step 7**). This process eventually leads to the release of the nucleocapsid into the cytosol (**Fig 1, step 8**).

The 5'-nontranslated region (5'-NTR) of HCV RNA harbours the IRES (Suzuki et al., 2007; C. Wang et al., 1993), a structurally complex region consisting of four domains which are critical for the regulation of the polyprotein translation and viral replication (**Fig 2**). The IRES binds sequentially the 40S ribosomal subunit, the eukaryotic initiation factors 3 and 2, and the 60S subunit *via* a GTP-dependent reaction. This eventually leads to the formation of the first peptide bond. The IRES activity appears to be further modulated by viral (core) (Boni et al., 2005) and host (La, heterologous ribonucleoprotein L, poly-C and pyrimidine track-binding) proteins. It was recently reported that the heterogeneous nuclear ribonucleoprotein D increases HCV IRES activity through binding to the stem loop region II element (Paek et al., 2008). Moreover, the domain I of IRES is capable of binding a liver specific microRNA (miR-122) that appears to enhance HCV RNA replication rate in a cell specific manner (Jopling et al., 2006; G. Randall et al., 2007). The polyprotein precursor generated by translation is cleaved by host and viral proteases (Suzuki et al., 2007). Host proteases generate the structural proteins core, E1, E2 and p7, while the viral proteases NS2/NS3 and NS3/4 generate respectively the non-structural proteins NS2 and NS3, and NS4A, NS4B, NS5A and NS5B (Bartenschlager et al., 1994; Failla, Tomei et al., 1995; Lin et al., 1994; Tanji et al., 1994). HCV genome replication is assumed to proceed within complexes associated with ER membranes and begins with the RNA-dependent RNA polymerase (NS5B)-mediated synthesis of a complementary replicative (negative) strand. The latter is then used as a template to synthesize the genomic (positive) strand by the same enzyme (**Fig 2**). Replication is thought to yield double stranded RNA (dsRNA) structures. In primary human hepatocytes, the rate of synthesis of the genomic strand appears to be 5 to 10 times greater than that of the replicative strand (Castet et al., 2002). Like other viral RNA polymerases, NS5B has a high error rate (averaging 10^{-4} to 10^{-5} per base site) and lacks proofreading activity, so that mutations accumulate in newly generated HCV genomes (quasispecies) (Farci et al., 2006; Pawlotsky, 2006). The building-up of the replication complexes is assumed to be initiated at ER membranes by NS4B, an integral membrane protein (Egger et al., 2002; Gao et al., 2004).

Analysis of replication complexes showed that the replicating HCV RNA and the NS proteins colocalize on cytoplasmic membrane structures. These structures appear to be resistant to detergent treatment and cofractionate with a lipid raft-associated protein, caveolin-2, suggesting that they are lipid rafts derived from intracellular membranes (Shi et al., 2003). It is now admitted that NS proteins form complexes in both ER and Golgi membranes, but that HCV RNA replication takes part in the Golgi membranes (Aizaki et al., 2004). Many viral and host proteins have been shown to interact with NS5B to regulate the rate of RNA replication (Suzuki et al., 2007). In the Huh-7.5/JFH1 HCVcc model, the E1/E2 heterodimer was retained in the ER and does not accumulate in other intracellular compartments or at the plasma membrane (Rouille et al., 2006). Core was found to be associated to lipid droplets but was not found in the nucleus or in association with mitochondria or other well-defined intracellular compartments. Moreover, core did not colocalize with E1/E2. Although subcellular structures similar to the previously reported “membranous web” were observed, they did not harbor virus-like bodies.

2.4. HCV virion release

The assembly of viral particules involves capsid protein oligomerisation and interaction with domains I and III as well as nucleotides 24-41 of the viral genomic RNA (Y. Tanaka et al., 2000). Core oligomerisation has been shown to be mediated by the 72-91 primary sequence of the protein (Matsumoto et al., 1996; Nakai et al., 2006). After nucleocapsid has been formed, it interacts with envelope proteins E1 and E2 and is secreted from the cell through plasmatic membrane budding so that host membrane constituents are also present in the virus membrane (Baumert et al., 1998; Ezelle et al., 2002; K. Murakami et al., 2006). NS5A has been shown to play a key role in regulating the early phase of HCV particle formation by interacting with Core through and its C-terminal serine cluster (Masaki et al., 2008). Huang et al. demonstrated that HCV assembles in ApoB and MTTP (microsomal triglyceride transfer protein) enriched vesicles and that the viral secretion is dependent on both ApoB expression and VLDL assembly from subdomains of the ER (H. Huang et al., 2007) (**Fig 2**). This suggests that HCV uses the lipoprotein/cholesterol export system at its own benefit to escape from hepatocytes, and this is consistent with the use of lipoprotein receptors such as LDLR and SR-BI as cellular entry sites. These results were confirmed recently (Nahmias et al., 2008). Brefeldin A, which blocks the communication between the ER and the Golgi, effectively inhibited ApoB protein secretion and HCVcc virion production in the Huh-7.5/JFH1 HCVcc model. Interestingly, HCV secretion was up-regulated by oleic acid and down-regulated by insulin, precisely mirroring ApoB secretion by the cells. Moreover, naringenin an inhibitor of MTTP strongly blocked virion release, while viral RNA replication was not affected. In addition, Chang et al. demonstrated that the level of apoE correlates with HCV infectivity in the Huh-7.5/JFH1 HCVcc model (Chang et al., 2007). ApoE-specific monoclonal antibodies against ApoE as well as ApoE-specific siRNA strongly decreased the release of infectious virions. These findings demonstrate that apoE is required for HCV virion infectivity and production, suggesting that HCV virions are assembled as apoE-enriched lipoprotein particles (**Fig 2**).

3. Mechanisms of host cell response to infection and modulation by HCV

The host response of cells to virus and other microorganism infections (so-called innate immune response) involves several strategies aimed at controlling and limiting the spread of infection. One of these is initiated by cellular sensors that recognise the presence of pathogen-associated molecular patterns and activate a cascade of signaling pathways leading

to the expression of signaling proteins (type I interferons -IFN α/β - and inflammatory cytokines) and genes (Interferon stimulated genes, ISG) (Gale & Foy, 2005). Another strategy by which the host may respond to viral infection is RNA interference (RNAi) (Voinnet, 2005).

3.1. Interferon-stimulating gene pathways

Two proteins are likely to play a major part as sensors of intracellular viral HCV RNA, TLR3 and RIG-I. TLR3 (Toll-like receptor) is a member of the TLR family that recognises dsRNAs (Alexopoulou et al., 2001). It is expressed in many tissues including the liver (Matsumoto & Seya, 2008). A recent report shows that TLR3 is localized not only on plasma membrane but also in the ER of unstimulated cells where it moves to dsRNA-containing endosomes in response to dsRNA, and colocalizes with c-Src kinase in the lumen of endosomes containing dsRNA (Johnsen et al., 2006). The acidic pH of endosomes (de Bouteiller et al., 2005) and association to the c-Src kinase favor dsRNA binding to and dimerisation of TLR3, and initiation of signal transduction, respectively. As reviewed by others (Bode et al., 2007; Matsumoto & Seya, 2008), TLR3 mediates signal transduction through an adaptor protein TICAM-1/TRIF. Once activated, TRIF recruits several proteins including TRAF3, TRAF6, NAP1 and RIP1 to form a multifunctional complex. Within this complex, TRAF3 and NAP1 recruit two kinases, IKK ϵ and TBK1, that phosphorylate IRF3 and IRF7, while RIP1 either mediates cell apoptosis or recruits IKK $\alpha\beta\gamma$ which in turn phosphorylates I κ B and activates nuclear translocation of NF κ B. Phosphorylated IRF3 and IRF7 translocate to the nucleus and cooperate with AP1 and NF κ B to activate the transcription of IFN β , while NF κ B activates the expression of inflammatory cytokines. The TLR3-mediated pathway appears to be dependent on the virus genome and entry process, and on the type of host cell and other anti-viral properties of host cells. Interestingly, TLR3 signaling pathway seems to be defective in Huh7 cells (Lanford et al., 2003; Preiss et al., 2008).

RIG-I (Retinoic acid Induced Gene-I), an interferon sensitive gene (ISG), is a ubiquitous cytosolic CARD-containing DexD/H RNA helicase (Yoneyama et al., 2004). RIG-I senses dsRNAs with secondary structures or with uncapped 5' triphosphate ends generated by several viruses with negative strand RNA, including HCV. A recent report identified the PolyU/UC region of the 3'-NTR of HCV as the major pathogen-associated molecular patterns of this virus (Saito et al., 2008). Recent work suggested that in the absence of RNA, RIG-I is inhibited by intra-molecular interactions between the CARD and C-terminus domains, while RNA binding strongly stimulates ATPase activity and signaling (Gee et al., 2008). RIG-I activates the mitochondria-bound protein, IPS-1/MAVS/VISA/Cardif protein (Hiscott et al., 2006) (referred to here as MAVS) through the N-terminal tandem CARD of RIG-I and the N-terminal CARD of MAVS (T. Saito et al., 2007; Vitour & Meurs, 2007). MAVS associates with several proteins including TANK, FADD and RIP1 (Lin et al., 2006; Meylan et al., 2005; Xu et al., 2005) and with proteins of the TRAF family, such as TRAF3 or TRAF6 (Saha et al., 2006). Within this multifunctional complex, MAVS activates the IKK $\alpha\beta\gamma$ complex and the TBK1/IKK ϵ kinases, thus provoking activation of NF- κ B and IRF3, respectively (Fitzgerald et al., 2003; Sharma et al., 2003). TBK1 and IKK ϵ present strong homologies and they both phosphorylate IRF3 (Matsui et al., 2006). Yet they differ in many aspects. IKK ϵ , but not TBK1, associates with MAVS at the mitochondria and induces a subset of IFN-induced genes through phosphorylation of STAT1 which then allows binding of the ISGF3 complex to their promoter elements (tenOever et al., 2004). In contrast to IRF3, IRF7 is an ISG the expression of which is induced by IFN α/β after IRF3-mediated IFN induction. It thus appears that IFN α/β induction has two phases, early and late. IRF3 plays a

major part in both phases while IFR7 plays its major part in the late process (Sato et al., 2000). Expression of IKK ϵ can be induced in response to several factors, such as inflammatory stimuli and viral infections through the conjugated action of NF- κ B and C/EBP transcription factors (Kravchenko et al., 2003). Interestingly, Huh-7.5 cells which are used for production of HCVcc particles (see section 4) appear to be deficient in RIG-I activity due to a mutation in the first CARD domain of the protein (Sumpter et al., 2005).

3.2. RNA interference

Micro RNAs (miR) constitute a class of non-coding RNA that are transcribed from miR-encoding genes by RNA polymerase II. miR transcripts are processed to mature 19-21 nucleotide length miR duplexes by two enzymes, Drosha in the nucleus and, after export to the cytosol by exportin 5, Dicer. The miR duplex then binds the RNA induced silencing complex (RISC) which targets the complementary mRNAs after hybridization to the antisense miR strand. Depending on the degree of homology between the antisense miR and the target mRNA, the latter is either degraded or its rate of translation is reduced, making RNAi a powerful tool for gene silencing. Most of miR binding sites are located in the 3'-NTR of mRNAs. This process of RNAi has been first recognized in plants and invertebrates as a mechanism of antiviral protection. However, the contribution of RNAi as another means of anti-HCV defence has only been recently explored (Pedersen et al., 2007). During HCV genome replication, dsRNA as well as hairpin structures are known to be formed (Pedersen et al., 2007; Y. Wang et al., 2006). It is likely that these species are processed by Dicer so that siRNAs are produced and may induce HCV RNA silencing. However, the possibility that RNAi has a potent impact on HCV replication has been questioned (Randall et al., 2007). Ironically, the first miR whose effect on HCV has been documented in detail, i.e. miR-122, was shown to *increase* and not decrease HCV RNA accumulation, suggesting that this virus is able to use the cellular miR machinery to its own benefit. Jopling et al. demonstrated that miR-122 which is expressed at a high level in the human liver (as well as in Huh-7 cells) is required for an efficient replication of the viral RNA of genotypes 1 and 2a, although neither the rate of translation nor the turnover of viral RNA were affected (Jopling et al., 2006; Jopling et al., 2005). They identified two sites of miR-122 binding separated by 14 nucleotides in the extreme 5'-NTR of HCV RNA and showed that occupation of both site is required for increased RNA accumulation (Jopling et al., 2008). Although the mechanism of this stimulation is not yet fully understood, it is possible that either the miR-122/RISC-HCV genome complex recruits factors that enhance viral RNA abundance, or that miR-122 stimulates HCV translation by enhancing the association of ribosomes with the viral RNA at an early initiation stage (Henke et al., 2008). Interestingly, the finding that ectopic expression of miR-122 in human kidney-derived cells substantially enhances HCV replication suggests that miR-122 may be responsible, at least in part, for the hepatic tropism of the virus (Chang et al., 2008). It has to be emphasized that miR-122 has been shown to play a significant role in promoting plasma cholesterol, lipid synthesis and inhibiting fatty-acid oxidation in the adult liver (Esau et al., 2006). Although this suggests that miR-122 is a possible therapeutic target for metabolic and cardiovascular diseases, it provides a direct functional link between HCV replication and lipid homeostasis. Recently, miR-199a* has been reported to target domain II of the IRES region of HCV and to strongly inhibit viral replication in HCVcc replicon systems of genotype 1b (Con1) and 2a (JFH1) (Murakami et al., 2009). This effect was shown to be IFN-independent since the expression of several genes known to be controlled by IFN was not affected by miR-199a*. Whether miR-199a* triggers the degradation of viral RNA or inhibits its translation is presently unknown. However, the fact that miR-199a* also targets the 5'-NTR of HCV RNA suggests that the unexpected effect of

miR-122 does not stem from the fact that it targets sequences in the 5'-NTR. miR-122 and miR-199a* appear therefore to produce opposite effects on HCV replication. The observation that miR-122 is expressed in the liver at a greater level than miR-199a* and the finding that inhibiting the RNAi machinery (notably Drosha and Dicer) reduces HCV replication and infection in the Huh-7.5/J6 (2a)/JFH1 HCVcc replicon system suggest that the proviral effect of miR-122 dominates on the anti-viral effect of miR-199a* (Randall et al., 2007). Interestingly, using the Huh-7 cells and the JFH1 replicon system Pedersen et al. demonstrated that IFN α/β upregulates several miRs including miR-196, -296, -351, -431, and -448 which inhibit HCV infection and RNA replication. Moreover and in the meantime, IFN downregulates miR122 (Pedersen et al., 2007). It appears therefore that IFN is capable of orchestrating a large arsenal of antiviral agents not only including ISGs, but miRs as well (**Fig 2**).

3.3. Interferon signaling pathway and viral resistance

At present the treatment associating pegylated IFN α and ribavirin represents the only available therapy against HCV infection (Chevaliez & Pawlotsky, 2007a; Meurs & Breiman, 2007). Several reports have shown that IFN α has a direct antiviral effect on HCV replication in different culture models including replicons (Huh-7), productive replicons (Huh-7.5), immortalized non-neoplastic hepatocytes (PH5CH8), fetal and adult primary human hepatocytes (Buck, 2008; Castet et al., 2002; Ikeda et al., 1998; Lanford et al., 2003; Lazaro et al., 2007; Lindenbach et al., 2005). On binding its receptor, IFN triggers the formation of a complex between a heterodimer of IFN receptors IFNAR1 and IFNRA2 and two members of the Janus kinase family, Jak1 and Tyk2. This initiates a cascade of phosphorylation reactions that leads to the activation of STAT1 and STAT2 which in addition to IRF9 form the ISGF3 complex. ISGF3 then behaves as a transcription factor. It translocates to the nucleus and transactivates a battery of ISGs that exhibit interferon sensitive responsive elements (ISRE) in their promoter. ISG proteins exhibit antiviral activity such as IRF7 (which participates to IFN α production amplification; see above), protein kinase R (PKR), 2'-5' oligoadenylate synthase (OAS), ISG56, ISG20, RIG-I, etc (Gale & Foy, 2005). Recently, strong evidence was provided that among other ISGs, viperin, a putative radical S-adenosyl-L-methionine (SAM) enzyme, is a potent inhibitor of HCV replicon (Jiang et al., 2008). This finding suggests that viperin represents a novel antiviral pathway that works together with other antiviral proteins, such as ISG20 and PKR, to mediate the IFN response against HCV infection.

However, the success of IFN therapy is limited to approximately 50% of patients in a genotype-dependent manner (Le Guillou-Guillemette et al., 2007). IFN signaling pathway as well as RNAi appear therefore to be subverted by HCV. Indeed, recent studies have demonstrated that the virus has developed strategies to block innate cell response and inhibit the antiviral effect of IFN (Bode et al., 2007; Gale & Foy, 2005; Randall & Goodbourn, 2008). Several HCV proteins interfere with the antiviral response of host cells to eventually repress the release of IFN β , and with the IFN α signaling. NS3/NS4A has been reported to block IRF3 activation by proteolytic cleavage of TRIF adaptor (which controls in part the TLR3-mediated signaling) and MAVS (in the RIG-I-mediated signaling), as well as the ISG56-mediated response (Foy et al., 2003; Li, Foy et al., 2005; Meylan et al., 2005), although some of these observations have been questioned recently (Dansako et al., 2007). HCV core inhibits STAT1 and induces the expression of SOCS3 which blocks ISGF3 formation (de Lucas et al., 2005; Yoshida et al., 2002), although SOCS3 increased expression has also been observed in IFN-resistant replicons that lack core protein (Zhu et al., 2005). E2 and NS5A have been shown to inhibit the activity of PKR which blocks translation by

phosphorylating eIF2 α , *via* protein-protein interaction (Gale et al., 1998; Taylor et al., 1999). NS5A has been reported as well to interact with and inhibit the HCV genome cleavage by the ISG OAS/RnaseL (Taguchi et al., 2004). In addition, NS5A induces the expression of IL8 which in turn negatively affects the antiviral effect of IFN (S. Girard et al., 2002; Khabar et al., 1997). While inhibiting the host innate or the IFN-induced defences, HCV proteins also protect infected cells from apoptosis (Bode et al., 2007). In this respect, HCV core protein has been shown to inhibit TNF α - or FAS-mediated activation of caspase 8, p21 and p53 expression, or to enhance Bcl-XL, Bcl2 and cyclin D1 expression or the Raf1/MAPK signaling pathways (Hassan et al., 2004; Saito et al., 2006). E2 was reported to inhibit apoptosis by negatively regulating the cytochrome c release from mitochondria after TRAIL activation (Lee et al., 2005). NS3/NS4A protects liver damage in animals treated with lethal doses of TNF α combined with D-galactosamine, while NS3 interacts with and affects p53 activity (Frelin et al., 2006; Tanaka et al., 2006). Finally, NS5A has been shown to attenuate liver damage in animals treated with TNF α , to decrease p53 DNA binding and to activate PI3K (Gong et al., 2004; Majumder et al., 2002; Street et al., 2005). However, some of these observations appear to be controversial depending on the cellular model used and this is not unexpected (see below). In a recent work (Hazari et al., 2007), HCV replicons with high and low activation of the IFN-promoter were cultured for a prolonged period of time in the presence of IFN α . Stable replicon cell lines with resistant phenotype were isolated and characterized for their ability to sustain viral replication in the presence of IFN. Reduced signaling and IFN-resistant phenotype was found in all Huh-7 cell lines even after eliminating HCV, suggesting that cellular factors were involved. Resistant phenotype in the replicons was not due to lack of interferon receptor expression but instead to defect in the JAK-STAT signaling pathway due to strongly reduced expression of Tyk2 and Jak-1 protein and phosphorylation of STAT 1 and STAT 2 proteins. These results emphasize the fact that depending on the cellular clone investigated, different cellular behavior may be observed in the resistance to IFN.

In addition to interfere with IFN signaling pathway, viruses in general (Hemmes et al., 2007) and HCV in particular are able to interfere with RNAi-dependent antiviral effect. Using cell lines that express constitutively green fluorescent protein (GFP) and in which HCV protein expression is inducible, Ji et al. demonstrated that silencing of GFP gene by siRNA was inhibited in the presence of the full length HCV polyprotein ; HCV E2 protein was shown to be responsible for this inhibition (Ji et al., 2008). Consistent with this observation, coprecipitation assays demonstrated that E2 binds to Argonaute-2 (Ago-2), a member of the RISC machinery, thus providing a basis for the suppression of RNAi by the virus. HCV core protein was shown to inhibit RNAi induced by shRNAs but not by synthetic siRNAs in various mammalian cells (Chen et al., 2008; Wang et al., 2006). These authors provided evidence that core directly interacts with Dicer which notably processes dsRNAs into siRNAs. Through deletion analysis, the N-terminal domain of core was shown to be responsible for inhibition of Dicer and RNA silencing activity. Thus, HCV core protein may abrogate host cell RNAi-mediated defense by suppressing the ability of Dicer to process precursor dsRNAs into siRNAs. It has been proposed that since pre-miRNA are also cleaved by Dicer, the inhibition of RNAi by HCV core may suppress the maturation of miRNAs in the host liver, thus influencing the expression of certain classes of gene regulatory molecules leading to liver pathologies including hepatocarcinogenesis (Wang et al., 2006).

4. Cellular models for HCV investigations

Cellular models for investigating *in vitro* HCV infection necessitate two major actors: an appropriate cell type and a practical form of the virus. HCV genome replication occurs

primarily within hepatocytes, although other cell types have been shown to be permissive as well although to a much lower extent, including peripheral blood mononuclear cells (Cribier et al., 1995; Lerat et al., 1996), biliary cells (Loriot et al., 1999) and enterocytes (Deforges et al., 2004). Hence, this review will focus on primary adult, fetal or immortalised hepatocytes and Huh-7 hepatoma cells and derivatives which have been largely used in recent developments. On the other hand, although the most natural form of the virus is present in the blood of infected patients as HCVser LVP (Andre et al., 2002; Kanto et al., 1995; Miyamoto et al., 1992; Prince et al., 1996; Thomssen et al., 1993), several surrogate forms of HCV virions have been developed including, transfected partial or full length HCV genomes, virus-like particles (HCV-LP) (Baumert et al., 1998), pseudotyped retroviral particles (HCVpp) (Bartosch et al., 2003; Hsu et al., 2003; McKeating et al., 2004), or cell culture particles (HCVcc) (Cai et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Recent data suggest that the nature of the virus is an important point to take into account regarding for example the cell entry process (Farquhar & McKeating, 2008). Several cellular models combining cells of various origin and various forms of virus have been developed in recent years. They all represent valuable tools for investigating HCV biology and antiviral therapeutics.

4.1. HCV-like particles (HCV-LP)

The production of HCV-LP in insect cells using recombinant baculovirus expressing the cDNA of the HCV structural proteins (HCV-J strain, genotype 1b), has been first reported by Baumert et al. (Baumert et al., 1998). These studies demonstrated that HCV core and envelope proteins E1 and E2 were sufficient for viral particle assembly, presumably from the ER, and that HCV RNA was selectively incorporated into the particles. These particles exhibited a density of 1.14 to 1.22 g/cm³ in sucrose density gradients (depending on studies), and a morphology (double-shelled particles 35 to 49 nm in diameter) similar to that of HCV particles detected in HCV infected chimpanzee liver. HCV-LPs were not secreted from cells but rather extracted from intracellular compartment, and were poorly infectious. This last point is a great advantage for the use of these particles in immunotherapeutic strategies (see section 7). A number of papers have been published on the use of these particles to investigate the structure and function of core and envelope proteins (Choi et al., 2004; Clayton et al., 2002; Owsianka et al., 2001) as well as the HCV-host cell interactions and virion entry, notably the interaction with and activation of dendritic cells (Barth et al., 2005). HCV-LP binding to and entry in cells is inhibited with anti-E1 or anti-E2 antibodies, demonstrating the critical role of E1 and E2 proteins in this process (Triyatni et al., 2002). In addition, these authors reported that HCV-LPs binding to several human hepatic cells (primary hepatocytes, HepG2, HuH7, and NKNT-3) and T-cells (Molt-4) was dose- and calcium-dependent but *CD81-independent*. Interestingly, preincubation of HCV-LPs with VLDL, LDL, or HDL inhibited their binding to cells, while preincubation of cells with VLDL, LDL, HDL, or anti-LDL-R antibody did not. Barth et al. demonstrated that binding of the HCV envelope glycoproteins to specific heparan sulfate structures on host hepatocarcinoma cells is critical for initiation of viral infection. This suggests that this interaction is a target for the antiviral host immune systems in responses to HCV infection *in vivo* (Barth et al., 2006). Conflicting results were reported on the role of the 5'-NTR on virion assembly and morphology in insect cells: the 5'-NTR of HCV genotype 1a is implicated in the assembly process of HCV structural proteins in insect cells, although no evidence for a direct interaction between the 5'-NTR and structural proteins has been reported (Girard et al., 2004). However, Zhao et al. reported that HCV 5'-NTR is not required for the assembly of HCV-like particles in insect

cells, that is, the core and envelope proteins are sufficient for viral particle formation (Zhao et al., 2003).

Other versions of HCV-like particles were engineered. Using a recombinant Semliki Forest Virus (SFV) subreplicon expressing genes encoding HCV structural proteins (genotype 1a), Blanchard et al. succeeded in producing HCV-LPs in mammalian BHK-21 cells (Blanchard et al., 2002). Ezelle et al. used a recombinant Vesicular Stomatitis Virus (VSV) vector expressing HCV core, E1, and E2 (genotype 1b) to generate HCV-LPs possessing properties similar to the ultrastructural properties of HCV virions (Ezelle et al., 2002). Both versions of SFV- and VSV-derived HCV-LPs were used to investigate the signal peptide peptidase processing of core protein, the interaction of structural proteins with the ER, and their role in HCV particle assembly and morphogenesis (Ait-Goughoulte et al., 2006; Blanchard et al., 2003a; Blanchard et al., 2003; Ezelle et al., 2002; Hourieux et al., 2007).

4.2. HCV pseudotyped particle (HCVpp)

Pseudotyped particles have been constructed on the basis of vesicular stomatitis virus or influenza virus with chimeric E1 and E2 proteins (Flint et al., 1999; Lagging et al., 1998; Matsuura et al., 2001). These proteins had to be modified in their transmembrane domain to allow transport and assembly on the plasma membrane of host cells. However, these modifications affected the conformation and function of the E1-E2 complex, so that their use has been controversial. In 2003, Bartosch et al. generated highly infectious HCVpp by assembling the full length E1/E2 glycoproteins on retroviral core proteins derived from mouse leukemia virus (MLV). These particles were produced in 293T cells following transfection with three expression vectors coding for E1/E2, the MLV gag-pol core proteins and a packaging-competent MLV-derived genome encoding GFP as a marker. HCVpp harvested from cell supernatant were purified and shown to contain both E1 and E2 glycoproteins as well as the gag-pol core MLV proteins. Although E1 and E2 are supposed to be sequestered in the ER, FACS analysis revealed that a small fraction of these proteins reaches the cell surface, the typical site of MLV budding, thus suggesting that in this system HCVpp do indeed bud from the cell surface. Long-term expression of GFP was demonstrated suggesting that infection of target cells leads to integration of genomic DNA in the host. HCVpp generated from functional E1/E2 of different genotypes (1 to 6) exhibited different infectivity, although the reason for these differences are not clearly understood (Lavillette et al., 2005a). However, HCVpp generated with only either E1 or E2 exhibited strongly reduced infectivity. HCVpp were shown to infect primary human hepatocytes and several hepatocarcinoma cell lines including Huh-7, HepG2, and Hep3B. Infection of hepatocarcinoma cells (i.e. Huh-7) with these particles was used to investigate the HCV entry step in host cells and data confirmed the role of CD81 in this process (Bartosch et al., 2003; Bartosch et al., 2003a; Hsu et al., 2003; Lavillette et al., 2005a; McKeating et al., 2004; Zhang et al., 2004). This Huh-7/HCVpp model was also used to evaluate the neutralising activity of antibodies raised in the serum of chronically infected patients (see section 7). Receptor competition assays indicated that CD81 is used by HCVpp of all different genotypes, whereas siRNA silencing assays revealed that the level of SR-BI needed for efficient infection varies between genotypes. HCV E1 is believed to mediate fusion, while E2 has been shown to bind members of the cellular receptor complex, including CD81 and SR-BI. Three putative CD81 interaction sites on HCV E2 have been previously localized in three regions : region 1 474–492; 2 522–551; and 3, 612–619. Rothwangl et al. generated a series of alanine substitutions in E2 within these three regions to identify those residues that are critical for viral entry (Rothwangl et al., 2008). The effect of each mutation was tested by challenging Huh-7 and Hep3B cells with HCVpp. Although most HCVpp mutants harboring

modifications within region 1 displayed greatly reduced infectivity ($\leq 50\%$ of wt HCVpp), the corresponding E2 proteins retained their capacity to bind soluble CD81, exhibited normal conformation, association with E1 and incorporation into HCVpp, suggesting that region 1 is not involved directly in the binding to CD81. In contrast, several mutants in regions 2 and 3 exhibited strongly reduced or no binding to CD81, suggesting a critical role of these regions in receptor binding, while the structure of the corresponding proteins was apparently correct as assessed by immunoprecipitation with appropriate antibodies. The lack of upstream peptides in proteins E1 and E2 is likely to affect their anchoring to the ER membrane, and in turn their glycosylation, folding, and heterodimerization, eventually leading to reduced infectivity. Using HCVpp, Bian et al. (Bian et al., 2009) demonstrated that at least 14 amino acids from the core C-terminus are required for E1 function and that at least 12 amino acids from the E1 C-terminus are required for E2 function. These peptides appear to influence the glycosylation of E1 and E2. Furthermore, the peptides upstream of E1 and E2 can be exchanged by those from another genotype.

Blanchard et al. studied the cellular mechanisms of HCV endocytosis using HCVpp (Blanchard et al., 2006). The data obtained with siRNA-mediated clathrin heavy chain depletion and with chlorpromazine (an inhibitor of clathrin-coated pit formation at the plasma membrane) suggest that HCV enters target cells *via* this pathway, followed by a fusion step within an acidic endosomal compartment. Several reports (Dreux et al., 2006; Voisset et al., 2005; Voisset et al., 2006) demonstrated that HCVpp entry in Huh-7 and subsequent endocytosis is accelerated by the presence of HDL (high density lipoprotein), although these lipoproteins do not interact directly with E1/E2. Notably, the time lag between HCV particles binding and internalisation, which is likely to reflect the receptor complex assembly, is reduced by HDL. The authors suggest that by interacting with SR-BI (an HDL as well as HCV receptor) HDL significantly increase CD81 recruitment that leads eventually to HCV internalisation. Thus, HDL likely acts by reducing the exposition of HCV to neutralising antibodies. The authors suggest that the HDL/SR-BI interaction interferes with antibodies blocking E2 binding to CD81, but not those antibodies targeting E1. Drugs inhibiting SB-BI activity (BLT4) or silencing of this receptor by siRNA strongly reduced the HDL-mediated effects on HCV entry. von Hahn et al. observed that oxidized (but not native) low-density lipoprotein (oxLDL), a ligand of SR-BI, inhibited HCVpp (gen 1a, 1b, 2a) entry in hepatoma (Hep3B, Huh-7 or HepG2/CD81) cells with a IC_{50} of $1.5 \mu\text{g/mL}$, that is within the range of oxLDL in human plasma (von Hahn et al., 2006). However, binding of soluble E2 to SR-BI or CD81 was not affected by oxLDL, suggesting that oxLDL does not act as a simple receptor blocker. At the same time, oxLDL incubation altered the biophysical properties of HCVpp, suggesting a ternary interaction of oxLDL with both virus and target cells. HCVpp systems have been widely used in the screening of anti-viral compounds and in immunological studies (see below section 6 and 7).

4.3. HCV subgenomic and genomic replicon systems

Yoo et al. and Dash et al. were the first to report the replication of transcripts of a putative full-length cDNA clone of hepatitis C virus type 1 (HCV-1) after transfection in Huh7 and HepG2 cell lines (Dash et al., 1997; Yoo et al., 1995). However, in both cases the HCV genome lacked the 3'-NTR region which appears to be mandatory for viral genome replication (Kolykhalov et al., 2000; Yanagi et al., 1999). Hence, the use of these replicons has been controversial (Bartenschlager & Lohmann, 2000). Only non structural proteins are involved in the control of virus genome replication. Thus, to construct a sub-genomic replicon system, Lohman et al. deleted the entire structural region of a cloned HCV consensus genome Con1 (genotype 1b) and inserted a selectable gene marker (neomycin phosphotransferase,

Neo) downstream of the HCV IRES; the translation of NS proteins was controlled by the IRES of the encephalomyocarditis virus which was located downstream of the Neo gene (Lohmann et al., 1999). This construct was transfected in Huh-7 cells and selection with G418 allowed to isolate resistant colonies. Positive colony examination revealed a very high level of HCV RNA synthesis with 1000 to 5000 copies per cell. Although this high level of replication did not apparently affect cell morphology, more extensive analysis revealed some alterations of the ER that were linked to the presence of non structural proteins (Gosert et al., 2003). In addition, the rate of viral RNA replication was dependent on the cell cycle, thus emphasizing the occurrence of functional interactions between the virus and the host cell (Pietschmann & Bartenschlager, 2003). Extensive analysis demonstrated that both viral and host cell determinants were important for the maintenance of a high level of replication. Among these, some mutations in all NS proteins (either individually or in combination) have been shown to confer a better efficiency to the replicon (Blight et al., 2000; Friebe et al., 2001; Guo et al., 2001; Krieger et al., 2001; Lohmann et al., 2001). These mutations appear not to reduce the cytopathic potential of the replicon, nor to affect the enzyme activity of NS3 and NS5B proteins. It is likely that they confer adaptation to the host cell. Interestingly, several mutations have been identified in the ISDR (interferon sensitivity-determining region) of NS5A (Bartenschlager & Sparacio, 2007). On the other hand, the host cell seems to contribute to the efficiency of replicon as evidenced by the strong changes in replication rate as a function of the number of passages of the cell line (Lohmann et al., 2003). Significant progress has been made recently in the development of efficient high throughput screening (HTS) HCV replication systems (Pietschmann & Bartenschlager, 2003), as well as replicons expressing green fluorescent protein (Ikeda et al., 2005; Moradpour et al., 2004). Kim et al. used a subgenomic HCV replicon (Huh7/Rep-Feo) derived from a chimpanzee infectious clone (strain HCV-N, genotype 1b) (Kim et al., 2007). Huang et al. developed another form of HTS subgenomic replicon (PLN5 and PLNCP54) based on Huh-7 cells and the HCV Con1b sequence (Huang et al., 2008). Replicon PLNCP54 included NS2 sequence allowing identification of compounds inhibiting NS2/3 cleavage through differential screening with PLN5. Each plasmid carried a firefly luciferase reporter under the control of the poliovirus IRES. The use of these replicons allows basic and applied studies on viral protein expression and subcellular localisation, the role of cis-acting RNA elements in replication, the antiviral effect of interferon, as well as on the screening of antiviral molecules targeting the IRES, protease (NS2/NS3, NS3/NS4), helicase (NS3) and polymerase (NS5B).

4.4. HCV production in Huh-7 cell culture models (HCVcc)

Apparently, the major reason for the lack of virus particle production in full length replicon systems is that adaptative mutations that increase RNA replication are deleterious for virus particle assembly or release. In other words, high levels of RNA and protein production do not necessarily lead to efficient virus production. In contrast to replicon of genotype 1, replicon JFH1 (genotype 2a) generated from the serum of a patient with a fulminant infection exhibited an unusual high rate of RNA replication (20 times greater than that of Con-1) when transfected in Huh-7 cells, without requiring adaptative mutations (Kato et al., 2003). When a full length genome HCV RNA JFH1 (2a) or intragenomic or intergenomic chimera (structural/non structural proteins) between J6 (2a)/JFH1, H77(1a)/JFH1 or S52(3a)/JFH1 were transfected in Huh-7-derived cells, notably the Huh-7.5 clone, several groups observed that infectious HCV particles (HCVcc) were secreted in the extracellular medium (Gottwein et al., 2007; Lindenbach et al., 2005; Lindenbach et al., 2006; Pietschmann et al., 2006; Rouille et al., 2006; Wakita et al., 2005; Yi et al., 2006; Zhong et al., 2005). The data suggest that determinants within the structural proteins have a major impact on kinetic and efficiency

of virus assembly and release. Sucrose density gradient analysis of HCVcc particles revealed that core, E1, E2 and HCV RNA co-sedimented at the same density, approximately 1.15-1.17. Using anti-E2 antibodies, spherical HCV particles of 50 to 65 nm could be visualised by immunoelectronic microscopy. These particles were shown to be infectious for Huh-7 cells as well as *in vivo* for chimpanzees. Interestingly, virions recovered from these animals exhibited a greater specific infectivity and lower density (i.e. 1.09) than those recovered in cell culture, suggesting increased association of virions with lipoproteins (Andre et al., 2005; Lindenbach et al., 2006). CD81 specific antibodies as well as serum from chronically infected patients significantly inhibited HCVcc-mediated infection of Huh-7 cells. Interestingly, confocal imaging of Huh7.5 cells infected with either J6/JFH1, or S52/JFH1 revealed that in HCV-infected cells lipid droplets are redistributed from the entire cytoplasm to a perinuclear area and colocalized with core. However, no difference in lipid content between infected and non-infected cells was detected. Recently, full-length HCV DNA constructs (genotypes 1a, 1b, and 2a) in HCV-ribozyme expression plasmids were shown to generate extracellular HCVcc particles when transfected into Huh-7.5 cells (Heller et al., 2005; Kato et al., 2007). As found for HCVpp entry (Blanchard et al., 2006), HCVcc entry was reported to be pH-dependent and sensitive to concanamycin A and NH₄Cl (Tscherne et al., 2006).

4.5. Human hepatocarcinoma and immortalized hepatocyte (IHH)-based systems

Several lines of immortalized human hepatocytes have been generated. PH5CH cell lines (clones PH5CH1 to PH5CH8) were produced by immortalisation of primary human hepatocytes by the large T antigen of the simian virus SV40 (Ikeda et al., 1998). HuS-E and HuS-T cell lines were generated by immortalisation of primary human hepatocytes with vectors expressing hTERT (telomerase) and either E6/E7 genes of human papilloma virus or the large T of SV40, respectively (Aly et al., 2007). Immortalized human hepatocytes (IHH) were generated by transfecting primary human hepatocytes with an HCV core (genotype 1a) expression plasmid. These cells exhibited a significant telomerase activity (Ray et al., 2000). FLC4 is a cell line derived from a human hepatocellular carcinoma (Aoki et al., 1998); these cells were maintained in culture for long periods in a radial flow bioreactor. All these cell lines were shown to express some hepatocyte phenotypic markers (as assessed by RT-PCR analysis) as previously observed with other immortalized hepatocyte preparations or hepatocellular carcinoma cells.

PH5CH, FLC4 and HuS-E were shown to be sensitive to HCV infection and permissive to viral genome replication after being inoculated with serum from chronically infected patients (HCVser). Anti-CD81 antibodies and IFN were shown to block infection in these models. PH5CH and FLC4 cell lines, but not HuS-E, were shown to produce virions in the extracellular medium for a long period (4 to 8 weeks) after infection. Hyper variable region quasispecies analysis in FLC4 cells *versus* inoculum revealed a different panel of sequences in the cellular viral RNA as compared with the inoculum RNA, suggesting adaptation of HCV to the conditions prevailing in these cells (Aizaki et al., 2003; Murakami et al., 2008). When FLC4 cells were transfected with HCV H77 (1a) RNA and cultured in a bioreactor, HCV RNA was detected in the extracellular medium almost two months post-transfection and remained at a level of 10⁵-10⁶ copies/day for another three months, consistent with the relative amount of core protein (13pg/day). Extracellular medium analysis revealed the presence of virus-like particles that sedimented in the range 1.03-1.06, that is, close to the range in which HCV particles sediment from human serum (1.03-1.12) (Andre et al., 2002). Finally, when the medium collected 93-95 days post transfection was incubated with naive FLC4 cells both HCV RNA and core protein were detected in the extracellular medium between 15 and 30 days post infection.

IHH were transfected by electroporation with the HCV H77 (1a) RNA. HCV RNA and protein expression were detected in cell cultures for up to 12 days, and the cultures were discontinued for lack of growth after 2 weeks. Similarly, IHH transfected with HCV RNA JFH1 exhibited signs of virus replication as assessed by intracellular RNA and localization of NS3 protein by immunofluorescence. The appearance of virus-like particles in H77 RNA-transfected IHH indicated that HCV 1a replicates and assembles as virus particles in these cells. Moreover, the presence of HCV 5'-NTR was detected in culture medium of IHH cells transfected with HCV genome. Interestingly, medium from cultured cells was able to infect naive IHHs as assessed by HCV RNA accumulation and immunodetection of NS proteins at day 4 post-infection. This infection was inhibited by serum from HCV-infected patient, displaying neutralizing activity against the vesicular stomatitis virus/HCV pseudotype. Interestingly, while JFH1 replicates with a higher efficiency than H77 at the RNA level in Huh-7 cells or its derivatives, similar levels of virus genome copies were detected in the supernatant of H77 and JFH1 RNA-transfected IHH cultures; the number of focus-forming units per milliliter (relative to H77 and JFH1) was also similar. This suggests an equal ability of IHH to respond to a wider range of HCV genotypes as compared to Huh-7 cells and derivatives. However, the production of HCV particles in these cells appears to be restricted to a short period not exceeding a few days. Interestingly, recent studies demonstrated that HCV infection triggers in these cells both IFN signaling pathway and an autophagic response (Ait-Goughoulte et al., 2008; Kanda et al., 2006; Kanda et al., 2007).

4.6. Human fetal hepatocyte cultures (HFH)

Carloni et al. and Iacovacci et al. investigated the infection of human fetal hepatocytes by hepatitis C virus (Carloni et al., 1993; Iacovacci et al., 1997). For this purpose, human fetal hepatocytes were grown in serum-free medium and inoculated with HCVser. Genomic and replicative viral RNA strands and viral proteins were detected in hepatocytes 5 days after inoculation. Virions were released from the infected cells into the medium and could be serially passaged three times into fresh liver cell cultures. More recently, Lazaro et al. (Lazaro et al., 2007) characterized long-term, serum-free primary and passaged cultures of nontransformed hepatocytes from human fetal liver. After transfection of these hepatocytes with genotype 1a HCV (p90/HCVFLpU of genotype 1a) or infection with HCVser of genotypes 1, 2, and 3, medium analysis revealed up to 10^6 copies/ml of HCV RNA exhibiting a cyclic pattern; virus-like particles (density 1.17) were detected in the medium of transfected hepatocytes. Viral infection (from transfected cells) could be transmitted to naive cells, and the infection was shown to be inhibited by IFN. Evidence for cell death in infected hepatocytes suggests that HCV has some cytotoxic effects in this model. However, the authors could not establish whether cell death is associated with HCV levels in the medium.

4.7. Primary human hepatocyte cultures (PHH)

Primary human hepatocytes are prepared by collagenase perfusion of either human liver segments resected for medical purpose (metastasis, adenoma, hepatocellular carcinoma) or from livers that have been refuted for transplantation. Plating substrates and chemically-defined media allow these cells to be maintained in primary culture for weeks with preservation of several hepatocyte phenotypic markers such as production of plasma proteins, blood coagulation factors, urea, expression of detoxication systems (cytochromes P450, conjugation enzymes, membrane transporters), response to physiopathological stimuli in terms of signaling pathway activation, etc (Guguen-Guillouzo et al., 1986; Guillouzo, 1998; Hewitt et al., 2007; Pichard et al., 2006; Pichard-Garcia et al., 2002). Ito et al. were the first to

demonstrate that hepatocytes isolated from an HCV-positive patient, could be cultured and continued to stably produce HCV infectious virions in their extracellular medium (Ito et al., 1996). Intracellular negative-strand RNA could be detected by a strand-specific RT-PCR method and the core protein could be detected by immunofluorescence microscopy. Fournier et al. were the first to demonstrate that hepatocytes isolated from non-infected patients were sensitive to HCVser infection and permissive to viral genome replication (Fournier et al., 1998). Since then, several groups have confirmed these findings. Although the performances of these primary culture models vary from one laboratory to another, depending on cell culture conditions, reported results are consistent and reproducible. HCV infection of primary human hepatocytes with natural different HCVser genotypes (from 1 to 5) has been assessed by detection of the replicative RNA strand, increased intracellular accumulation of genomic RNA, production of infectious virions, generation of new quasispecies in culture, and inhibition of replication by IFN (Buck, 2008; Carriere et al., 2007; Castet et al., 2002; Chong et al., 2006; Fournier et al., 1998; Molina et al., 2007; Molina et al., 2008; Rumin et al., 1999). This model of primary and highly differentiated human hepatocytes infected with HCVser is clearly the one that most closely mimics the physiological situation. In addition, primary human hepatocytes were also shown to be sensitive to HCVpp (Codran et al., 2006; Meertens et al., 2008; Regeard et al., 2008) and HCVcc (JFH1) infection (Molina et al., 2008; Reynolds et al., 2008). Freshly isolated primary human hepatocytes have been recently used to evaluate comparatively the transcriptome profiling induced by core, NS3 and NS5A, or the HBV HBx protein as a control (via infection with adenovirus) (Budhu et al., 2007).

5. Comparative analysis of HCV cellular models

In order to be used with confidence for predictive antiviral effect in man, HCV cellular models must meet at least two major criteria: i) to mimic as closely as possible the *in vivo* situation in man at both the cellular and virion levels, ii) to be practical, reliable and adaptable to high throughput screening. When considering the panel of models described in section 4, it is clear that none of them appears to meet these two criteria (**Table 1**).

HCV-LP and HCVpp represent practical and flexible forms of virions and can be easily obtained in large amounts. These particles appear to be very different from the natural virions that circulate in the blood of patients and are almost exclusively used for assessing viral entry and neutralisation of infection by patient-derived antibodies. However, recent results obtained with the model of primary human hepatocytes infected by HCVser (Molina et al., 2007) do not confirm some of the data generated with HCV-LP or HCVpp. Notably, using these particles, several authors questioned the role of LDLR in the cellular entry of HCV. Triyatni et al. observed no inhibition of HCV-LP entry into MOLT4 cells by anti-LDLR antibodies (Triyatni et al., 2002). Bartosch et al. and Hsu et al. observed no inhibition of HCVpp infection of Huh-7 cells by LDL, or by anti-LDLR antibodies, respectively (Bartosch et al., 2003; Hsu et al., 2003). In contrast, LDLR was shown to play a part in the entry of HCVser in primary human hepatocytes (Molina et al., 2007). It is likely that these differences result from the fact that HCV-LP and HCVpp do not fully mimic the natural virions, especially in terms of association with lipoproteins (Andre et al., 2002; Andre et al., 2005). On the other hand, HCVcc particles exhibit a density of 1.15 to 1.17 g/ml, that is significantly greater than that characterizing infectious HCVser LVP in human plasma (<1.06 g/ml) suggesting that HCVcc exhibit minor association with lipoproteins and lipids. It was recently reported that CD81 was indispensable for infection of primary human hepatocytes with HCVser of different genotypes (Buck, 2008; Molina et al., 2008). However, while a soluble form of CD81 large extracellular loop is capable of preventing infection of Huh7.5 cells by JFH1-HCVcc, it did not prevent infection of cultured primary hepatocytes by HCVser

(Molina et al., 2008). In addition, soluble CD81 was much more effective in blocking HCVcc infection in Huh7.5 cells than in primary hepatocytes. These inconsistencies show that both the form of virus particle and the host cell are important determinants for HCV replication.

Subgenomic replicon systems appear to be very practical by combining simplicity, rapidity, robustness, and reproducibility. These systems lack the virion partner and are primarily used only for the screening of antiprotease and antipolymerase drugs under conditions that are far from the *in vivo* situation (see below). Although these replicons have been used to reveal drug resistance mechanisms (Bartenschlager, 2005), recent investigations for example on the effect of HCV protein E2 on the modulation of TJs in infected cells clearly emphasized the limitations of these models (Benedicto et al., 2008).

The Huh-7.5/HCVcc models provide greater advantages as they can be used to investigate every aspects of HCV biology from cellular entry to secretion. Hence, these models are ideal candidates for specific testing of therapeutics targeting viral entry, assembly, and release, as well as neutralizing antibodies and other drugs acting on cellular entry, such as fusion inhibitors. However, all these systems too often incorrectly referred to as *human hepatocytes*, are based on hepatocellular carcinoma cells which although being of human origin *are not hepatocytes*. These cells are dedifferentiated and exhibit abnormal hepatic phenotype with deregulated proliferation, gene expression, signaling pathways, host anti-viral response, endogenous metabolism, etc. In addition, these systems are based on the replication of synthetic HCV RNAs expressed from selected or engineered clones. For example, JFH-1 is a rare virus isolated from a patient with fulminant hepatitis C (Kato et al., 2003). Moreover, these cells cannot be infected with natural HCVser. Another problem with hepatoma or other cell lines including immortalized human hepatocytes is that their proliferation may change the cell population by adaptation to both the virus and the therapeutic compounds being tested. This is obviously not the case in primary human hepatocytes and hepatocytes *in vivo* which are known to proliferate at a very low rate, especially in seriously damaged liver with fibrosis and cirrhosis (Roskams, 2006). Such drastic difference in phenotype, notably proliferating *versus* quiescent cells (but this is not the only difference) may clearly lead to false or unappropriated anti-viral responses that will not be observed *in vivo*. A further problem encountered with cultured hepatoma cells (including HepG2, Hep3B and Huh-7) is their impaired poly(I-C)- and virus-activated IFN responses and the considerable variations they exhibit in the IFN response, notably in different Huh7 cell lines originating from different laboratories (Keskinen et al., 1999; Lanford et al., 2003; Li et al. 2005). This suggests that studies carried out with these cells must be interpreted with caution. Notably, TLR3 and RIG-I signaling pathways have been shown to be defective respectively, in Huh7 cells (Lanford et al., 2003; Preiss et al., 2008) and in Huh7.5 cells because of a point mutation within the RIG-I CARD-like homology domain (Sumpter et al., 2005). It is likely that these defects account for the high permissiveness of Huh-7.5 cells for HCV RNA replication. In contrast, the PH5CH8 immortalized cells that were established from non-neoplastic hepatocytes retain robust IFN responses to extracellular and intracellular poly(I-C) (Preiss et al., 2008). In this respect, these cells are likely to mimic normal hepatocytes *in vivo*, and thus may represent a valuable model for investigation of antiviral responses in hepatocytes.

Cultured fetal human hepatocytes appear to be as functional as adult hepatocytes in terms of natural virus infection, replication and release. However, some of their enzyme machinery are known to be different from those found in the adult, notably in terms of drug metabolizing enzymes (Hines, 2008). Thus, care must be taken when using these cells for anti-viral screening because prodrug activation, if required, may be catalytically inactive or not expressed, and drug metabolism which limits drug availability may be absent.

Primary adult human hepatocyte models infected with natural HCVser of any genotype closely mimic the *in vivo* situation. In addition, these models offer wide possibilities

to investigate antiviral drugs: i) the cells can be maintained in a differentiated phenotype, ii) they can be cultured in such a way as to maintain their drug metabolizing capacities (Pichard-Garcia et al., 2002), iii) they can be infected with HCVser of any genotype (including HCVpp and HCVcc particles), iv) the innate immune response is fully preserved, in contrast to what is observed in Huh-7 cells and derivatives, and other hepatoma cell lines (Keskinen et al., 1999; Lanford et al., 2003; Li et al., 2005; Sumpter et al., 2005); indeed, fetal and adult human hepatocytes produce IFN β and ISGs in response to IFN and HCV infection (Buck, 2008; Castet et al., 2002; Lazaro et al., 2007) a process that is likely to account for the low level of viral infectivity in these cells; iv) finally, hepatocytes can be prepared from different patients so that the contribution of the interindividual variability of the host biology to antiviral drug response can be evaluated, in contrast to hepatoma cell lines of immortalized hepatocytes that are issued from a single individual; indeed previous analysis show that such diversity exists in terms of natural anti-viral response, response to IFN, receptor expression, etc. (Chevaliez & Pawlotsky, 2007a).

Metabolic phosphorylation of nucleoside analogs is an essential step for the pharmacological activity of these compounds against NS5B (see section 6). The question of whether the rate and extent of nucleoside phosphorylation are similar or different in hepatoma cell lines and primary human hepatocytes has to be addressed in any investigation aimed at screening antiviral molecules. Indeed, significant differences have been noted between different hepatoma cell lines as well as between hepatoma cell lines and primary human hepatocytes (Hernandez-Santiago et al., 2004; Klumpp et al., 2008; Paeshuyse et al., 2008). Interestingly, it has been reported that the phosphorylation of some nucleoside analog derivatives that is observed in primary human hepatocytes does not occur in replicon cells (Huh-7) either because the compounds cannot enter the cells or because of a deficient conversion (Ma et al., 2007). This clearly emphasizes the risk of false negative in the screening of anti-HCV compounds using solely the replicon systems. Drug metabolism plays a major role in controlling the therapeutic effects of drugs. It is clear that the battery of drug metabolizing enzymes (including cytochromes P450 and conjugation enzymes) and transporters are expressed at very low level or quite differently in the hepatoma cell lines as compared with differentiated hepatocytes (Hewitt et al., 2007; Jover et al., 1998). Thus, false responses may be obtained as a consequence of either the absence of prodrug activation or the absence of drug metabolism. The same caveat arises with immortalized hepatocyte cell lines. This suggests that antiviral candidates that require a metabolic activation (other than phosphorylation) may be tested negative in cell lines. On the other hand, drugs that are rapidly biotransformed in hepatocytes to inactive metabolites may eventually appear to be inactive *in vivo* but will be tested as positive in cell lines (in which no inactivation takes place). Another important aspect to consider is that HCV chronic patients are (and will be in the future) polymedicated so that drug-drug interactions have to be anticipated (Thompson et al., 2009). Primary human hepatocyte cultures are the only *in vitro* system that allows one to predict such interactions with full confidence (Gomez-Lechon et al., 2007; Hewitt et al., 2007; Pichard et al., 2006). Last but not least, mounting evidence suggests that host genes significantly modulate HCV infection and replication (Ikeda & Kato, 2007; Moriishi & Matsuura, 2007; Randall et al., 2007; Sheikh et al., 2008). Since gene expression is highly dependent on the differentiation status of cells, this provides a further and strong argument in favor of the use of primary human hepatocytes for investigating HCV biology.

However, primary human hepatocyte culture models suffer from several major drawbacks. First, procurement and use of human tissue face ethical concerns that require necessary autorisations from regulatory agencies and informed consents of donors or donor's family. Adult human liver tissue suitable for the isolation of hepatocytes is obtained either from patients undergoing surgical liver resection for the removal of different types of lesions

(metastasis, biliary cyst, etc.), or from brain-dead organ donors whose liver has been refuted for transplantation (tissue traumatism, high level of steatosis, presence of a tumor, etc.). Since the liver tissue is strongly sensitive to ischemia and deteriorates rapidly after death, human hepatocyte isolation requires an appropriate coordination and logistics between the laboratory, surgeons, and anatomopathologists. The samples are transported to the laboratory as quickly as possible but often arrive late in the afternoon or in the evening meaning that the initial part of the work including hepatocyte isolation and plating is often overnight. Second, availability of human liver is limited and unpredictable. Indeed, there may be several weeks between the obtention of one liver sample of adequate quality and the next one, while in other cases, several liver samples may be obtained within the same day meaning that at least two teams have to work in parallel. Third, the quality of the tissue is variable from one liver sample to another due to the health conditions of the donor, nature of lesion, pre-surgery treatment or to the duration of transportation. For instance, a level of steatosis greater than 60% or pre-surgery chemotherapy (which is very toxic to the liver) results in poor quality and yield of cells. The amount of tissue is also variable from one liver sample to another (i.e. from 50 grams to 500 grams or more) making difficult to standardize the isolation procedure. Thus, the amount of hepatocytes isolated from a liver sample may therefore vary between a few millions to billions of cells. Fourth, the phenotype of hepatocytes has a tendency to exhibit more or less drastic changes immediately after cells isolation and plating, due to adaptation to the culture conditions. This process is often referred to as de-differentiation. Indeed, a number of phenotypic markers may be lost more or less rapidly after plating. Within the last decades, however, extensive investigations have allowed to design culture conditions that considerably limit this de-differentiation process by using chemically defined culture media, specific cell substrates (such as collagen sandwich or matrigel), specific cytokine/growth factor cocktails, co-cultures with other liver cell types, or 3D-cell configurations (Hewitt et al., 2007). Fifth, another problem is the high level of autofluorescence of primary human hepatocytes in culture which makes extremely difficult, if not impossible, to investigate intracellular events such as nuclear translocation of proteins, or to visualize intracellular molecules or proteins, including notably HCV proteins by immunofluorescence-based methods. Thus, in most reports, HCV infection is mainly investigated by RT-PCR measurement of viral RNA. Sixth, and as a consequence of all the drawbacks listed above, it is evident that these cultures are quite inappropriate for high throughput screening explorations. Therefore, primary human hepatocyte cultures should be used as a secondary screening system to confirm, invalidate, or complement data obtained with the other more practical systems described above.

6. Cellular models for the development of anti-HCV agents

The current therapy for HCV infection is a combination including pegylated interferon- α (IFN) and a nucleoside analog ribavirin (Chevaliez & Pawlotsky, 2007b). This therapy allows permanent eradication of circulating HCV virions (undetectable level of viral RNA in the blood) in only approximately 50% of patients infected with HCV of genotype 1, while the success of this therapy is greater (80% of patients cured) with other genotypes such as 2 and 3. In addition, IFN/ribavirin treatment is associated with many side effects (including depression, headache, fever, hemolytic anemia) that not only worsen the patient's quality of life but also lead to non-compliance with prescribed treatment in a significant number of cases (Hayashi & Takehara, 2006; Shepherd et al., 2007; Younossi et al., 2007). It is therefore evident that new therapeutic approaches that combine new viral targets, better efficiency and safety are crucially needed. Excellent reviews have been published in this respect (Neyts, 2006; Soriano et al., 2008; Stauber & Kessler, 2008; Wakita, 2007). In this section, we are going to summarize a number of experiments and investigations that have been carried out

£ To the best of their knowledge, authors have no real or perceived conflicts of interests.

using the HCV cellular models described in section 4. We shall focus on the use of these models in the screening of new chemical entities and RNA interference strategies. The use of such models for vaccine research and for the characterization of the neutralising activity of antibodies from chronically infected patients will be discussed in section 7.

6.1. HCV targeting by new chemical entities

Most pharmaceutical research on new chemical entities against HCV focus on inhibitors of viral entry, E2 binding inhibitors, NS2/NS3/NS4A protease inhibitors, NS5A inhibitors, NS3 helicase inhibitors, IRES inhibitors, NS5B polymerase inhibitors, inhibitors of virus assembly, inhibitors of virus release (Neyts, 2006; Soriano et al., 2008; Stauber & Kessler, 2008; Wakita, 2007). Of these, inhibitors of NS3/4A and NS5B are the most extensively developed therapeutic molecules since these enzymes play a highly strategic role in the virus life (Thompson et al., 2009). Huh-7/HCVpp models, subgenomic replicons, Huh-7/HCVcc replicon systems, immortalized hepatocytes and primary human hepatocytes have been used in this respect. Several reviews aimed at describing the use of the subgenomic replicons and the Huh-7.5/HCVcc systems for the screening of anti-HCV drugs have been published (Bartenschlager, 2005; Bartenschlager & Sparacio, 2007; Horscroft et al., 2005; Pietschmann & Bartenschlager, 2003). We have therefore decided to focus the present review on the most recently published data (since 2007 in most of cases). A list of potential anti-HCV compounds is shown in **Table 2**. This list is by no means extensive but merely intended to provide examples of recently characterized inhibitors using various HCV cellular models. In general, these investigations are based on the analysis of viral markers such as HCV RNA and antigens as indicators of treatment efficacy; occasionally, viral genome analysis of resistant cellular clones allows to identify/confirm the target of antiviral compounds. Of note, the use of primary human hepatocyte infected with HCVser is still quite limited. Some of the inhibitors previously discovered in cellular systems are now being tested in patients (Ali et al., 2008; Paeshuyse et al., 2008).

Targeting NS3/4A protease is particularly important because this viral enzyme not only orchestrates the processing of viral proteins, but also because it participates to the virus-mediated inhibition of the host response by cleaving TRIF and MAVS (see section 3). This has been referred to as the “double whammy” effect (Farley, 2003). Several types of NS3 serine protease inhibitors have been discovered. These include substrate-based peptide inhibitors targeting the active site of the enzyme, strong electrophiles located at the position of the scissile amide bond, phenanthrenequinones, thiazolidines, benzoylamides (Trahtenherts et al., 2008). BILN 2061 was the first HCV protease inhibitor to enter clinical trials, and early results were highly promising showing a rapid and significant decline in plasma HCV RNA virus load in all treated patients infected with HCV genotype 1 (Lamarre et al., 2003). Unfortunately, further development of the compound had to be stopped due to cardiac toxicity (Reiser et al., 2005). In recent studies, a comparative analysis of the ability of two potent macrocyclic inhibitors of NS3/4A protease (TMC435350 and TMC380765, **Table 2**) to inhibit HCV RNA replication and to restore IFN β production was carried out using a genome length NNeoC-5B/2-3 cell replicon system (Liang et al., 2008). The results showed that the concentrations of inhibitor necessary to restore the RIG-I signaling pathway leading to IFN production were much greater (2 orders of magnitude at least) than those necessary to inhibit the viral genome replication, in spite of the finding that the level of full-length MAVS protein expression was recovered. No definitive explanation has been provided for these observations. It is suggested that the rescue of the host response by NS3/4A inhibitors may not contribute significantly to their therapeutic effect. However, it reminds to be verified that similar observations are observed in primary hepatocytes infected with HCVser.

NS5B inhibitors are classified into nucleoside and nonnucleoside inhibitors (Beaulieu, 2007; Pauwels et al., 2007). Nucleosidic inhibitors compete with the natural ribonucleoside triphosphate substrates at the catalytic site of NS5B, while nonnucleosidic inhibitors exhibit different chemical structures and inhibit the initiation and/or elongation step by targeting different sites of the enzyme, including domains close to the active site and allosteric sites. Several new compounds have been tested including derivatives of nucleosides, naphthalenones, benzothiadiazepines, pyridazinones, and benzodiazepines.

Treatments of replicon systems with antiviral molecules directed against NS3/4 or NS5B generally lead to the emergence of mutants with reduced sensitivity to the treatment. The duration of treatment required to observe the emergence of such mutants varies considerably with the nature of the antiviral molecule. For instance, Ali et al. showed that short term (less than one month) treatment of replicon did not generate mutants to PSI-6130, NM107 or R1479 (two other nucleoside analogs) while resistant variants emerged during treatment with a non nucleotide analog NNI-1 (Ali et al., 2008). In contrast, long-term treatments (several months) resulted in the generation of mutants (notably S282T) with reduced sensitivity to PSI-6130 and its prodrug R7128. Interestingly, while these two molecules generate the same active species, wide differences in resistance and nature of mutations were observed presumably due to cell-dependent ability to generate the active species. These results suggest that the extent of resistance observed during a drug treatment is not only linked to the virus adaptation through the selection of mutants, but also to the host cell through the selection of cell populations with a modified nucleoside metabolism. A panel of HCV NS3 mutants conferring resistance to protease inhibitors (BILN2061, VX950 and ITMN191) were investigated by transient replication assay in a genotype 1b N strain replicon (He et al., 2008). The results showed that the selective advantage conferred by drug-resistant mutants depends on their relative drug susceptibility, relative replication capacity compared to the wild-type virus, and the concentration of drug. Different protease inhibitors caused unique selective advantage profiles that might correlate with their resistance profiles. Bassit et al. evaluated combinations of nucleoside analogues beta-D-2'-C-methylcytidine (2'-C-MeC; NM-107) or beta-D-2'-deoxy-2'-fluoro-2'-C-methyleytidine (PSI-6130) with IFN α 2b or triple combination with ribavirin using the Huh-7 subgenomic HCV replicon (Clone B) (Bassit et al., 2008). Double and triple combinations of 2'-C-MeC or 2'-F-C-MeC with IFN α 2b or of IFN α 2b plus ribavirin with either 2'-C-MeC or 2'-F-C-MeC demonstrated synergistic antiviral effects.

Other inhibitors have been investigated in addition to those described above. Some interferon variants display EC_{50} (the concentration that inhibits HCV replication by 50%) significantly smaller than that relative to the routinely used IFN α 2b (Erickson et al., 2008; Escuret et al., 2006). Using an HTS replicon system, Huang et al. screened a library of 230,000 compounds (P. Huang et al., 2008). They defined hits as compounds with an EC_{50} below 10 μ M and a CC_{50} (the concentration that inhibits cell viability by 50%) of 10-fold over the EC_{50} . Two diphenyl heterocyclic compounds were discovered R706 and R803 that contained a dichloroacetamide moiety (**Table 2**). Both inhibited HCV replication at protein and RNA levels. However, it was not possible to identify a target for these compounds of either viral or cellular origin. Interestingly, R803 was shown to be able inhibit HCV RNA replication that was refractory to IFN-mediated inhibition, and to induce only few resistant clones with respect to NS3/4 or NS5B inhibitors.

Medicinal herbs or natural compounds are being used increasingly in the search for safe and efficient drug candidates for hepatitis C virus infection. Lee et al. investigated the anti-HCV effect of compounds from Mori Cortex Radicis (Lee et al., 2007). The screening of a library of extracts from medicinal plants (173 species) for compounds with anti-HCV affinity led to the selection of a methanol extract from Mori Cortex Radicis. Fractionation of

the extract by monitoring antiviral activity with a replicon model revealed five compounds including mulberroside C, moracin P, O, and M and mulberrofuran K that behaved as potent inhibitors of the replicon assay. This was confirmed by NS3 helicase inhibitory activity for moracin P, and O. Using the Huh-7.5/JFH1 HCVcc system, Nahmias et al. observed that naringenin, an abundant flavonoid in citrus fruits, is an inhibitor of VLDL secretion and of the MTP activity as well as the transcription of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and acyl-coenzyme A:cholesterol acyltransferase 2 in infected cells (Nahmias et al., 2008). Most interestingly, these authors observed that naringenin reduces HCV secretion in Huh-7.5 infected cells by 80%. Supplementing HCV patient diet with this compound should therefore be an option that deserves to be tested in the future. However, this compound is a potent inhibitor of several human liver cytochrome P450s (Breinholt et al., 2002; Brill et al., 2009) and its use will have to be tightly controlled. Several compounds have been shown to inhibit HCV entry or E2-CD81 interaction including cyanovirin-N, arbidol, a peptide of lactoferrin (C-s-3-33), serum amyloid A (Abe et al., 2007; Cai et al., 2007; Helle et al., 2006; Lavie et al., 2006; Pecheur et al., 2007). Using the model of primary human hepatocyte infected with HCVser (genotypes 1, 2 and 3), Molina et al. demonstrated that the human recombinant soluble LDLR (the LDL binding domain consisting of the 7 repeats) is a potent inhibitor of HCV entry (Molina et al., 2007). This peptide which mimics the LDLR binding site apparently competes with membrane bound LDLR and thus inhibits HCVser binding to hepatocytes.

The screening of most of these inhibitors has necessarily been carried out with a limited number of HCV cellular systems, notably for many of these with genotype 1b subgenomic replicon systems. One may therefore wonder whether the candidate anti-HCV compounds will be as efficient on the other HCV genotypes and subtypes; the existence of multiple quasispecies that prevail *in vivo* further amplifies this concern (Le Guillou-Guillemette et al., 2007; Paeshuyse et al., 2008). In this respect, cyanovirin-N, an inhibitor of HCV entry offers a promising new contribution to the arsenal of anti-HCV drugs (Helle et al., 2006). Using the Huh-7/HCVpp and Huh-7.5/JFH1 HCVcc systems, these authors showed that by interacting with N-linked glycans associated with HCV envelope glycoprotein E2, this compound is likely to inhibit the E2-CD81 interaction and cellular entry of HCV of various genotypes. Interestingly, a comparative analysis of the anti-HCV activities of a selection of compounds with distinct molecular targets (NS3, helicase and NS5B inhibitors, cyclophilin binding compounds, IFNs) was reported using several genotype 1b replicon systems using Huh-5-2, Huh-9-13, Huh-Mono and Huh-6 cells (Paeshuyse et al., 2008). These authors observed no significant difference in the response of the different replicon cell lines to the different inhibitors. A recent study demonstrated the utility of chimeric replicons for broad-spectrum activity determination of HCV inhibitors. Herlihy et al. created a panel of intergenotypic chimeric replicons harboring NS5B sequences from different genotypes (2b, 3a, 4a, 5a, 6a) (Herlihy et al., 2008). Stable cell lines were generated with replication-competent chimeras in order to evaluate the antiviral activity of HCV nonnucleoside polymerase inhibitors (NNIs) that target different regions of the protein. Very wide ranges of inhibitory effects of selected compounds were evidenced against NS5B chimeric replicons harboring non-genotype 1b sequences. Similarly, Huang et al. observed widely variable responses (> 30-fold shift in EC50) of genotypes 1 and 2a to inhibitor R803 (Huang et al., 2008). In addition, and as indicated above, HCV variants resistant to selective inhibitors are expected to emerge. Thus, a drug should be effective against several (if not all) genotypes and have a high barrier to resistance selection to be used in antiviral therapy against HCV. Alternatively, when the tested compound exhibits a genotype-dependent action, analysis of intergenotypic replicon systems or the use of human hepatocytes infected with serum derived HCV of different genotypes may provide additional hints regarding its mechanism of action.

Host proteins that cooperate with the virus represent other classes of therapeutic targets (Ikeda & Kato, 2007; Moriishi & Matsuura, 2007; Sheikh et al., 2008) (**Table 2**). In contrast to viral inhibitors, cellular protein inhibitors have the advantage of being insensitive to the virus genetics (genotype and subtype), although their use may lead to side effects. The use of cellular models that are as close as possible to the differentiated human hepatocyte should be mandatory in this respect. Using an HTS subgenomic HCV replicon (Huh7/Rep-Feo) and OR6 cells stably expressing the full-length genotype 1 replicon ORN/C-5B/KE9 as a secondary screening system, Kim et al. screened an appropriately formatted library of 2568 bioactive molecules including FDA-approved drugs (Kim et al., 2007). Corticosteroids (triamcinolone, dexamethasone, prednisolone and methyl prednisolone) were found to be promoters of HCV replication, irrespective of their immunomodulatory effect, as well as some PPAR γ agonists (notably troglitazone, but not clofibrate). In contrast, phosphodiesterase inhibitors (trequinsin), MAP38 inhibitors (SB 203580), and HMG-CoA reductase inhibitors (atorvastatin, simvastatin, and fluvastatin - while the inhibitory effect of lovastatin and pravastatin was weaker) as well as other compounds demonstrated antiviral activity in these systems. Cholesterol synthesis inhibitors reduce the production of mevalonate, a precursor in isoprenoid and geranylgeranyl pyrophosphate synthesis. It has been reported that geranylgeranylation inhibition, rather than cholesterol synthesis inhibition *per se* is responsible for the inhibition of HCV RNA replication (Kapadia & Chisari, 2005; Ye et al., 2003). Indeed, the anti-HCV effect of the statins was rescued by addition of geranylgeraniol. Because the HCV genome does not encode a geranylgeranylated protein, it is likely that a host geranylgeranylated protein must play an important role in HCV replication. Wang et al identified FBL2 as such a protein required for HCV replication through formation of an active complex with NS5A (Wang et al., 2005). In contrast, using primary human hepatocytes infected with HCVser we reported that another statin, squalestatin, *increased and not decreased* the entry and genome replication of HCV most likely through up-regulation of LDL receptor (Molina et al., 2007). This opposite effect of squalestatin as compared to other statins is most likely due to the fact that this compound which blocks squalene synthesis, a step downstream of farnesyl pyrophosphate, does not interfere with isoprenoid and geranylgeranyl pyrophosphate synthesis. As any other statins squalestatin induces the expression of the LDLR and this is likely the reason for its stimulating effect on hepatocyte infection by HCVser. On the other hand, and interestingly, 25-hydroxycholesterol inhibited LDLR expression and HCV genome replication in the same model (Molina et al., 2007).

Lipid metabolism is likely to play an important role in HCV RNA replication. Using replicon systems, Leu et al. and Kapadia et al. reported that polyunsaturated fatty acids (PUFAs) such as arachidonic acid, docosahexanoic acid and eicosapentaenoic acid inhibit HCV replication by a mechanism that appears to be independent of their antagonist effect on LXR (for example T0901317 an agonist of LXR had no effect on HCV replication), while linoleic and linolenic acids reduced HCV RNA levels only slightly, and saturated fatty acids enhanced HCV RNA levels (Kapadia & Chisari, 2005; Leu et al., 2004). Interestingly, using primary human hepatocytes infected with JFH1/HCVcc we observed that linoleic and linolenic acids inhibit the replication of HCV RNA by 50 to 80% (Molina & Maurel, unpublished results). Sphingolipid biosynthesis pathway is another locus of anti-HCV strategy. Together with cholesterol, these lipids as well as glycosphingolipids enter into the composition of lipid rafts, the suspected site of HCV replication (Shi et al., 2003). Screening a natural product library with an HCV1b HTS replicon system, Sakamoto et al reported that inhibitors of serine palmitoyltransferase (the first step enzyme in sphingolipid biosynthesis) such as myriocin or NA255, inhibitors of dihydroceramide synthase (a further step in this pathway) such as fumonisins B1, and inhibitors of glycosphingolipid (GSL) synthesis such as PPMP all inhibited HCV replication (Sakamoto et al., 2005). The mechanism of this effect is

not yet clear but could involve the NS5B-membrane web association.

Another pathway that is likely to play a major part in HCV replication is the GTP biosynthesis pathway. The first enzyme involved in this pathway is the inosine monophosphate dehydrogenase (IMPDH). Inhibitors of this enzyme such as ribavirin, mizoribine, micophenolic acid and VX-497 (in association with ribavirine) were all shown to inhibit HCV replicon systems (Henry et al., 2006; Naka et al., 2005; Zhou et al., 2003).

The N-glycosylation pathway that controls proper folding of proteins has also been identified as a possible locus of anti-HCV action. Derivatives of deoxynojirimycin (DNJ) iminosugars, which are inhibitors of α -glucosidase, have been shown to prevent the correct folding and assembly of E1 and E2 HCV glycoproteins (Branza-Nichita et al., 2001). By using the Huh-7/HCVpp model Chapel et al. showed that the inhibition of α -glucosidases by iminosugars results in the misfolding and misassembly of HCV glycoprotein pre-budding complexes (Chapel et al., 2007). This leads to a reduction in the incorporation of E1/E2 complexes into HCVpp and a subsequent reduction of infectivity of the secreted particles. This suggests the potential usefulness of DNJ derivatives in targeting HCV infection.

Several problems must be anticipated when screening chemical libraries with cellular models, including protein binding and cytotoxicity of compounds. It has been demonstrated that drugs including certain antiviral compounds show markedly reduced activity *in vivo* or in cellular models because of their natural tendency to bind serum proteins (Molla et al., 1998). To evaluate this possibility, the inhibitory effect of the antiviral candidates should be measured in cells cultured in media containing increasing concentrations of human serum (Huang et al., 2008). Many small molecules are cytotoxic, and hepatoma replicon cell lines are highly sensitive to cytotoxic or cytostatic agents. This may generate false positives. Indeed, cytotoxic effects can be mistaken for antiviral effects by decreasing the end-point signal by reducing cell number, while not affecting the rate of HCV replication. Primary human hepatocytes are generally less sensitive to drug toxic effects (Huang et al., 2008), because they express higher level of detoxication enzymes (Jover et al., 1998). It is therefore mandatory to run cytotoxicity tests in parallel to antiviral tests in all antiviral analysis using cellular systems.

Finally, the possibility that *in vitro* cellular models do not correctly predict drug effects *in vivo* cannot be ruled out (Henry et al., 2006). Indeed, the case of various immunosuppressors provides a typical example in this respect. Cyclosporin A and mycophenolic acid were shown to inhibit HCV replication in replicon or immortalised cell lines (El-Farrash et al., 2007; Henry et al., 2006; Nakagawa et al., 2004). However, treatment of liver transplanted recipients with immunosuppressors does not eradicate the virus but in contrast *activates* re-infection of the grafted liver (Pollard, 2004). It is likely that the suppressive effect of these drugs on the host immune response (which is obviously absent from the HCV replication cellular models) largely dominates and actually masks the decrease in viral load resulting from their antiviral activity.

6.2. HCV targeting by RNA interference and related approaches

Given the critical role played by miRs in the control of both HCV infection and replication, and host gene regulation, therapeutic strategies using RNAi offer promising possibilities (Watanabe et al., 2007). In addition and in contrast to the long and high cost development of new chemical entities, designing and validating an siRNA that targets a specific gene is a relatively fast process. Synthetic siRNA or shRNA use the same machinery as miRs to downregulate their target genes. HCV targeting by RNAi strategies can be envisaged both on HCV RNA itself (for instance the 5'-NTR) and on host genes that are mandatory for viral infection and/or replication (for instance the CD81 receptor). Subgenomic

replicons, HCVcc replicon systems and primary human hepatocytes have been used in this respect, siRNAs or shRNAs being either transfected directly or expressed after transduction of host cells with lentiviruses. Almost all regions of HCV (genotypes 1 and 2) have been targeted by RNAi including the 5'-NTR, E2, core, NS3, NS4B, NS5A, NS5B and the 3'-NTR using both subgenomic replicon and Huh-7/HCVcc systems (Korf et al., 2007; Watanabe et al., 2006) (**Table 2**). In most of cases, 80 to 95% inhibition of both HCV genome replication and protein synthesis was obtained with low concentrations (in the μM -nM range) of siRNA. However, as observed with anti HCV drugs, the virus is likely to escape or resist to siRNAs through mutations of the sequences which are specifically targeted. Several strategies have been tested to tentatively overcome this problem with convincing results (Watanabe et al., 2007). The 5'-NTR which displays the greatest homology between different genotypes has been targeted in many studies. However, this region is highly structured and, as a consequence, rather reluctant to hybridization. Another strategy is based on the use of long dsRNA that can be degraded by Dicer in a number of siRNAs that can be used to target multiple sites of a heterogeneous population of viral genomes. Thus, siRNA raised against the 5'-NTR of HCV 1b were able to inhibit the replication of HCV 2a (Watanabe et al., 2006). Another strategy consists to target different regions of HCV genome by combining several siRNAs (De Francesco & Migliaccio, 2005; Korf et al., 2007).

Ribozymes and DNAzymes have also been used to inhibit HCV replication (Bhindi et al., 2007; Usman & Blatt, 2000). Jarczak et al. combined hairpin ribozymes targeting the 3'-NTR and siRNAs targeting the 5'-NTR of HCV with significant inhibition (Jarczak et al., 2005). Trans-splicing ribozymes have been used to repair mutant RNAs responsible for human genetic diseases and cancers (Ryu & Lee, 2004). These authors designed a series of such ribozymes to target the HCV IRES region, that is, to replace HCV transcripts with a new RNA exerting or not anti-HCV activity. Gonzalez-Carmona et al. tested a series of hammerhead ribozymes targeting the 5'-NTR of HCV (Gonzalez-Carmona et al., 2006). Ribozyme (Rz1293) targeting the GCA 348 cleavage site in the HCV loop IV displayed a significant inhibitory activity (>70%).

DNAzymes are preferred to ribozymes because they are easier to prepare, less sensitive to chemical and enzymatic degradation and easier to deliver into cells. Roy et al. designed DNAzymes targeting different regions of HCV IRES of different genotypes (Roy et al., 2008). The RNA cleavage and translation inhibitory activities revealed that the majority of DNAzymes efficiently and specifically inhibited HCV RNA translation. Interestingly, DNAzymes exhibited genotype-specific activities, some of them targeting genotype 1b and others targeting genotype 2a, more efficiently. Trepanier et al. designed a series of 2'-O-methyl-modified DNAzymes to target highly-conserved RNA sequences located within the HCV core-E1 coding region (Trepanier et al., 2008). One of these, Dz858-4-Ome, produced a decrease of 63, 87 and 84% of intracellular HCV RNA, core protein and HCV antigen expression, respectively, 6 h post-transfection. DNAzymes can thus be used as selective and effective inhibitors of HCV RNA replication and represent potent therapeutic agents against HCV infection.

Targeting of cell factors that are mandatory for HCV infection and replication is another potent RNAi strategy because these genes are not (or much less) subjected to mutations. Such a strategy has been used successfully by targeting La, eIF2B, hVAP-33, proteasome α -subunit 7 and Hu antigen R proteins, as well as proteins interacting with the NTRs of HCV (Zhang et al., 2004). We reported on the downregulation of CD81 in primary human hepatocytes by RNAi strategy. Our results showed a significant reduction of HCVser entry and replication in a genotype-independent manner (Molina et al., 2008). Henry et al., combined shRNAs directed to IRES, NS5B and CD81. Interestingly, although these various shRNAs were active on their own, their combination resulted in an additive effect (Henry et

al., 2006). Using a systematic RNAi screening and the Huh-7.5/HCVcc (genotype 1) model, Randall et al. identified a panel of host genes that regulate HCV replication, either by direct interaction with the viral genome or by controlling signaling pathways (Randall et al., 2007). Proteins that exhibited a strong potential for HCV replication included CD81, DDX3X (dead box helicase), SEC11L1 and HM13 (signal peptidases), ATF6 (unfolded protein response), VAPA and VPS35 (vesicular traffic), ACTN1 (cytoskeleton), and various kinases that bind NS5A including PDPK1, RAF1, EIF2AK2. Interestingly, inhibition of ATF6 expression stimulated HCV replication. Similarly, Superkova et al. screened a panel of siRNAs that preferentially target human protein kinases using Huh-7 and a selectable tricistronic HCV genotype 1b subgenomic replicon expressing firefly luciferase gene as reporter (Supekova et al., 2008). They identified three kinases, Csk, Jak1, and Vrk1 which promote HCV replication as assessed by viral RNA and protein levels. Interestingly downregulating Fyn, a kinase that is negatively regulated by Csk, induced HCV replicon, suggesting that Csk mediates its effect on HCV replication through Fyn. In another study, Ariumi et al. hypothesized that kinases such as the ataxia-telangiectasia mutated kinase (ATM), ATM- and Rad3-related kinase (ATR), poly(ADP-ribose) polymerase 1 (PARP-1), and checkpoint kinase 2 (Chk2) that control the host response to genomic stress might affect HCV replication (Ariumi et al., 2008). Using Huh-7 cells and subgenomic and full length genotype 1b replicon as well as the genotype 2a HCVcc system, they observed that shRNA-mediated downregulation of ATM or Chk2 significantly inhibited HCV replication. NS3-NS4A interacts with ATM and NS5B interacts with both ATM and Chk2, suggesting that the ATM signaling pathway is critical for HCV RNA replication. Using the Huh-7.5/JFH1 HCVcc system, Yang et al. reported that expression of fatty acid synthase (FASN), a cytosolic protein, increases in the extracellular medium of HCV-infected cells (Yang et al., 2008). Whether this increase coincides with increased lipogenesis is presently unknown. Interestingly, shRNAs directed to FASN mRNA, as well as C75 a pharmacological inhibitor of FASN blocked HCV RNA synthesis in the replicon (genotype 1b) and Huh-7.5/HCVcc (2a) systems. This coincided with a reduction of CLDN1 (but not CD81) expression. These results provide an interesting link between HCV infection and the lipid metabolic disorder (steatosis) that appears to be induced by this infection.

7. Cellular models for the development of anti-HCV vaccine and the characterization of neutralizing antibody activity

Although HCV-LPs derived from insect cells are noninfectious, they display some properties of the native virions and are able to confer protection in animal models. Indeed, virus-LPs have been used successfully as vaccines in papilloma, rotavirus, and Norwalk virus infections (Baumert et al., 1999). Similar strategies for HCV vaccine development have been tested using insect cell-derived HCV-LPs. Such particles were shown to be highly immunoreactive to anti-HCV antibodies in serum of patients infected with various HCV genotypes. Moreover, HCV-LPs derived from insect cells were safe and immunogenic in different animal models including BALB/c and HLA-A 2.1 transgenic (AAD) mice, and two non human primates baboon and chimpanzees (Baumert et al., 1999; Elmowalid et al., 2007; Jeong et al., 2004; Murata et al., 2003; Steinmann et al., 2004). Mice immunized with VSV-HCV-C/E1/E2 generated cell-mediated immune responses to all of the HCV structural proteins, and humoral responses notably to E2 were also readily evident. Thus, engineered VSVs expressing HCV Core, E1, and E2 and/or HCV-LPs represent useful tools in vaccine and immunotherapeutic strategies designed to address HCV infection (Ezelle et al., 2002). Jeong et al. and Elmowalid et al. characterized the immunogenicity and induction of protective immunity by HCV-LPs in baboons and chimpanzees (Elmowalid et al., 2007;

Jeong et al., 2004). Animals were immunized with HCV-LPs or HCV-LPs plus AS01B adjuvant. After immunization, they developed an HCV-specific immune response and proliferative lymphocyte responses against core, E1, and E2. When submitted to infectious HCV inoculum, one chimpanzee developed transient viremia with low HCV RNA titers (10^3 to 10^4 copies per ml) in the third and fourth weeks. Other animals exhibited high levels of viremia (10^4 to 10^5 copies per ml) which however became undetectable by week 10 after infection. After infection, all animals demonstrated a significant increase in peripheral and intrahepatic T cell and proliferative responses against the HCV structural proteins. These responses correlated with the decrease in HCV RNA levels. Interestingly, when non-immunized chimpanzees were infected with the same HCV inoculum, three developed persistent infection with viremia in the range of 10^5 to 10^6 copies per ml. These results show that HCV-LP immunization induces HCV-specific cellular immune responses that can control HCV challenge in primate models.

The factors leading to spontaneous clearance of HCV or to viral persistence are elusive. Understanding virus-host interactions that enable acute HCV clearance represent a major step in the development of more effective therapeutic and prophylactic strategies. In this respect, several groups used HCV particle infection systems to monitor the neutralizing activity of antibodies collected from acutely or chronically infected patients. Steinman et al developed an HCV-LP-based model system using hepatoma cell lines Huh-7 and HepG2 (Steinmann et al., 2004). They observed that cellular HCV-LP binding was specifically inhibited by antibodies from patients with acute or chronic hepatitis C in a dose-dependent manner. Interestingly, the presence of neutralizing antibodies was not associated with viral clearance *in vivo*. Using a library of homologous overlapping envelope peptides covering the entire HCV envelope, they identified an epitope in the N-terminal E2 region as a target of human neutralizing antibodies. Using a sensitive neutralization assay based on infectious HCVpp, it was reported that sera from haemodialysis infected patients (genotype 1b) either inhibited or enhanced HCVpp (E1/E2 genotype 1b or 1a) infectivity (Lavillette et al., 2005). The inhibitory effect inversely correlated with the HCV RNA level in the serum suggesting that inhibitory antibodies may be involved, at least in part, in the control of virus replication *in vivo*. However, using a similar system, Meunier et al. observed that the presence of neutralizing antibodies is not necessarily required to clear the virus (Meunier et al., 2005). On the other hand, enhancement of infectivity was observed preferentially with serum from patients or chimpanzee with sustained high replication rate, as well as with serum from non infected individuals. Apolipoprotein C1 alone or as a component of HDL was shown to be responsible for this enhancement (Meunier et al., 2005). Owsianka et al. used HCVpp to assess the reactivity and neutralizing capability of antisera and monoclonal antibodies raised against portions of the HCV E2 envelope protein (Owsianka et al., 2005). Rabbit antisera raised against either HVR1 or ectodomain of E2 showed limited and strain specific neutralization. By contrast, the monoclonal antibody (MAb) AP33 (recognizing E2 residues 412-423, a region highly conserved across different genotypes) demonstrated potent neutralization (inhibition of CD81 binding) of infectivity against HCVpp carrying E1E2 representative of all genotypes tested. Identification of neutralizing antibodies is likely to be of significant benefit to future vaccine and therapeutic antibody development. Boo et al. investigated the capacity of antibodies in patients submitted to antiviral therapy to neutralize HCVpp (Boo et al., 2007). No correlation was observed between baseline viral load, quasispecies population, neutralizing antibody levels and the response of patients to treatment with PEG-IFN and ribavirin. More recently Haberstroh et al. using HCVpp in kinetic and fusion assays observed that after binding of the virus to the target cell, neutralizing antibodies in the majority of HCV-infected individuals target an early step of HCV entry, most likely related to HCV-CD81, SR-BI interactions as well as membrane fusion (Haberstroh et al.,

2008). Furthermore, they identified viral epitopes in E1 and E2 representing potential targets for neutralizing antibodies.

8. Conclusion

A number of *in vitro* cellular models have been generated to investigate different aspects of HCV biology including cell entry, replication, assembly, excretion, host antiviral and IFN responses and viral resistance to treatments. These models are based on a range of host cells and a range of forms of virion particles. All these models exhibit advantages and disadvantages at the biological and experimental levels. On the one hand, investigations aimed at basic research should favor the systems that most closely mimic the *in vivo* situation for the step of virus biology that is the object of the study. On the other hand, investigations aimed at applied therapeutics and pharmacology should first be based on the most adapted HTS systems for the screening of chemical libraries or other antiviral agents. A number of new chemical entities and new therapeutic strategies (RNAi) have been discovered and identified with these cellular models. In many cases, it will be necessary to subsequently confirm the efficacy of selected positive candidates with the model of primary human hepatocytes infected with natural HCVser, using different hepatocyte preparations (from different liver donors) and different serum samples (from different infected patients with different load and genotype). This model will even allow predicting pharmacokinetics of the new chemical entities as well as possible drug interactions and liver toxicity. These last points are important because it is now clearly anticipated that in the near future anti-HCV treatments will involve a series of different drugs/agents.

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Legends for figures

Figure 1. The various steps of HCV entry in hepatocytes. Refer to text (sections 2.2. and 2.3.) for detail.

Figure 2. HCV genome replication, assembly and release. Refer to text (sections 2.3 and 2.4) for detail.