Study of cell host factors involved in Hepatitis C virus tropism

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« Même un chemin de mille lieues commence par un premier pas »

Proverbe japonais

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ABBREVIATIONS

aa	amino acid
apoB, apoE	Apolipoprotein B or E
ARF	Alternative reading frame
ARFP	Alternative reading frame protein
CD81	Cluster of differenciation 81
CLDN	Claudin
CMV	Cytomegalovirus
CsA	Cyclopsorin A
CyP	Cyclophilin
Da	Dalton
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule three grabbing non integrin
DGAT-1	Diacyl glycerol acyltransferase 1
ECL	Extra cellular loop
ECMV	Encephalomyocarditis virus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EphA2	Ephrin receptor A2
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell culture derived HCV
HCVpp	HCV pseudo-typed retroviral particles
HDL	High density lipoprotein
HIV	Human immunodeficient virus
HS	Heparan sulfate
HVR	Hypervariable region
hVAP-33	Human homologue of the 33-kDa vesicle-associated membrane protein-associated protein
IFN	Interferon

IFNAR	Interferon alfa receptor
IRES	Internal ribosomal entry site
IRF-3	Interferon regulatory factor 3
JFH1	Japanese fulminent hepatitis
KO	Knock-out
LD	Lipid droplet
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LEL	Large extracellular loop
LNA	Locked nucleic acid
LSEC	Liver sinusoidal endothelial cells
L-SIGN	Liver and lymph node specific SIGN
LVP	Lipo-viro-particles
MAVS	Mitochondrial antiviral signaling protein
MEF	Mouse embryonic fibroblast
miRNA	microRNA
MTP	Microsomal triglyceride transfer protein
NANBH	Non-A Non-B hepatitis
NPC1L1	Niemann Pick C1- Like 1
NS	Non structural proteins
NTR	Non translated region
OCLN	Occludin
ORF	Open reading frame
PEG-IFNα	Pegylated interferon alfa
PKI	Protein kinase inhibitor
PKR	Protein Kinase R
RC	Replication complex
RdRp	RNA dependent RNA polymerase
RE	Reticulum endoplasmic

RIG-I	Retinoic acid inducing interferon gene I
RISC	RNA-interference-silencing complex
RNAi	RNA interference
RTKs	Receptor tyrosine kinase
sE2	Truncated soluble form of the glycoprotein E2
SL	Stem loop
SP	signal peptidase
SPP	signal peptide peptidase
SR-BI	Scavenger receptor class B type I
TC-PTP	T-cell protein tyrosine phosphatase
TGF-α	Transforming growth factor alfa
TLR3	Toll-like receptor 3
TRIF	Toll-IL-1 receptor domain-containing adaptor inducing IFN-beta
uPA-SCID	urokinase-type-plasminogen activator (uPA)-Severe combined immunodeficiency
VLDL	Very low density lipoprotein

INTRODUCTION

Hepatitis C virus (HCV) infection affects more than 160 million individuals worldwide. HCV infection is most often asymptomatic, leading to an evolution of the disease toward chronic hepatitis in 70% of the cases. Chronic hepatitis evolves silently to cirrhosis in 15 to 20% of the infected patients, among which 5% will develop hepatocellular carcinoma.

The treatment for hepatitis C infection is based on pegylated interferon alfa and ribavirin. This treatment, little effective and expensive, is not prescribed to all infected patients, due to its strong side effects. The absence of a vaccine and new medications for the treatment of hepatitis C renders primordial the development of alternative therapies.

New molecule development to treat HCV has been hampered by the lack of *in vitro* and *in vivo* models. Since its discovery in 1989, the first model system allowing the study of the full HCV life cycle *in vitro* has been available from 2005 only. Furthermore, among the rare cellular models supporting the entire HCV life cycle, all have hepatic origin, revealing the rigorous hepatic tropism of HCV.

Currently, only chimpanzees and mice, repopulated with human hepatocytes, allow the *in vivo* study of HCV infection, mouse hepatocytes being naturally resistant to HCV infection. So far, no mouse model easily handleable is available to the scientific community, which slows down the preclinical studies and the launch on the market of effective and more tolerated molecules. Its hepatotropism and its species specificity make HCV a difficult to study virus.

The PhD work presented in this manuscript first focused on factors restricting HCV infection to hepatocytes. In a second part, we have studied HCV infection in mouse hepatoma cells. After a bibliographic summary of the current knowledge on HCV, the original results emanating from our work will be presented and finally, the last part of this manuscript will be dedicated to the discussion of the results as well as conclusions and perspectives of the subject.

A. Hepatitis C virus

HCV has been isolated in 1989 from a complementary DNA bank constructed using the plasma of patients suffering from a non-A and non-B hepatitis (NANBH) (Choo et al., 1989).

i. Structure of hepatitis C virus

HCV belongs to the *Flaviridae* family. This family contains single strand RNA viruses of positive orientation which are classified in three different genera: *Flaviviruses* (Yellow fever virus, West Nile virus and Dengue virus), Pestivirus (classical swine fever virus, Bovine Viral Diarrhoea virus) and Hepacivirus in which the unique member is HCV. However, very recently a virus isolated from dog's respiratory samples has been shown to be a dog homolog of HCV (CHV, canine hepacivirus) and it has been suggested, according to their similarities that this virus could be a new member of the Hepacivirus genus (Kapoor et al., 2011).

HCV is an enveloped virus with a diameter of 55 to 65 nm (Kaito et al., 1994) containing a nucleocapsid protecting a single positive strand RNA genome of 9600 bases. The HCV genome is constituted of a non-translated region at its 5' end (5' NTR) containing the internal ribosomal entry site (IRES), an open-reading frame (ORF) encoding the structural and the non-structural proteins and a non-translated region at the 3' end (3' NTR). The HCV envelope derives from the cellular lipid bilayer and contains the viral glycoproteins E1 and E2. The nucleocapsid is composed of the capsid protein (core) which protects the genomic RNA (**Figure 1**).

HCV circulates within the blood of infected patients under different forms. The viral particle can circulate freely, associated to lipoproteins of different sizes or associated to immunoglobulins. These different forms confer the virus a heterogeneous distribution on a sucrose gradient. The particles found in the low density fractions (1,03 to 1,08 g/ml) and associated to lipoproteins of low density (LDL) or very low density fraction (VLDL), are more infectious than those found in fractions of higher density (1,17 to 1,25 g/ml), which are most often associated to immunoglobulins (Agnello et al., 1999; Andre et al., 2002).

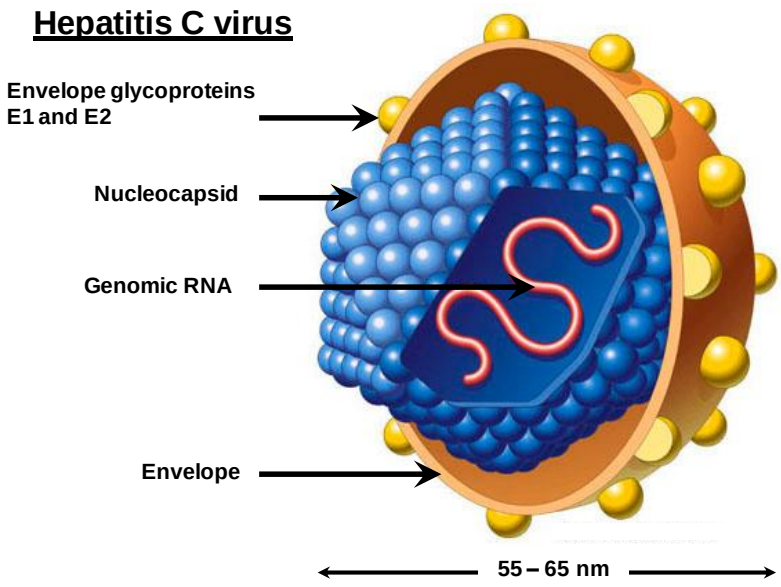


Figure 1: Schematic representation of HCV. *HCV is an enveloped virus with a diameter of 55 to 65 nm, containing an icosahedric capsid protecting the positive strand genomic RNA of 9.6 kb. The glycoproteins E1 and E2 are embedded into the lipid bilayer of the viral particle. (© 2001, James. A. Perkins)*

The low density particles are called lipo-viro-particles (LVPs) due to their association to lipoproteins. These LVPs are rich in triglycerides and contain, besides the viral capsid containing the HCV genomic RNA, the glycoproteins E1 and E2 and the apolipoproteins B and E (apoB, apoC and apoE) (Diaz et al., 2006).

ii. From genome to viral proteins

The HCV genome is translated into a precursor polyprotein of 3000 amino acids in the endoplasmic reticulum (ER). This polyprotein is cleaved by cellular and viral proteases in a co and post-translational manner, into 10 viral proteins; (i) **the structural proteins**, viral capsid or C protein or core and the envelope glycoproteins E1 and E2 as well as (ii) **the non-structural proteins,** p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (**Figure 2A**) (Ploss and Dubuisson, 2012).

1. The non-translated regions

The **5' NTR region,** of 341 nucleotides, well structured and conserved, contains elements from the IRES that ensure the translation of the polyprotein and domains important for replication. The IRES corresponds to the domain II. to IV. of the 5' NTR region as well as the initial part of the ORF sequence (**Figure 2B**). This structured

organization of the RNA within the IRES is indispensable to assure the cap-independent translation of the viral RNA. Studies have revealed that the 5' NTR sequence would contain important elements for the translation but also for RNA replication (Astier-Gin et al., 2005; Friebe et al., 2001). Thus, it has been shown that (i) the domain I. of the 5' NTR sequence is fundamental for the viral RNA replication (Friebe et al., 2001), (ii) IRES regions are important for the RNA replication efficiency (Friebe et al., 2001) and finally (iii) the stem loop of the domain II. of the IRES is essential for replication, in addition to its role in the translation process (Appel and Bartenschlager, 2006). It has also been shown that the 5' NTR of the RNA represents an interaction site for the microRNA-122 (miR-122) which binds to two sequences present between the domain I. and II. of the 5' NTR region (Jopling et al., 2005). This interaction is so important that its inhibition leads to a drastic decrease in viral load in infected chimpanzees (Lanford et al.). This interaction will be the subject of a following paragraph in the present manuscript.

The **3' NTR region** has a variable size according to the genotypes. It can be subdivided into three distinct domains: (i) a poorly conserved region, (ii) an internal sequence uracil/pyrimidin (poly(U/UC)) of an average length of 80 nt and (iii) the X region of 100 nt, which constitutes the most conserved domain and the most structured of the 3' NTR region. The X region includes three loops (SL1, SL2 and SL3) and plays a primordial role in the negative strand RNA synthesis during replication (**Figure 2B**). A new particular structure of the viral RNA has been found within the NS5B coding sequence, this structure called 5BSL3.2 interacts with the stem-loop SL2 during replication of the viral RNA and this interaction is essential for this step of the viral life cycle (**Figure 2B**) (Friebe et al., 2001; You et al., 2004).

2. The viral proteins

The **core protein** (capsid protein or C protein), the first protein to be translated and cleaved from the polyprotein, constitutes the unique component of the viral capsid. Its maturation follows a process involving two cellular proteins. During translation, the nascent polyprotein is directed toward the ER. The core protein is then cleaved a first time by the signal peptidase (SP) at the protein junction core-glycoprotein E1, releasing core from the polyprotein to give an immature protein of 23 kDa (Yasui et al., 1998).

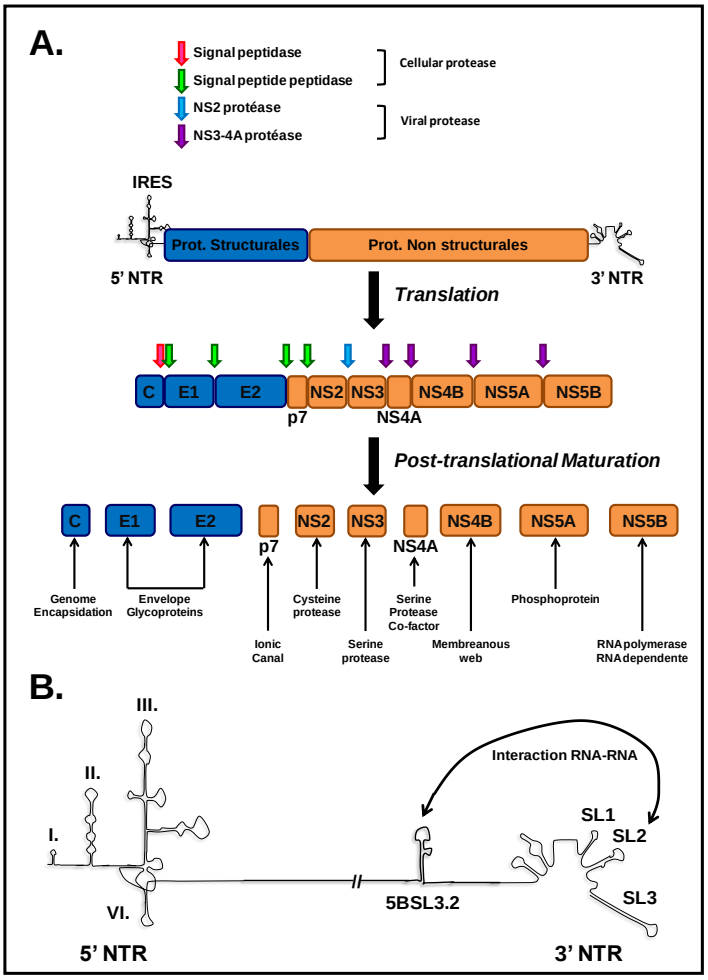


Figure 2: A. Organization of HCV genomic RNA. *The HCV genome is a positive single strand RNA. IRES, present in the non coding 5' end (5'NTR), is upstream of the open reading frame which codes for a polyprotein of 3000 amino acids. The polyprotein is cleaved in a co and post-translational manner by cellular and viral proteases (colored arrows) releasing structural proteins (blue) and non-structural proteins (orange).* ***B. Schematic representation of non-translated regions of HCV RNA.*** *The domains of the non-translated regions (NTR) are indicated in each region.*

The sequence between amino acids 179 and 191, anchored in the ER membrane, is further recognized and cleaved by the signal peptide peptidase (SPP) to give the mature form of the 21 kDa core protein (McLauchlan et al., 2002). The maturation of the core protein by SPP is essential since the insertion of mutations within the recognized sequence or the inhibition of SPP activity by specific drugs, reduce considerably the production of infectious viral particles (Targett-Adams et al., 2008). The mature core protein remains anchored at the ER membrane by its hydrophobe domain present at the C-terminal part, which gives stability to the core protein. The protein moves then to the lipid droplets (LD) at the ER level (McLauchlan et al., 2002). Furthermore, it has been recently shown that diacyl glycerol

acyltransferase 1 (DGAT-1), a factor important for the assembly and release of infectious viral particles and involved in LDs biogenesis, recruits core protein to LDs (Herker et al., 2010). Apart of its structural role, the core protein plays an important role in the assembly of the viral particles. Its N-terminal domain, rich in basic amino acids, interacts with the 5' NTR region of the viral RNA allowing the formation of the nucleocapsid. It has also been shown that the protein core recruits the non structural proteins involved in the viral RNA replication around the LDs, suggesting a central role of core protein in the morphogenesis of the virions (Miyanari et al., 2007).

The protein core coding sequence encodes, on an alternative reading frame (ARF) the frame +1, another protein of 17 kDa called **protein core+1, protein F (Frameshift) or ARFP (alternative reading frame protein)**. This protein is localized in the ER and its half-life does not overpass 10 min because it is rapidly degraded by the proteasome (Xu et al., 2003). A truncated form of the ARFP protein of 8 kDa has been isolated and its expression is inversely proportional to the core protein, suggesting that its expression decreases when the replication increases (Wolf et al., 2008). The presence of antibodies and T cells specific to the ARFP protein insinuated that it is produced *in vivo* (Dalagiorgou et al., 2011; Gao et al., 2011). The precise function of the ARFP protein is not yet known, but it has been suggested that some effects attributed to the core protein could in fact be due to the expression of the ARFP protein (Branch et al., 2005). Furthermore, Fiorucci *et al.* have shown that ARFP could modulate the expression of cytokines (Fiorucci et al., 2007).

The **envelope proteins E1 and E2** are the constituents of the viral particle envelope. They interact with the host factors present at the cell surface and induce the fusion between the viral particle envelope and the host endosomal membrane. During the polyprotein maturation, E1 and E2 are released by the SP (Dubuisson et al., 2002). E1 and E2 contain each a large N-terminal extracellular domain of respectively 160 and 334 amino acids and a C-terminal transmembrane domain of 30 hydrophobic amino acids (Cocquerel et al., 2000). To be functional, these proteins associate to each other in a heterodimer through their transmembrane domain (Op De Beeck et al., 2001). E1 has a molecular weight of 31 kDa. Its precise role in HCV entry into host cells remains not well understood but it seems that the E1 protein plays a role in the fusion process between the viral envelope and the host endosomal membrane (Lavillette et al., 2007). E2 has a molecular weight of 70 kDa. The role of

this protein is more characterized because it is the primary target of the immune responses, suggesting an important role in the HCV entry process into host cells. E2 includes three hypervariable regions (HVR). HVR1, present at the N-terminal part, is constituted of 27 amino acids. HVR2 is composed of 9 amino acids and more recently a third region has been identified the HVR3 region, encompassed between the HVR1 and the HVR2 regions (Troesch et al., 2006). HVR1 is a hotspot of extreme genetic variability, responsible for the differences in viral particle infectivity in patients and also within a same patient. This high variability allows the virus to escape from host immune responses (von Hahn et al., 2007). The deletion of HVR1 in the E2 glycoprotein renders the virus much less infectious *in vivo* (Forns et al., 2000), suggesting that this region has a functional role, essentially in the virus attachment to the CD81 entry factor (Bankwitz et al., 2010). Furthermore, it has been noticed that the HVR1 region also interacts with the SR-BI for an efficient entry. Indeed deletion of this region from the E2 protein reduces the susceptibility of HCVpp and HCVcc entry to anti-SR-BI neutralization (Bankwitz et al., 2010; Bartosch et al., 2003b; Scarselli et al., 2002). The structure of HVR1 is relatively well conserved, which suits to its roles in HCV entry into the host cell and as a target of the immune system (Penin et al., 2001). The function of the HVR2 region is less characterized. It seems that it is engaged in the interaction of the glycoprotein E2 with host cell entry factors such as CD81 (Roccasecca et al., 2003). The third hypervariable region, HVR3, is 35 amino acids long and seems to be also implicated in the viral binding to host cell factors (Troesch et al., 2006).

The glycoproteins E1 and E2 form complexes stabilized by disulfide bonds (Vieyres et al., 2010). These proteins are highly glycosylated. Glycan residues are added to E1 and E2 during the polyprotein elongation at the ER level. These glycosylations play a primordial role in the viral particle infectivity (Helle et al., 2010; Lavie et al., 2007). Furthermore, the presence of glycosylations at the envelope protein surface plays a role in the dissimulation of functional domains to neutralizing antibodies (Falkowska et al., 2007; Helle et al., 2011).

The **p7 protein** is the smallest viral protein of HCV. It is 63 amino acids long and arises from an imperfect cleavage of the E2 protein. Its precise role in the HCV life cycle is not well characterized. It has been shown that p7 has the property to oligomerize thus forming an ion channel (Carrere-Kremer et al., 2002). This protein

could be involved at an early step of the morphogenesis of viral particles upstream of the virions assembly (Jones et al., 2007). Besides, Steinmann *et al.* have shown that p7 plays a role in the assembly and release of viral particles (Steinmann and Pietschmann, 2010). P7 also appears to be important for HCV infection of chimpanzees (Sakai et al., 2003). Despite its presence in the ER membrane and its role in virion assembly and in viral particle infectivity, it is not clear whether this protein is present at the viral particle surface (Dubuisson, 2007). Furthermore, recently it has been shown that p7 interacts with NS2 for HCV particle assembly, and this interaction regulates core localization (Boson et al., 2011; Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011; Stapleford and Lindenbach, 2011; Tedbury et al., 2011). Interestingly, its involvement in virion assembly was independent of its ion channel activity suggesting that p7, apart of its ion channel activity, has another function in HCV particle assembly (Boson et al., 2011; Tedbury et al., 2011).

The **NS2 protein** is a 21-23 kDa protein. NS2 is a cystein protease associated to the ER membrane (Lorenz et al., 2006). The N-terminal domain of NS2 consists of one or several transmembrane domains; the exact number is still controversial (Jones et al., 2007). The C-terminal domain of NS2, in association with the N-terminal domain of NS3, forms the NS2-3 protease, an enzyme catalyzing the unique cleavage between these two proteins (Lorenz et al., 2006). NS2 is not essential for viral replication but is involved in the assembly of infectious viral particles (Charrin et al., 2009; Jones et al., 2007). There have been several studies very recently supporting the importance of NS2 in viral assembly. It has been shown that NS2 interacts with the ion channel p7, and this interaction regulates the intracellular localization of core (Boson et al., 2011; Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011; Stapleford and Lindenbach, 2011; Tedbury et al., 2011). Interestingly, all the mentioned studies could demonstrate that NS2 is able to interact with non-structural and structural proteins, thus it has been proposed that NS2 serves as a scaffold for the assembly of viral particles (Ma et al., 2011). The studies have shown that NS2 interacts with the structural proteins E1 and E2 bringing them to the assembly sites close to lipid droplets. They also could detect interactions between NS2 and NS3 and NS5A and found that NS2 was close to replication complex. These results provide a new function for NS2 as a conductor of HCV particle assembly.

The **NS3 protein** assures several functions during the HCV life cycle. NS3 is a 70 kDa protein associated at its N-terminal domain to the **NS4A protein,** acting as a co-factor, to form a protein complex having a serine protease activity (Morikawa et al., 2011). The C-terminal end of the protein has a helicase activity indispensable to the viral RNA replication, by contributing to the double strand RNA and the RNA secondary structure unwinding (Raney et al., 2010). It has been recently shown that the helicase activity of NS3 could have a role in the early phase of the viral particle assembly. It seems that this protein recruits NS5A to the LDs, presumed site for viral particles assembly (Ma et al., 2008). The protease activity of the protein complex NS3-4A allows the cleavage of the non-structural proteins present downstream of the NS4A protein. The protease activity is better characterized than the helicase activity, probably due to its importance in the escape from the host cell innate immune response. Indeed, it has been shown that NS3-4A has the capability to cleave several cellular proteins, in particular adaptive molecules of the innate immune response. Foy *et al.* have shown in 2003 that this protein has the capacity to disrupt the phosphorylation of the transcription factor interferon regulatory factor 3 (IRF-3), the major factor for the induction of the interferon (IFN) response (Foy et al., 2003). The factors recognized and cleaved upstream of IRF-3, have been identified later on and are the mitochondrial antiviral signaling protein (MAVS or IPS-1, VISA or CARDIF) (Li et al., 2005b) and the Toll-IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF or TICAM-1) (Li et al., 2005a), which are the adaptive molecules of retinoic acid inducing interferon gene I (RIG-I) and Toll-like receptor 3 (TLR3) respectively. More recently, a third protein has been identified as being a target of NS3-4A, this latter is called T-cell protein tyrosine phosphatase (TC-PTP). The cleavage of this protein could have the effect of stimulating the activation of the epidermal growth factor receptor (EGFR) and thus increase HCV replication. However the mechanism by which the inhibition of TC-PTP could have a positive effect on HCV replication is not yet known (Brenndorfer et al., 2009). With its versatility and its numerous cellular targets, the protein complex NS3-4A is a great therapeutic target. Many molecules have been developed to inhibit the protease activity of NS3-4A, such as the FDA-approved protease inhibitors telaprevir and boceprevir, but the genetic variability of the virus induces resistance to these newly developed inhibitors (Morikawa et al., 2011).

The **NS4B protein** is a non-structural protein that, few years ago, was not yet characterized. NS4B is a 27 kDa hydrophobic protein anchored in the ER membrane (Hugle et al., 2001). This protein was initially described as being able to induce by its own, drastic rearrangements in the conformation of ER membranes, known as the membranous web (Egger et al., 2002). This membranous web is known to be the scaffold necessary for the replication complex (Gosert et al., 2003). The NS4B protein contains at least 4 transmembrane domains and has the capacity to oligomerize, facilitating the replication complex formation (Yu et al., 2006). Despite the advances on the understanding of the viral life cycle, the function of the NS4B protein remains to be better characterized.

The **NS5A protein** exists in the host cell as two phosphorylated forms. A basal phosphorylated form of 56 kDa and a hyperphosphorylated form of 58 kDA. This hyperphosphorylation of NS5A is due to cellular kinases such as casein kinase 1 (CK1) and CK2. The N-terminal extremity of NS5A adopts an amphiphil α helix structure which allows it to anchor into the ER membrane. It could also allow it to form protein/protein interactions essential to the formation of a functional replication complex. NS5A is constituted of three domains I, II and III. The domain I and II are involved in viral replication (Tellinghuisen et al., 2005). Moreover, it has been recently reported that the domain III intervenes into the assembly of the viral particles (Appel et al., 2008) and more recently, it has been demonstrated that NS5A interacts with apoE, a primordial factor for viral particles assembly (Benga et al., 2010; Jiang and Luo, 2009). NS5A has also a role in the regulation of the balance between viral RNA replication activity and virion assembly (Alvisi et al., 2011). It has been suggested that its phosphorylation rate could be the factor which regulates viral RNA replication (Evans et al., 2004; Neddermann et al., 2004).

The **NS5B protein** is a RNA dependant RNA polymerase (RdRp). Its C-terminal region (21 amino acids) forms an α helix transmembrane domain allowing the protein to anchor into the cytosolic part of the ER. This domain is not primordial for the polymerase activity *in vitro* but is essential for HCV RNA replication in cell culture (Moradpour et al., 2004). The NS5B structure is similar to most of the polymerases, which means a structure in right hand, with subdomains thumb-palm-

finger (Bressanelli et al., 2002). A particularity of the HCV RdRp is that the intense interaction between the subdomains thumb and finger results in a closed conformation of the active site (Moradpour et al., 2007). Furthermore, NS5B oligomerization has been shown to be important in the RNA synthesis. The importance of this protein in the viral RNA replication makes it a major target of new therapeutic molecules for the treatment of HCV infection (Patil et al., 2011).

iii. The genetic variability of HCV

HCV is known to present a high genetic variability. The absence of a NS5B proof reading frame activity explains in part this high variability. However, the strong *in vivo* viral replication (10^{10} to 10^{12} newly produced virions per day) may also contribute to this variability (Neumann et al., 1998). Despite the high error rates which can reach 10^{-3} to 10^{-4} per nucleotide, the 5' NTR region is one of the most conserved region of the viral RNA with a homology superior to 90% between the different genotypes (Bukh et al., 1992). According to this high variability, HCV variants are classified into four classes which are genotypes, sub-genotypes, isolates and quasi-species (Farci and Purcell, 2000). To date, HCV is classified in 7 major genotypes (1 to 7) and subdivided into sub-genotypes, classified by letters (1a, 1b, 2a…). Within a same patient, the virus can have a variability of 1 to 5% and be distributed as a quasi-species. The genotype 1a is frequent in North America and in Europe. The genotype 1b has a worldwide repartition and is the most frequently encountered genotype. The genotypes 2a and 2b are present in North Italy and in Japan. The genotype 3 is more frequently encountered in India and in South-East Asia. The genotypes 5 and 6 are relatively rare but can be found in South Africa and South-East Asia, respectively (**Figure 3**). The genotype identification is fundamental because certain genotypes (1 and 4) are less sensitive to the pegylated interferon-α and ribavirin-based treatment than others (2 and 3); and the length of the treatment is also dependent on the genotype (Maekawa and Enomoto, 2009).

iv. Hepatitis C : the disease

HCV is the principal cause of chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma. With more than 160 million people infected worldwide (2,3% of the world population), HCV is a major health burden (Negro and Alberti, 2011). The parenteral

route is the principal way of HCV transmission. Before the systematic screening tests of the blood bags, transfusion and transplantation were the principal causes of contaminations.

Currently, the principal cause of contamination in developed countries is the use of drugs through intravenous injections and nasal absorption. In developing countries, the contamination overcomes during surgical acts with contaminated material (Alter, 2007). Contaminations have also been reported through the use of piercings, tattoos or acupuncture (Kim et al., 2011; Tohme and Holmberg, 2012).

Hepatitis C is a progressive disease which evolves from an acute hepatitis to a chronic hepatitis if the infection is not diagnosed. In the long term, chronic hepatitis evolves towards cirrhosis and in 5% of the cases, the cirrhosis leads to hepatocellular carcinoma (HCC).

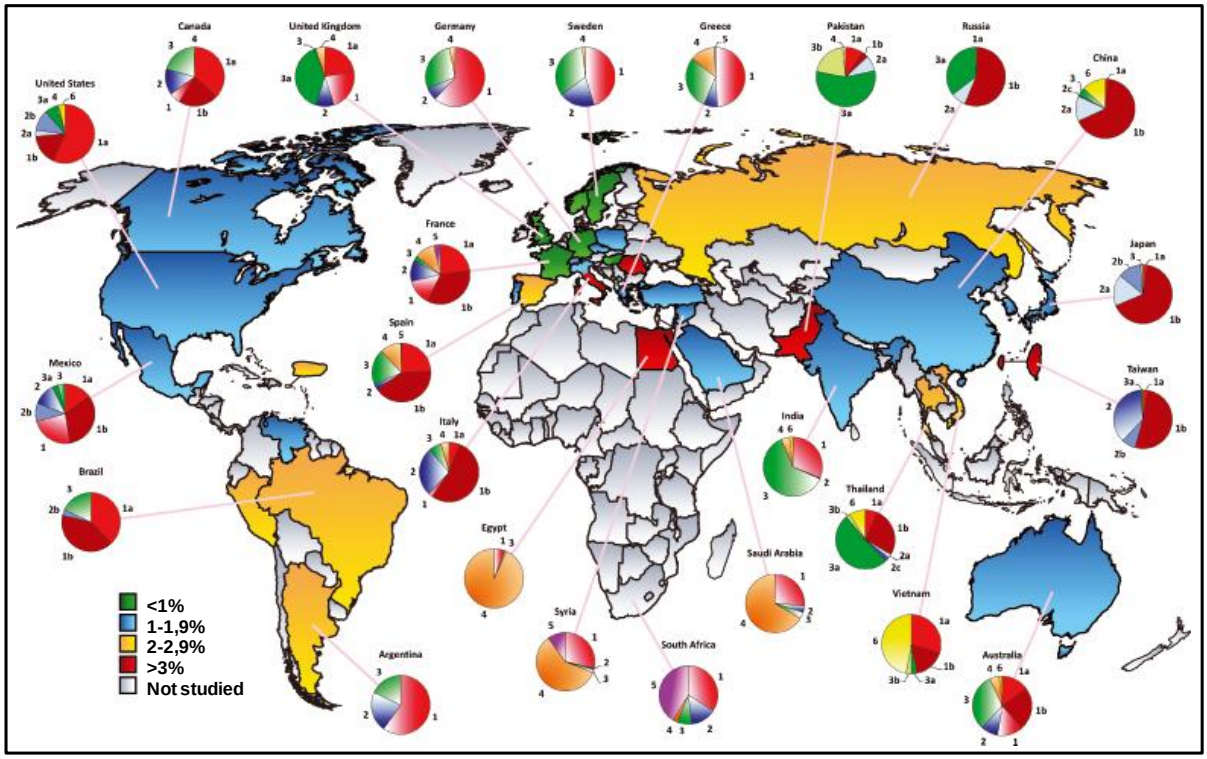


Figure 3: Worldwide repartition of the different HCV genotypes and prevalence among adults. ***(adapted from (Negro and Alberti, 2011))***

The acute phase of HCV infection is most often asymptomatic, but 20 to 30% of the patients present symptoms such as tiredness, nausea, jaundice and anorexia. The incubation time is relatively short (4 to 12 weeks), this phase is followed by an

increase of the transaminases (ALAT), first signs of a hepatic injury. In 25% of cases, an immune response, fast and efficient, is launched and leads to the natural clearance of the virus (Grebely et al., 2012). Fulminent hepatitis C exists, but these cases remain really rare (1% of the patients). In 70% of the cases, the virus persists within the organism which leads to a chronic hepatitis. This latter is defined by the presence of viral RNA in the blood six months following the primary infection. The chronic infection remains most of the time asymptomatic but can be associated to extra-hepatic syndromes such as cryoglobulinemia, nephropathie or thyroïdian pathologies (Zignego et al., 2007). Approximately a quarter of the chronically infected patients will evolve to cirrhosis. In 1 to 4% of the cases, the disease will evolve to **hepatocellular carcinoma (HCC)** (Lauer and Walker, 2001) for which the unique treatment remains currently the hepatic transplantation, followed by a systematic re-infection of the liver graft (Germani et al., 2012).

B. The viral life cycle

i. HCV entry

HCV entry is the first step of virus-host cell interactions which leads to a robust infection. HCV entry is a finely orchestrated process involving many viral and cellular factors. So far, HCV entry is one of the best characterized steps of the viral life cycle most likely due to its attractiveness as a target for antiviral therapy (Zeisel et al., 2011b).

1. HCV entry factors

The viral factors involved in HCV entry are the glycoproteins E1 and E2. These proteins have been described previously (cf. A. ii. 2. The viral proteins).

HCV attachment to hepatocytes and viral entry are complex processes including several steps. Using several tools and HCV models, different cell surface factors have been identified as interacting directly or indirectly with HCV. These factors are CD81 (Pileri et al., 1998), the low density lipoprotein receptor (LDLR)

(Agnello et al., 1999), heparan sulfates (HS) (Barth et al., 2003), the scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002), DC-SIGN (dendritic cell-specific intercellular adhesion molecule three grabbing non integrin)/ L-SIGN (DC-SIGNr, liver and lymph node specific) (Lozach et al., 2004) (Pohlmann et al., 2003), claudin-1 (CLDN1) (Evans et al., 2007) and occludin (OCLN) (Liu et al., 2009; Ploss et al., 2009; Yang et al., 2008c). *In vivo*, HCV enters into the liver through the blood stream via the sinusoids. The interception of viral particles by the liver sinusoid endothelial cells (LSEC) could facilitate the infection of neighboring hepatocytes which are not in direct contact with the circulating blood. This process could involve DC-SIGN, which is expressed in Kupffer cells present next to hepatocytes, and L-SIGN which is highly expressed in LSECs. It has been shown that DC-SIGN and L-SIGN are able to bind the E2 glycoprotein with high affinity (Gardner et al., 2003; Pohlmann et al., 2003).

At the hepatocyte surface, HS are the first attachment sites for the virus (Barth et al., 2003; Barth et al., 2006). This non-specific binding could facilitate virus access to the host entry factors.

It is known that HCV circulates in the blood stream associated with different lipoproteins such as VLDL and LDL. Thus, the LDLR has been suggested as a binding molecule and/or a host factor for HCV entry (Agnello et al., 1999; Wunschmann et al., 2000). Since retroviral pseudo-particles bearing the HCV glycoproteins (HCVpps, detailed later) are not associated with lipoproteins, studies assessing the role of LDLR using HCVpps did not uncover any major role of LDLR in HCV entry (Bartosch et al., 2003a). Furthermore, no direct interaction between the HCV E2 glycoprotein and the LDLR could be determined (Wunschmann et al., 2000). However, it has been shown that LDLR allows the internalization of HCV, derived from patient's sera, in HepG2 cells deficient in CD81 expression by interacting with viral particles associated to LDL (Agnello et al., 1999). More recently, Albecka *et al.* have shown that LDLR has a role in virus binding to cells, but more remarkably, its physiological function plays a role in HCV replication (Albecka et al., 2012). This group also suggests that internalization of HCV through LDLR binding leads to a non-specific internalization and to a non-productive infection, but the physiological function of LDLR is important is HCV replication. Further studies are required to assess the precise role of the low density lipoprotein receptor in both HCV entry and

HCV RNA replication, but it is clear that this receptor does not play a role in the post-binding steps of HCV entry.

CD81 was the first HCV entry factor identified. CD81 is a ubiquitously expressed protein of 25 kDa including a small and a large extracellular loop (LEL). CD81 has been the first described molecule as interacting with a soluble form of the E2 glycoprotein and shown to be essential for HCV entry (Pileri et al., 1998) (McKeating et al., 2004). It has been shown that CD81 LEL is the domain involved in the interaction with viral glycoprotein E2 since a soluble form of this domain could inhibit both HCVpp entry and the cell-culture derived HCV (HCVcc) infection (Zhang et al., 2004). Several amino acids have been identified in E2 and CD81 as crucial for E2 binding to the CD81 receptor (Bertaux and Dragic, 2006; Owsianka et al., 2001; Patel et al., 2000; Pileri et al., 1998). The *in vitro* models developed these last years, the HCVpp (Bartosch et al., 2003a) and HCVcc (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) brought remarkable information on the E2-CD81 interactions and allowed to highlight important amino acids present in the E2 glycoprotein at postions 415, 420, 529, 530 et 535 (Dhillon et al., 2010; Owsianka et al., 2006). Furthermore, functional analysis of HCV entry factors required to over passing the species specific barrier of HCV, revealed that human CD81 and human OCLN, is the minimal set of human factors necessary to render mouse cells permissive to HCV entry (Ploss et al., 2009).

SR-BI also named CLA-1, CD36 or LMPII analogous-1 is highly expressed in steroidogenic tissues (ovaries and adrenal glands) as well as in the liver (Krieger, 2001). This 82 kDa glycoprotein is composed of a LEL which binds to several lipoproteins (HDL, LDL and oxidized LDL (oxLDL)), and is involved in the bi-directional transport of cholesterol to the plasma membrane as well as the selective uptake of cholesteryl ester (CE) from HDL and catabolism of VLDL. SR-BI was first identified as another putative HCV receptor based on its interaction with the soluble form of the E2 glycoprotein and this interaction was localized to the HVR1 domain of E2 (Scarselli et al., 2002). Recent studies indicate that the amino acids responsible for this interaction are amino acids 70 to 87 of the SR-BI protein which interact with the amino acid E210 of the E2 glycoprotein (Catanese et al., 2010). It seems that SR-BI could have a dual role in the HCV entry process, during the attachment phase but

also in a post-attachment step of the HCV entry process (Catanese et al., 2010; Zeisel et al., 2007). Recently, it has been shown that SR-BI has multiple function in HCV entry. It seems that SR-BI plays an attachment function, an access function and an HCV entry enhancement function and only the enhancement function requires E2 binding (Dao Thi et al., 2012). Natural ligands of SR-BI have been shown to modulate HCV infection, HDL are able to increase both HCVpp and HCVcc infectivity while the oxidative form of LDL (oxLDL) inhibit their infection (Bartosch et al., 2005; Voisset et al., 2005; von Hahn et al., 2006). Recent evidence suggests that the physiological properties of SR-BI (lipid transfer and HDL binding) are required for the proper role of SR-BI as an HCV entry factor (Dreux et al., 2009). Altogether, these data indicate that a complex interaction between HCV glycoproteins, lipoproteins and SR-BI is involved in the HCV entry process.

None of the factors previously cited could explain the limited HCV tropism suggesting that other factors were involved in HCV entry. Thus, in the effort to identify the remaining HCV entry factors, Evans *et al.* have performed a lentiviral based delivery of a complementary DNA bank derived from the highly HCV-permissive human cell line Huh7.5 into the HCVpp non-permissive 293T cell line. This system allowed identifying **CLDN1** as another HCV entry factor (Evans et al., 2007). Upon CLDN1 expression, the HCVpp non-permissive cell line 293T, become permissive to HCV entry. CLDN1 is a 23 kDa protein which contains 4 transmembrane domains. It belongs to the claudin family which includes 24 members in humans. It has been reported that CLDN6 and 9 are also able to support HCV entry (Meertens et al., 2008; Zheng et al., 2007). Claudins are essential components of the tight junctions and they regulate paracellular permissivity and maintain epithelium and endothelium polarity. CLDN1 is expressed in all the epithelial tissues but predominantly in the liver (Furuse et al., 1998). It has to be taken into account that CLDN1 is expressed at the tight junctions of the hepatocytes but is also present at the basolateral surface of these cells (Reynolds et al., 2008). It has been recently suggested that the CLDN1 proteins which are not involved in the tight junctions could play a critical role in HCV entry (Cukierman et al., 2009; Evans et al., 2007) and these proteins could intervene in a post-binding step of the HCV entry process (Krieger et al., 2010). To date, there is no evidence showing an interaction between CLDN1 and HCV. Studies have shown that the first extracellular loop of CLDN1 and

more particularly the residues within the conserved motifs of the claudins W(30)-GLW(51)-C(54)-C(64) are important for HCV entry (Cukierman et al., 2009; Evans et al., 2007). It has been shown that CLDN1 interacts with CD81 in several cell lines and the formation of this complex is essential for HCV infection (Harris et al., 2010; Harris et al., 2008). Mutations within the residues 32 and 48 in the ECL1 domain of CLDN1 disrupt CLDN1 and CD81 association and its function as an HCV entry factor (Benedicto et al., 2009). While expression of CLDN1 in 293T cells renders these cells permissive to HCVpp, its expression in HeLa or HepH cells which both express CD81 and SR-BI do not allow rendering them permissive to HCV entry (Evans et al., 2007), suggesting that other factors are involved in HCV entry process and that remained to be identified.

OCLN was recently identified as another host cell factor critical for HCV entry (Benedicto et al., 2009; Liu et al., 2009; Ploss et al., 2009). OCLN is a transmembrane protein of 65 kDa containing 4 transmembrane domains. Like CLDN1, OCLN is present in the tight junctions of polarized cells. So far, it remains a matter of discussion whether HCV directly interacts with OCLN at the cell surface or intracellularly (Benedicto et al., 2009). It has been suggested that OCLN, with CD81, was responsible of the species specificity of HCV. Indeed the expression of human OCLN in combination with human CD81 renders mouse cells permissive to HCV entry (Ploss et al., 2009). Of note, a study has demonstrated that glucocorticoïds lead to an increase in OCLN expression in hepatocytes and an increase in HCV entry (Ciesek et al., 2010) while HCV infection induces a down-regulation of OCLN expression thus preventing a super-infection (Liu et al., 2009).

Further studies are required to discriminate the precise role of OCLN in the HCV entry process and its possible interaction(s) with the other known HCV entry factors.

Among all HCV entry factors involved in HCV entry, it has been shown that the minimal set of cell factors allowing HCV entry into non permissive cells are CD81, OCLN, CLDN1 and SR-BI (Ploss et al., 2009).

More recently, through a functional siRNA screen, our laboratory identified 58 kinases as important novel HCV entry cofactors, among which two receptor tyrosine

kinases (**RTKs**) **ephrin A2 (EphA2)** and **epidermal growth factor receptor (EGFR)** (Lupberger et al., 2011). Both EphA2 and EGFR are transmembrane proteins with a large extracellular domain involved in ligand binding and intracellular regulatory domains which contain phosphorylation sites for the regulation of cellular processes. EphA2 regulates cell proliferation, motility and cell proliferation (Lackmann and Boyd, 2008). Its natural ligand is ephrin A1. EGFR, present at the cell surface, can bind to two ligands which are transforming growth factor alfa (TGF-α) and epidermal growth factor (EGF), and regulates cell proliferation, survival, differentiation during development and tumorigenesis (Schneider and Wolf, 2009). The implication of RTKs in HCV entry was studied using the well characterized protein kinase inhibitors (PKIs) dasatinib (EphA2 inhbitor) and erlotinib (EGFR inhitor). The inhibition of EGFR and EphA2 activity in human hepatocytes decreased the susceptibility of these cells to HCVpp entry, suggesting that these RTKs were involved in HCV entry (Lupberger et al., 2011). Further silencing of these RTKs, using specific siRNAs, confirmed the functional role of these RTKs in the HCV entry process, but interestingly did not affect binding of the soluble form of the E2 glycoprotein to human hepatocytes. These results suggest that these RTKs have an important role for HCV entry but this role does not require direct RTK-HCV interaction. Thus it has been proposed that RTKs play a role in post-binding steps of the HCV entry process (Lupberger et al., 2011). It has been further demonstrated that RTKs regulate the interaction between CD81 and CLDN1 to form complexes which are crucial for HCV entry and erlotinib and dasatinib inhibit HCV entry by disrupting CD81-CLDN1 complex formation (Lupberger et al., 2011). Using a kinetic assay to determine the steps where RTKs act on HCV entry, Lupberger *et al.* demonstrated that these RTKs are involved at late steps of HCV entry and involved in pH-dependent fusion of the viral membrane (Lupberger et al., 2011). Finally, the relevance of these RTKs was confirmed *in vivo* by delivering PKIs to HCV infected uPA-SCID mice repopulated with human hepatocytes and this demonstrated the clinical potential of erlotinib as a novel antiviral strategy (Lupberger et al., 2011).

While the HCV entry process is becoming more and more characterized, a recent study has identified another HCV entry factor required for HCV infection. Because HCV is naturally associated to cellular lipoproteins, Sainz *et al.* have assessed the role of the cholesterol uptake receptor **Niemann-Pick C1-Like 1**

(NPC1L1) as another putative HCV entry factor. NPC1L1 is a cholesterol uptake receptor containing thirteen transmembrane domains and is about 1332 amino acid long (Yu, 2008). NPC1L1 is present on the apical face of enterocytes and on the canalicular membrane of hepatocytes (Gao et al., 2011; Sainz et al., 2011; Yu, 2008). It is known that NPC1L1 is involved in the cholesterol absorption in enterocytes and is involved in the transfer of cholesterol from canalicular bile to hepatocytes (Sainz et al., 2011; Temel et al., 2007; Yu, 2008). Sainz *et al.* have shown that the inhibition of NPC1L1 activity, using an antibody and RNAi silencing, leads to a drastic decrease of HCV infection (Sainz et al., 2011). They have identified the first LEL (LEL1) to be the domain important for HCV infection (Sainz et al., 2011). Further drug-mediated inhibition of NPC1L1 using ezetimib, a direct inhibitor of NPC1L1 internalization, demonstrated that NPC1L1 acts at post-binding step(s) of HCV entry but before viral membrane fusion (Sainz et al., 2011). Finally, they have shown that NPC1L1 action on HCV entry is cholesterol dependent, since NPC1L1 had no effect on HCVpp, known not to be associated to lipoproteins, but had more drastic effect on HCVcc bearing a mutation that enhances the association of the viral particles to cholesterol compared to wild-type HCVcc (Sainz et al., 2011). These data suggest that the cholesterol uptake properties of NPC1L1 could reveal important binding domains of the E2 glycoprotein when the lipoviroparticles bind to the host cell surface (Sainz et al., 2011), but this remains to be demonstrated. Thus, the identification of NPC1L1 as a putative HCV receptor brings more information on the complex mechanism of HCV entry. However, it is not yet known how this receptor is involved in HCV entry process, since NPC1L1 is present at the bile canalicular side of hepatocytes while it is believed that HCV entry occurs at the basolateral side of hepatocytes (Farquhar and McKeating, 2008; Lupberger et al., 2012; Zeisel et al., 2011b).

2. HCV entry: a multi-factor process

In a physiological context, HCV, associated with lipoproteins and coming from the bloodstream, interacts with hepatocytes at the basolateral surface of hepatocytes. HS are the first interacting molecules to which HCV binds in a non-specific manner (Barth et al., 2003) (Barth et al., 2006). This step is the first one of a complex process

involving several host factors: SR-BI (Scarselli et al., 2002) (Bartosch et al., 2003b) (Zeisel et al., 2007), CD81 (Pileri et al., 1998), CLDN1 (Evans et al., 2007; Krieger et al., 2010) and OCLN (Ploss et al., 2009) (Liu et al., 2009). It is of importance to note that all the cited entry factors are required and important for a persistent HCV infection. These data suggest that HCV entry follows a finely regulated process through the formation of a HCV-entry factors complex(es) at the host cell surface (Farquhar and McKeating, 2008; Krieger et al., 2010; Zeisel et al., 2007). The formation of such complexes has been first shown using the fluorescence resonance energy transfer (FRET) where the role of the formation of the CD81-CLDN1 complex has been demonstrated to be crucial in HCV entry (Harris et al., 2010; Harris et al., 2008). The fact that only CLDN-1, -6 and -9 are able to interact with CD81 and support HCV entry, suggests that this complex is important for HCV entry (Harris et al., 2010; Krieger et al., 2010). At present, the formation of complexes between other HCV entry factors is not yet known. It has been shown that most of the CLDN1 molecules present at the plasma membrane interact with OCLN, but the relevance of such interaction in HCV entry has not yet been demonstrated (Harris et al., 2010). Moreover, it has been shown that cellular contacts influence CLDN1 and SR-BI expression and favors the formation of HCV entry factor complexes, facilitating HCV internalization (Schwarz et al., 2009).

Up to now, the different events involved in HCV-entry factor interactions, internalization, fusion and replication remain unknown. Using the HCVpp and HCVcc model system, studies have shown that HCV entry into human hepatoma cells and primary human hepatocytes is dependent on clathrin-dependent endocytosis (Blanchard et al., 2006; Codran et al., 2006; Coller et al., 2009).

Furthermore, it has been demonstrated that efficient HCV entry requires an actin-clathrin association (Coller et al., 2009). According to the complexity of HCV entry, it is likely that the internalization of HCV particles is associated with HCV entry factor internalization and it has recently been shown that CD81 and CLDN1 internalize together with HCV (Coller et al., 2009; Farquhar et al., 2012). It is established that the polarization restricts HCV entry and that HCV entry factors involved in the entry process are mostly expressed at the basolateral face of hepatocytes and not those present at the tight junction (Reynolds et al., 2008).

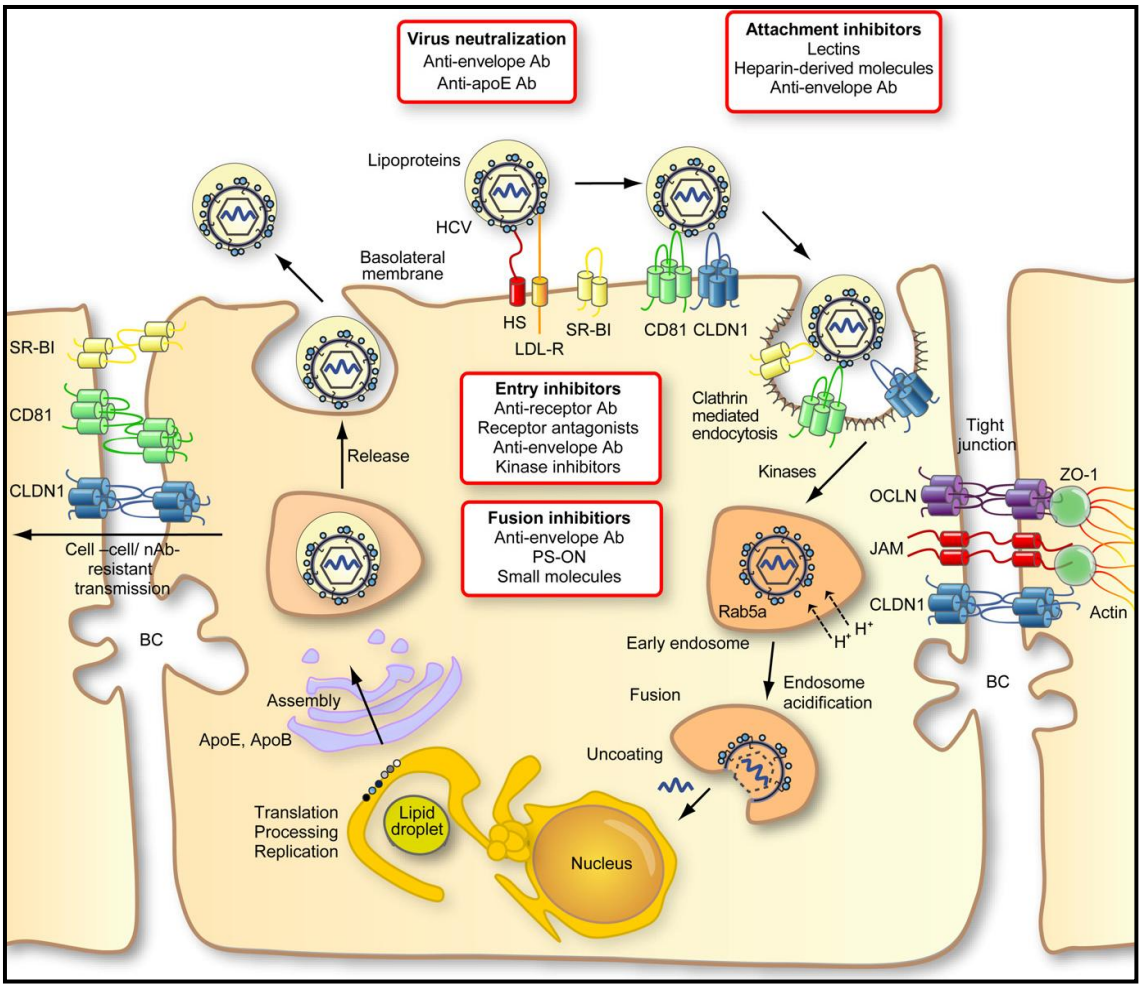


Figure 4: HCV entry into hepatocytes. *HCV binds to hepatocytes via highly sulfated heparan sulfates (HS) and the LDL receptor (LDLR) at the basolateral surface of hepatocytes. Following this interaction, a sequence of events involving several host factors such as SR-BI, CD81, CLDN1 and OCLN takes place. The virion is then internalized in a clathrin-dependent manner. The fusion between the vial membrane and the endosomal membrane leads to the release of the viral RNA into the cytoplasm where the translation and the replication will take place. The HCV viral particles are then assembled and released out of the host cell at the level of ER-lipid droplet interactions. Cell-to-cell transmission is an alternative route of infection of hepatocytes by HCV, allowing escape from neutralizing antibodies present in the extracellular environment. This multistep entry process offers several interesting targets for antiviral therapy as indicated by red boxes. In the figure BC means bile canaliculi. Figure and legend from (Zeisel et al., 2011b).*

In agreement with these data, imaging studies suggest that HCV internalization does not occur at tight junctions (Coller et al., 2009). In clathrin-dependent endocytosis, viruses are transported, together with their receptors, in early and late endosomes (Marsh and Helenius, 2006). It has been shown that HCVpp were directed to early and not late endosomes (Meertens et al., 2006). This result is in agreement with recent imaging studies where colocalization between Rab5, an early endosome marker, and HCV particles has been shown (Coller et al., 2009).

Enveloped virus entry is mediated by fusion of the membranes catalyzed by peptide fusion present into viral enveloped glycoproteins (Smith and Helenius, 2004).

Up to now, the mechanisms mediating HCV fusion has not been elucidated, but it has been suggested that the fusion mechanism occurring for other *Flaviviridae* viruses may apply to HCV (Moradpour et al., 2004). Observations on HCVpp (Bartosch et al., 2003a; Lavillette et al., 2006) and HCVcc (Blanchard et al., 2006; Tscherne et al., 2006), show that membrane fusion is pH-dependant, thus allowing HCV RNA delivery into cytoplasm, supporting the hypothesis of similar fusion mechanisms between HCV and other *Flaviviridae* viruses. Although HCV entry requires an acidification step, it is worth noting that extracellular HCV is resistant to acid pH treatment (Meertens et al., 2006; Tscherne et al., 2006). Compared to other viruses, HCV fusion is delayed, thus it has been suggested that HCV requires an additional, post-internalization step to deliver its genomic RNA such as: additional low-pH-dependent protein interaction, enzymatic modifications or further HCV particle trafficking to other compartments for efficient fusion (Meertens et al., 2006). Membrane fusion is the last step of the HCV entry process and different fusion assays have been developed to better understand the fusion requirements. Based on an artificial liposome/HCVpp fusion assay, it has been shown that HCVpp fusion is dependent on low-pH, on temperature and is facilitated by cholesterol (Lavillette et al., 2007). Furthermore, the role of the envelope glycoproteins in the fusion process has been highlighted by discovering patient-derived anti-HCV antibodies able to inhibit post-binding steps and membrane fusion (Haberstroh et al., 2008; Kobayashi et al., 2006). However, these fusion assays did not evaluate the role of HCV entry factors in the fusion process. In order to assess the role of viral and cell factors in the fusion process, Kobayashi *et al.* have developed a cell-cell fusion assay, where viral glycoproteins are expressed on a cell type and the host cell factors on a second one (Kobayashi et al., 2006). This assay confirmed that HCV fusion was dependent on low pH, but more interestingly, it highlighted for the first time the importance of CLDN1 and CD81 in the fusion process. Furthermore, it has been demonstrated that the RTK inhibitors erlotinib and dasatinib, inhibiting EGFR and EphA2 respectively, decreased significantly HCV fusion in a cell-cell fusion assay (Lupberger et al., 2011). So far, the question remains whether HCV entry factors act directly on the fusion process or if they participate in early events for an efficient fusion **(Figure 4)**.

In addition to the primo-infection of hepatocytes via the previously described process, also called “cell-free entry”, it has been demonstrated that another route of infection exists, called “cell to cell transmission” (Timpe et al., 2008). Indeed, it has been shown that HCV can be directly transmitted from an infected cell to an adjacent cell through a mechanism also requiring HCV entry factors. SR-BI, CD81, CLDN1 and OCLN seem to be implicated in this transmission mode (Brimacombe et al., 2011; Schwarz et al., 2009; Timpe et al., 2008). It has to be noted that a CD81-independent route of cell-to-cell transmission has been reported (Brimacombe et al., 2011; Timpe et al., 2008; Witteveldt et al., 2009). The cell-to-cell transmission allows the virus to escape from most of the neutralizing antibodies (Brimacombe et al., 2011), which allows the virus to persist in the liver. This transmission mode should be taken into account for the development of future therapeutic molecules and more particularly the development of antibodies targeting HCV host entry factors.

ii. HCV replication

HCV RNA replication follows a process involving several viral and host factors. As for all positive strand RNA viruses, the replication begins with the synthesis of a negative strand complementary to the positive strand which will be the template for the replication of the genome into multiple copies.

1. The replication complex

Infections by a positive strand RNA virus leads most of the time to a rearrangement of the intracellular membranes, a pre-requisite for the formation of a replication complex (RC) which will associate viral proteins and cellular components. The formation of the negative strand and of a complementary positive strand RNA is catalyzed by the NS5B protein, the HCV RdRp (cf the previous chapter on NS5B). The recombinant NS5B protein shows an RdRp activity *in vitro* but the activity lacks specificity and fidelity to the template. It is thus conceivable that cellular and/or viral factors are required for an optimal replication of the viral RNA and for the formation of the RC.

Upon HCV genome delivery into host cell cytoplasm, the genomic RNA is translated and the polyprotein cleaved at the ER membrane where the replication complex will be localized (Bartenschlager et al., 2004). The rearrangement of the membranes takes place at the ER to form a membranous web. It seems that the NS4B protein is able by its own to induce these rearrangements (Egger et al., 2002). It is not clear whether NS4B recruits cellular proteins to induce the formation of vesicles or if the protein can do it by its own. So far, the proteins composing the RC are not yet defined.

It is known now that HCV life cycle, from entry to assembly and release, is tightly linked to the host lipid metabolism. In cell culture, HCV RNA replication is stimulated by saturated and mono-unsaturated fatty acids while poly-unsaturated fatty acids inhibit HCV replication (Alvisi et al., 2011; Yang et al., 2008b). These data suggest that membrane fluidity is an important requirement for an efficient function of the RC. It has been shown that HCV RNA replication occurs in detergent resistant membranes and co-localizes with caveoline 2, an essential component of lipid rafts (Banaudha et al., 2011). Indeed, lipid rafts are involved in RC formation, through a protein-protein interaction between hVAP-33 and the two viral proteins NS5A and NS5B (Gao et al., 2004). Generally, the membranous web consists of small interlocked vesicles in the ER membrane scaffold, forming a membrane associated multi-protein complex which contains all the non structural HCV proteins (Egger et al., 2002). The role of this compartmentalization remains unclear; it has been proposed that the formation of the membranous web could protect HCV RNA replication from the innate immune response. Several host factors are involved in an efficient HCV RNA replication and it has been demonstrated that HCV RNA replication is closely linked to the assembly and release of the viral particles (Miyanari et al., 2007).

2. Host cell factors associated to HCV RNA replication

HCV replication is a complex process that is not fully understood. For several years, the lack of cellular model systems to study the full HCV life cycle has hampered the identification of cell host factors involved in the replication of HCV RNA. Sub-genomic replicons, where only the non-structural proteins of the virus are expressed in cells, have been a great tool to better characterize HCV replication. The discovery of a

HCV clone, JFH1, isolated from a patient suffering of a Japanese fulminant hepatitis (JFH) and able to reconstitute the entire HCV life cycle in human hepatoma cells in culture has been a breakthrough in HCV research (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

a. microRNAs

MicroRNAs (miRNAs) are small non-protein-coding RNAs of about 22 nucleotides. They are encoded as single or clustered transcription unit. These transcription units can be found in non-protein-coding regions, protein-coding genes or as independent transcription units. These small non–coding RNAs are a novel class of gene regulators identified this last decade. MiRNAs encoding transcription units are transcribed, as long mono-, bi- or poly-cistronic transcripts called primary-miRNAs (pri-miRNA) by the RNA polymerase II. Pri-miRNAs are recognized and cleaved by Drosha (associated to DGCR8) to give a shorter RNA transcripts called pre-miRNAs that are actively exported to the cytoplasm. Dicer (associated to TRBP) will recognize and cleave pre-miRNAs to release a duplex of miRNAs. One of the two strands of the duplex will be loaded onto the RNA-interference-silencing complex (RISC). MiRNAs have a sequence called the seed sequence, of about 6 nucleotides, that will bind to the seed-match sequence at the 3’ end of the target mRNA. According to the matching between the seed sequence and the seed-match sequence, the target mRNA will be either degraded if the matching is perfect or its translation repressed if the matching is imperfect (**Figure 5**).

Among the host cell factors involved in HCV replication, **miRNA-122** is the most surprising factors that is hijacked by the virus. MiRNAs are known to repress gene expression through binding to the 3’UTR of the messenger RNA. The identification of miRNA-122 as enhancing HCV RNA replication is so far, the unique example of a miRNA-virus interaction which benefits to the virus. MiRNA-122 is highly expressed in the liver and represents about 70% of all the liver-expressed miRNAs (Chang et al., 2004). Looking for liver specific factor that could explain the liver tropism of HCV, Jopling *et al.* have inspected HCV RNA genome for potential miR-122 binding sites. They identified two sites, present at the 5’NTR and 3’NTR of the HCV RNA genome.

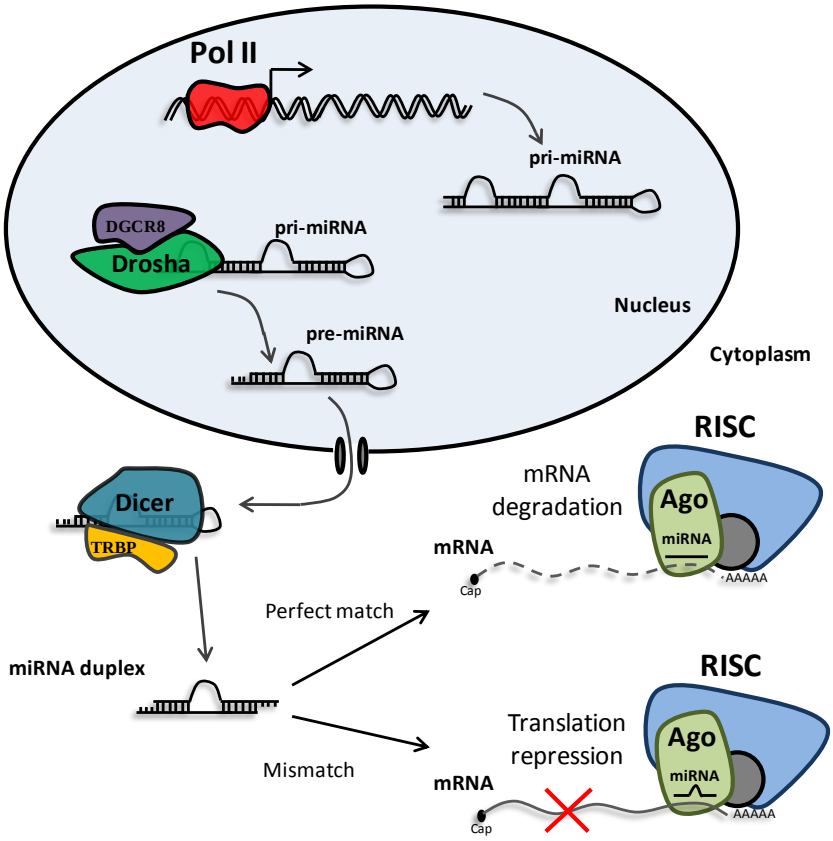


Figure 5: Biogenesis of microRNAs and function. *microRNAs (miRNAs) originate from specific genes present in the nucleus of eukaryotic cells. They are transcribed through the RNA polymerase II in long transcripts, the pri-miRNAs (~70 nucleotides long, which are cleaved a first time by Drosha, a type III RNase enzyme (associated to DGCR8 and other co-factors) in pre-miRNAs. These shorter transcripts are actively transported to the cytoplasm to be further cleaved by Dicer (associated with TRBP) to release a miRNA duplex. After dissociation of the complex, one of the two strands is loaded on the RNA-induced silencing complex (RISC). If the miRNA sequence match perfectly to the target mRNA, this latter will be degraded, in the other case, the mRNA translation will be repressed.*

To better characterize the functionality of these potential miR-122 binding sites, they have mutated the seed match sequences present on HCV RNA and identified the 5'NTR miR-122 binding site to be crucial for HCV replication (Jopling et al., 2005).

Indeed, expressing miR-122 holding the corresponding mutations in its seed sequence allowed recovering a robust HCV RNA replication, suggesting that miR-122 interacts physically with HCV RNA and this interaction has an essential role for HCV infection (Jopling et al., 2005).

The identification of miR-122 as a key HCV RNA replication host factor highlighted this miRNA as a new therapeutic target for HCV infection. This hypothesis has been studied in chimpanzee by administration of a locked nucleic acid (LNA)-

modified ologigonucleotide complementary to the 5' end of miR-122. This strategy led to a marked decrease of HCV RNA viral load and cholesterol in chimpanzee's blood and has shown no viral resistance to the treatment, fact which is observed when using viral proteins as target for therapy (Lanford et al., 2010). It is worth noting that drug mediated miR-122 antagonism is efficient on the 7 major HCV genotypes (Lanford et al., 2010). The antiviral effect observed by the delivery of the miR-122 antagonist was associated with concomitant improvement in liver histology (Lanford et al., 2010). Using miR-122 as a potential target for viral clearance is a promising therapy to treat HCV infection, nevertheless, few information is available on the host genes regulated by this miRNA and its high expression in the liver suggest an important role in hepatic function regulation.

The first target of miR-122 identified is the mRNA of Cat-1 (Cationic amino acid transporter-1) (Chang et al., 2004). This protein is involved in amino acid cell starvation. Under starvation, miR-122 binding to Cat-1 mRNA is abrogated allowing then the transport of amino acids (Jopling, 2012). Studies using mice have revealed that miR-122 regulates a number of genes involved in lipid metabolism. By miR-122 antagonist delivery in mice, Krutzfeldt *et al.* have shown that miR-122 down-regulated cholesterol biosynthesis genes (Krutzfeldt et al., 2005). These data were later confirmed by another study performed on mice, where miR-122 was also inhibited using another modified LNA. Esau *et al.* could show that fatty-acid synthesis rates decreased while fatty acid oxidation rates increased in treated mice. Furthermore they observed a marked decrease in cholesterol levels upon miR-122 activity inhibition in normal of diet-induced obese mice. Among the identified target genes, there is aldolase A (AldoA), N-myc downstream regulated gene3 (Ndgr3), hemochromatosis (Hfe) and hemojuvelin(Hjv). Altogether, these interesting results suggest that miR-122 could be a target for both HCV infection but also in diet-induced obesity. But so far, no study could show long term effect of miR-122 inhibition on human subject.

In addition, miR-122 has been implicated in the regulation of HCC development by acting on cell proliferation and inhibiting tumorigenesis. Indeed, it has been demonstrated that miR-122 expression is down-regulated in liver cancers (Kutay et al., 2006). Furthermore, ectopic expression of miR-122 in human hepatoma cell lines reverses their tumorigenic properties, such as growth, replication potential,

clonogenic survival, migration and sensitizes tumorigenic cells to anticancer drugs (Bai et al., 2009; Ma et al., 2010; Xu et al., 2011). It seems that miR-122 acts on factors involved in cell movement, cell morphology, cell-cell signaling and transcription, thus acting as a tumor suppressor miRNA regulating intrahepatic metastasis formation (Tsai et al., 2009). It has been recently shown that miR-122 acts on a specific signaling pathway involved in embryogenesis and carcinogenesis, leading to suppression of cell proliferation and induction of hepatoma cell apoptosis (Xu et al., 2012). Altogether, these results suggest that targeting miR-122 for a prolonged period of time, could have negative effects and enhance hepatocarcinogenesis. Indeed, a recent study has shown that miR-122 knock-out (KO) mice were more susceptible to HCC development (Hsu et al., 2012). Furthermore, adenovirus mediated delivery of miR-122 in a HCC mouse model significantly decreased the development of HCC in miR-122 receiving mice compared to control mice (Hsu et al., 2012). These data have to be taken into account when developing therapeutic protocols for targeting miR-122, in HCV treatment.

So far, miR-122 is the unique example of a positive cooperation between a miRNA and a viral infection, but the precise mechanism of miR-122 mediated HCV RNA regulation remains to be clarified. It has been shown that the miRNA interacts physically with the 5'NTR of HCV RNA, within the first domain of the IRES. But the function of this interaction is still a controversy. It has been shown that miR-122 does not modulate the elongation phase of HCV RNA (Villanueva et al., 2010). Because the miR-122 binding is close to the domains involved in RNA recognition and translation by ribosomes, it has been suggested that miR-122 could has a positive effect on HCV RNA translation (Henke et al., 2008; Niepmann, 2009) or modulate HCV RNA abundance in human hepatocytes (Norman and Sarnow, 2010). Jopling *et al.* have identified 2 nucleotides within the seed sequence, important for HCV RNA replication (Jopling et al., 2005). Recently, a study has identified other unexpected miR-122 nucleotides outside of the seed sequence interacting with HCV RNA. These results are interesting since mutation of these nucleotides at position 15 and 16 of miR-122 had no effect on miR-122 mediated inhibition of miR-122 target mRNA, while these mutation abolished HCV RNA replication (Machlin et al., 2010). The authors suggest that this unusual interaction between miR-122 and HCV RNA could

play a role in masking HCV RNA from sensors of the innate immune response such as protein kinase R (PKR) or retinoic acid inducible gene I (RIG-I). Further studies are required to better characterize the precise role of miR-122 interactions in HCV RNA replication. Even if this interaction is not yet well characterized, promising pre-clinical studies show that drug-mediated antagonism of miR-122 is well tolerated by monkeys (Elmen et al., 2008; Hildebrandt-Eriksen et al., 2012). More recently, a phase IIa clinical study has been presented at the 62th Annual Meeting of the American Association for the Study of Liver Disease (AASLD), demonstrating that the delivery of a miR-122 LNA (Mirvirsen®, Santaris Pharma a/s) in HCV infected patients leads to a decrease in the viral load of 2 to 3 log from the baseline after 10 weeks of monotherapy. Interestingly, out of the 9 patients involved in the group receiving the highest dose of Miravirsen® (7mg/Kg), 4 became HCV RNA negative during the study (A Randomized, Double-blind, Placebo Controlled Safety and Anti-viral Proof of Concept Study of Miravirsen, an Oligonucleotide Targeting miR-122, In Treatment Naïve Patients with Genotype 1 Chronic HCV Infection, Abstract AASLD, San Francisco, California).

Apart from miR-122, other miRNAs have been shown to interact with HCV RNA and among those; there is miR-199a, miR-196b and miR-29.

miR-199a binding sites have been identified in the 5'UTR of HCV RNA, suggesting that this miRNA interacts physically with this latter (Murakami et al., 2009). Overexpression of this miRNA has a robust inhibitory effect on HCV replication while chemical inhibition of the miR-199a leads to an increase of HCV replication (Murakami et al., 2009). Mutation analysis showed that inhibitory activity of miR-199a on HCV replication is dependent of the complementarities between the HCV RNA sequence and miR-199a suggesting that miR-199a interacts physically with HCV RNA (Murakami et al., 2009). While this miRNA is not highly expressed in the liver, a study has shown that its expression was up-regulated during HCV replication (Banaudha et al., 2011). Further studies are required to determine the role of this miRNA in HCV infection of hepatocytes.

miR-196b has a binding site in the NS5A coding region of HCV RNA. It has been shown that the expression of this miRNA, as well as miR-122, is modulated upon IFNβ treatment (Pedersen et al., 2007). These authors suggested that the IFNβ

inhibitory effect on HCV infection could be due to the regulation of important miRNAs for HCV infection. However, another study has demonstrated on infected patients undergoing IFN therapy, that there was no correlation between miR-122 expression and viral load (Sarasin-Filipowicz et al., 2009). A mechanism of miR-196b action on HCV infection has been shown by Hou *et al.* In this study, the authors have identified that the mRNA of Bach1, a repressor of the anti-oxidative and anti-inflammatory heme oxygenase 1 (HMOX1), was down-regulated, leading to the up-regulation of HMOX1 expression and inhibition of HCV infection (Hou et al., 2010).

miR-29 family interaction with HCV infection has been shown recently. MiRNA profile analysis of infected Huh7.5 cells and biopsies from chronically infected patients' liver showed that the level of the three members of the miR-29 family decreased by two-fold compared to control (Bandyopadhyay et al., 2011). Furthermore, they have shown that over expression of this miRNA inhibited HCV replication in Huh7.5 cells. It is worth noting that overexpression of miR-29 in hepatic stellate cells leads to a decrease in collagen production and in the inhibition of their proliferation. This study suggests that miR-29 is a novel miRNA impacting HCV infection. However, the mechanism by which miR-29 acts on HCV replication is not yet known.

b. Cyclophilins

Cyclosporine A (CsA) is a well characterized molecule derived from fungus and used in transplantation as an immunosuppressor. Interestingly, even before the identification of HCV as the causative agent of the NANBH, it had been noticed that CsA inhibited NANBH in liver transplant recipients. Indeed, CsA has a broad antiviral activity and its potential anti-HCV activity has been demonstrated *in vitro* by Watashi *et al.*, who have shown that CsA reduces drastically HCV RNA replication (Watashi et al., 2003). Furthermore, the anti-HCV activity of CsA was not dependent on its immunosuppressor activity, since other immunosuppressors did not affect HCV infection. It has been shown that, in chronic hepatitis C patients, CsA in combination with interferon is more effective than interferon monotherapy (Inoue et al., 2003). CsA exerts its immunosuppressor activity by binding to cyclophilins. The **cyclophilin** (CyP) family contains about 16 members, among which cyclophilin A (CyPA), CyPB and CyPC. Since, CsA primary targets are cyclophilins, it has been proposed that

these proteins could be new host cell factors for HCV RNA replication (Nakagawa et al., 2005). The precise member of the family involved in HCV RNA replication was not yet clear for several years. It has been shown that both CyPA (Yang et al., 2008a) and CyPB (Watashi et al., 2005) play a role in HCV replication. It is likely that several members of the cyclophilin family are involved in this step of HCV life cycle but contradictory data have been published these last years. Indeed, Nakagawa *et al.* identified the cyclophilins as the factors responsible for the CsA-mediated anti-HCV activity and they have shown by transient and stable knock-down expression that CyPA, B, C and D led to a decrease in HCV RNA replication (Nakagawa et al., 2005). While other studies confirmed the role of CyPA and B in HCV RNA replication, no-one could confirm the involvement of CyPC and D in this step of the HCV life cycle. Recently, a study has demonstrated, using sub-genomic replicons and full-length viruses, that CyPA and not B, plays a role in HCV replication (Kaul et al., 2009). This study suggested also that CyPA has a broader role in HCV infection as the authors identified a role for CyPA in virus production (Kaul et al., 2009). In contrast, it has been shown that the HCV NS5A protein is able to interact *in vitro* with both CyPA and B and this interaction involves catalytic residues of the cyclophilins (Fernandes et al., 2010). While many studies have confirmed and characterized the role of CyPA in HCV infection (Chatterji et al., 2009; Ciesek et al., 2009; Foster et al., 2011; Yang et al., 2008a) only few could show a role of CyPB in HCV infection (Heck et al., 2009; Morohashi et al., 2011). Although it is unclear how cyclophilins impact HCV RNA replication and which protein is involved in this process, it is clear that targeting cyclophilins is a novel alternative for HCV therapy. The use of CsA for the treatment of HCV looks attractive, but the immunosuppressive action of CsA renders difficult the use of this molecule in HCV treatment. A novel synthetic non–immunosupressive cyclosporine, DEBIO-025 has been developed and showed potent anti-HCV activity in both sub-genomic replicating hepatoma cells and in the context of HCVcc infection (Paeshuyse et al., 2006). Targeting cyclophilins with DEBIO-025 is promising since the anti-immunosuppressive action has been precluded and DEBIO-025 is currently in phase III clinical trial for HCV treatment (Sarin and Kumar, 2012).

c. Other host factors involved in HCV replication

Since HCV RNA is a complex mechanism, it is not surprising that this step of the HCV life cycle also involves several other host factors. Randall *et al.* have identified, through a siRNA screening on HCV replicating Huh7.5 cells, a set of 26 genes required for an efficient HCV replication. They identified several genes involved in the RNA interference machinery such as DICER and proteins of the RISC complex (Randall et al., 2007). They have also confirmed the role of VAP-A and VAP-B in HCV RNA replication through a direct NS5A interaction (Gao et al., 2004; Hamamoto et al., 2005). They identified several kinases which interact with NS5A, such as RAF1, EIF2AK2 or GR2, as important for HCV RNA replication (Randall et al., 2007). Other studies have revealed that HCV replication is tightly linked to the lipid metabolism (Kapadia and Chisari, 2005). Indeed, SREBPs, a transcription factor required for the transcription of genes involved in cholesterol biosynthesis, is stimulated both by HCV infection and the expression of individual viral proteins suggesting that the host cell lipid metabolism is crucial for HCV infection (Oem et al., 2008; Park et al., 2009; Waris et al., 2007). Since HCV is able to hijack host cell machineries to sustain its own life cycle, it is likely that a lot of host factors are required for an efficient HCV replication. A recent study performed a genome wide genetic screen and identified 44 host factors decreasing HCV propagation (Li et al., 2009). The identification of potential host factors that are implicated in HCV infection will allow defining new clinical targets for the treatment of HCV infection. A deeper investigation of the newly identified host factors should clarify whether these factors directly interact with viral proteins or if they are indirect co-factors.

iii. Assembly and release of virions

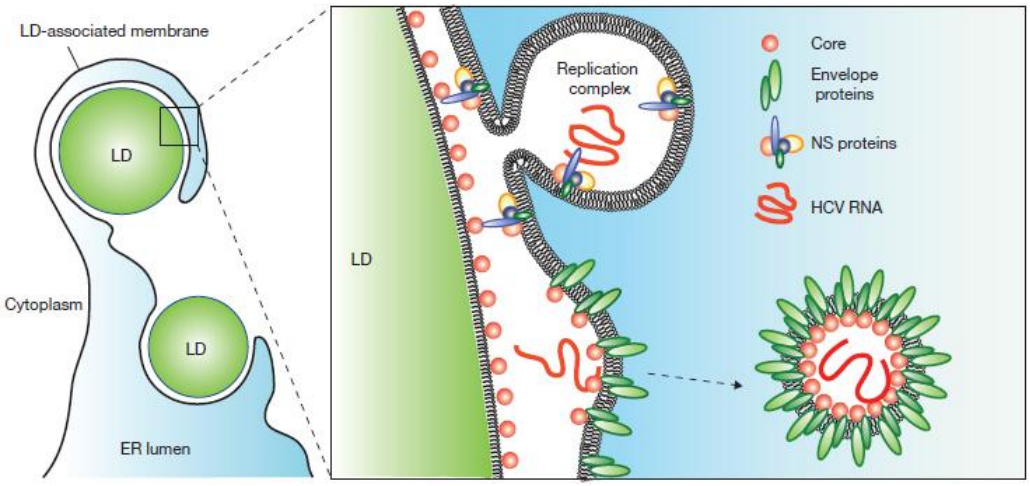
During HCV replication, several positive strand RNA will be newly synthesized which will be used for the synthesis of viral proteins or will be encapsidated by the core protein to form new virions. The late steps of viral assembly and release are not well characterized yet. It is thought that assembly takes place when the viral RNA interacts with the core protein, which leads to the nucleocapsid formation through unknown mechanism (Roingeard and Hourioux, 2008). So far, no encapsidation

signal has been identified yet. It is believed that the virions bud from the ER membrane at ER-LD junctions and then virions are released by exocytosis. The assembly and release process are both tightly linked to the secretion of VLDL (Gastaminza et al., 2008). It is assumed since a while that HCV circulates in the blood of chronically infection patients associated to LDL and VLDL (Andre et al., 2002). The circulating virus represents a heterogenous population of virions with different densities from 1.03 to 1.25 g/ml (Nielsen et al., 2006). These viral particles are enriched with apoB and apoE and have physico-chemical properties of VLDL (Andre et al., 2005). Monoclonal anti-apoB and apoE antibodies recognized specifically patient serum-derived LVPs, suggesting that virions are indeed associated with VLDL (Andre et al., 2005; Owen et al., 2009). The recent development of full length viruses able to replicate in cell culture, the HCVcc (further explained later), allowed to further study the association of HCV with lipoproteins *in vitro*. Gastaminza *et al.* have shown that intracellular viral particles are less dense and infectious than secreted viral particles, suggesting that the association of viral particles with VLDL occurs inside the cell during the maturation process of VLDL (Gastaminza et al., 2006). The process by which viral particles are assembled and associate to VLDL remains unclear. It has been demonstrated that the core protein plays an important role in this process, since the core protein associates to ER-associated LDs after its release from the polyprotein (McLauchlan et al., 2002) and disruption of this interaction lead to a marked decrease in HCV viral production (Boulant et al., 2007). More recently, Miyanari *et al.* have shown that the core protein has the capability of recruiting HCV non-structural proteins close to LDs (Miyanari et al., 2007). Using confocal and electron microscopy imaging and mutational analysis, they have demonstrated that the core protein was able to interact with non-structural proteins (such as NS5A) and recruit the RC to the site of HCV assembly, the LDs (Miyanari et al., 2007). Furthermore, they have shown that this interaction is important for the assembly process. They also suggest that the non-structural proteins known to be involved in HCV replication could have an unexpected role in HCV assembly and release by creating a micro-environment that would facilitate HCV assembly or by providing newly synthesized genomic HCV RNA in proximity to nucleocapsid for viral assembly (Miyanari et al., 2007). Thus they proposed a model for HCV viral production where the core protein recruits non-structural proteins and the replication complex to lipid droplet-ER interaction sites where core binds to viral

RNA and forms the nucleocapsid which will incorporate E1 and E2 glycoproteins to be released in the ER lumen (Miyanari et al., 2007). The identification of these core-non-structural protein interactions highlights a close collaboration of the viral proteins involved in HCV replication and assembly, but the model proposed does not explain how the virus associates with lipoproteins intracellularly.

Several host factors have been identified as playing a role in HCV assembly and release. While the role of apoB is still controversial (Bartenschlager et al., 2011; Jiang and Luo, 2009), several studies confirmed the role of apoE in the HCV assembly and release process. It has been shown that apoE is present at the surface of the virions, and the expression level at the virions surface correlates with the HCV viral particle infectivity (Chang et al., 2007). Furthermore, using a knock-down assay, Jiang *et al.* confirmed the role of apoE in the HCV assembly and release process and they have shown that silencing apoB expression had no effect on HCV production (Jiang and Luo, 2009) while Gastaminza *et al.* found that HCV assembly and maturation depend on apoB and the microsomal transfer protein (MTP) (Gastaminza et al., 2008). More recently, our laboratory has identified an interaction between NS5A and apoE, using a yeast two-hybrid system. Our laboratory has demonstrated that this interaction is important for HCV assembly, since HCV NS5A mutants known to be defective in HCV assembly and production failed to bind apoE (Benga et al., 2010). Thus, a model has been proposed for the formation of HCV particles involving apoE **(Figure 6B)**. The presence of apoE at the virions surface allows the virus to bind in an easier way to the hepatocytes surface through natural apoE binding molecules such as HS, SR-BI and LDLR, thus suggesting that apoE has a dual role in HCV infection during HCV assembly and HCV entry (Jiang et al., 2012)(for review see (Bartenschlager et al., 2011)). Furthermore, apoE is expressed in human hepatocytes as different isoforms, apoE2, apoE3 and apoE4 - apoE3 representing the most common isoform. A recent study suggested that HCV is able to associate to different apoE isoforms and the apoE isoform associated to the viral particle influences the infectivity of the latter (Hishiki et al., 2010): indeed the expression of the apoE2 isoform in apoE knock down cells lead to a poor recovery of infectious HCV viral particle production while HCV RNA production in the supernatant was equivalent to the other isoforms tested.

A.



B.

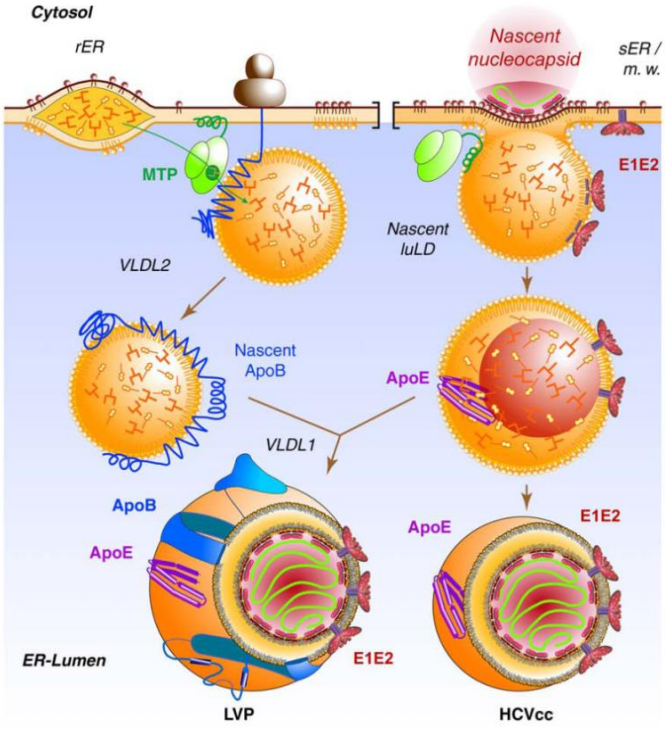


Figure 6: Schematic representations of models for the HCV virion assembly process. ***A.*** *HCV assembly model proposed by Miyanari et al. (Miyanari et al., 2007). HCV core protein recruits non-structural proteins and the replication complex to lipid-droplet-ER interaction sites where viral RNA binds to the core protein to form the nucleocapsid that will bud from the ER membrane.* ***B.*** *During translation at the rough ER, nascent apoB (blue line) is translocated into the ER lumen and loaded by MTP with phospholipids and triglycerides (left panel). This leads to the formation of a neutral lipid core that is converted into a spherical particle (VLDL2) acquiring exchangeable apoE. In the smooth ER (sER) or membranous web, a second precursor (the luminal LD; luLD) is formed from the ER membrane and by MTP-mediated triglyceride enrichment (right panel). E1 and E2 retained at the ER membrane might slide onto this luLD prior to pinching-off. The nucleocapsid would be inserted into the hydrophobic lipid core of the pinching-off luLD due to the hydrophobic nucleocapsid surface. In VLDL competent cells such as primary human hepatocytes, this precursor could fuse with VLDL2 to form the LVP. Alternatively in Huh-7 cells where VLDL1 formation is inefficient, HCVcc is secreted predominantly as particles lacking apoB. A. Schematic representation from (Miyanari et al., 2007)(A) and B. Legend and schematic representation from (Bartenschlager et al., 2011).*

In opposite to these results, an even more recent study has shown, using mouse cells replicating sub-genomic replicons and trans-complemented to express HCV structural proteins, that all human isoforms as well as the mouse homologue of apoE were similarly capable in supporting infectious HCV particle production (Long et al., 2011).

Among the other host cell factors required for proper HCV viral particle assembly, are MTP, heat shock cognate protein 70 (Hsc70) and diacylglycerol acyltransferase-1 (DGAT-1).

MTP is a key enzyme for VLDL production, so it is not surprising that drug-mediated inhibition of MTP lead to reduce HCV production (Gastaminza et al., 2008; Huang et al., 2007). Hsc70 has been shown to colocalize with HCV viral proteins and with LDs and influence HCV production (Parent et al., 2009). DGAT-1 is an enzyme involved in lipid droplet formation. Drug mediated inhibition of DGAT-1 activity drastically reduced HCV release (Herker et al., 2010). Herker *et al.* have shown that DGAT-1 recruits core protein to LDs suggesting that HCV assembly requires DGAT-1-mediated lipid droplet synthesis (Herker et al., 2010).

C. Model systems to study HCV

The development of more efficient and more tolerated antiviral drugs has been slowed down by the lack of good model system to study the HCV life cycle. These last years, several *in vitro* and *in vivo* models have been developed but better models are required to better understand the intimate virus-host interactions.

i. In vitro models

Based on the knowledge on HCV structure and infection, several *in vitro* systems have been either developed or isolated. The *in vitro* systems include patient serum-derived HCV, recombinant HCV envelope glycoproteins, HCV-like particles (HCV-LPs), HCV pseudo-particles (HCVpp), subgenomic replicons and cell-culture derived

HCV (HCVcc). They all improved significantly the knowledge on HCV infection, but all have limitations that prevent a full understanding of HCV infection in hepatocytes. The next section will present major models developed for the study of HCV infection and their limitations.

➢ Recombinant E2 glycoprotein

The truncated soluble form of the glycoprotein E2 (sE2), where the transmembrane domain of the glycoprotein has been deleted, allowed the identification of host cell factors involved in either HCV binding or entry such as CD81 (Pileri et al., 1998), SR-BI (Scarselli et al., 2002) and HS (Barth et al., 2003). However, since in the infectious virion the E2 glycoprotein is associated to the E1 glycoprotein, this model is limited to the study of sE2-host factor interaction and does not allow the study of the entire HCV entry process.

➢ HCV-like particles

HCV-like particles (HCV-LPs) are defined as non-infectious particles composed of the structural proteins core, E1, E2 and p7. Their production is allowed by the delivery of the genes encoding the viral proteins through a bacculovirus system into mammalian or insect cells (Baumert et al., 1998). Their biochemical, biophysical and antigenic properties are similar to patient derived infectious virions. The structure of the heterodimeric glycoprotein complexes resemble to those on the native particles and their capability to bind and enter hepatoma cell lines and primary human hepatocytes makes them an attractive tool to study HCV-hepatocytes interaction during the entry process (Barth et al., 2005; Triyatni et al., 2002; Wellnitz et al., 2002). Furthermore, HCV-LPs have similar antigenic properties as those of patient-derived viral particles and thus have been suggested as a potential vaccine (Baumert et al., 1999). And recently, it has been shown that injection of HCV-LPs to chimpanzees induces a HCV-specific cellular immune response which protects the animals of persistent HCV infection following a second HCV challenge (Elmowalid et al., 2007). However, this model is limited since no viral genome or reporter gene is present inside the particle, thus this model requires specific microscopy or flow cytometry techniques to study HCV-host cell interactions.

➢ HCV pseudo-particles

HCV pseudo-particles (HCVpp) are chimeric viruses composed of the HCV envelope glycoproteins E1 and E2, the human immunodeficient virus (HIV) or murine leukemia virus (MLV) core protein and a reporter gene encoding either GFP or luciferase (Bartosch et al., 2003a). This model system was the first robust system allowing the study of all HCV entry steps from binding to membrane fusion. The presence of functional heterodimer E1E2 complexes allowed identifying host cells receptors involved in HCV entry, such as CLDN1 (Evans et al., 2007) and OCLN (Ploss et al., 2009). HCVpp production relies on co-transfection of 3 different expression vectors into human embryonic kidney (HEK-293T) cells, each one encoding the above cited components of the pseudo-particles. HCVpp are released into the supernatant of transfected cells to a level of 10^5 infectious units/ml (Bartosch et al., 2003a). HCVpp infection is measured through the expression of a sensitive reporter gene and this system allows high throughput screenings. However, a limitation of this system is the fact that HCVpp are not associated with lipoproteins since 293T cells do not synthesize VLDL and that this system precludes the study of viral replication and assembly.

➢ HCV genomic and sub-genomic replicons

Ten years after the first identification of HCV genome, few *in vitro* models have allowed the study of HCV infection and no model was able to replicate *in vitro*. To overpass this restriction, Lohmann *et al.* developed sub-genomic replicons (Lohmann et al., 1999). These systems are bicistronic RNAs which are able to replicate autonomously and allow selecting cells that support the replication of these sub-genomic replicons. They are composed of (i) the HCV IRES which drives the translation of a selection marker (usually neomycin) and (ii) the ECMV IRES that drives the translation of the HCV non-structural proteins and the RNA is flanked by (iii) the 5' and 3' NTR of HCV. The sub-genomic replicons were the first *in vitro* model allowing the study of HCV RNA replication. In this system, the selection pressure of the antibiotic allows to select for cells that are able to replicate the virus and it has been shown that adaptive mutations arise, mainly in the non-structural proteins NS3, NS4B and NS5A (Bartenschlager et al., 2004) and these adaptive mutations lead to an increase of the replication rate (Lohmann et al., 2001). These subgenomic

replicons allowed selecting cell lines supporting high replication rate of HCV RNA. One of these cell lines, called Huh7.5, was obtained by curing HCV replicating Huh7 cells with interferon alfa (Blight et al., 2002). While sub-genomic replicons allow studying HCV RNA replication, the absence of the structural proteins was a limitation in understanding their influence in the HCV life cycle and in their involvement in HCV viral particle formation. Thus, based on the sub-genomic replicon strategy, genomic replicons have then been developed (Pietschmann et al., 2002). However, although HCV structural proteins were expressed and properly folded, no infectious viral particle could be detected in the supernatant of Huh7 cells replicating full-length genomic replicons (Pietschmann et al., 2002).

- **Cell culture derived HCV**

During the development of sub-genomic replicons, a Japanese team isolated a viral clone from a patient suffering of fulminent hepatitis and created a sub-genomic replicon out of this clone, which is able to replicate in human hepatoma cell lines without any selection pressure (Kato et al., 2001). Using this original clone, called JFH1 for Japanese Fulminent Hepatitis 1, three different laboratories have been able to develop a full length virus that is able to infect, replicate and produce infectious viral particles, thus reconstituting the full HCV life cycle *in vitro* (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). This cell culture-derived HCV (HCVcc) *in vitro* model system is based on the transfection of the JFH-1 viral RNA into Huh7 cells that will produce infectious HCV viral particles that are able to infect naïve Huh7 cells as well as chimpanzees and mice repopulated with human hepatocytes (described in more detail later) (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The detection of the infection was easily monitored using sensitive techniques which rely on the presence of viral antigens (viral RNA or viral proteins) (Lindenbach et al., 2005; Wakita et al., 2005). To facilitate the infection monitoring, viruses bearing a reporter gene (luciferase or fluorescent proteins) have been further developed (Koutsoudakis et al., 2006; Schaller et al., 2007). The development of this model system could confirm previous data obtained using more artificial *in vitro* model system such as the involvement of the glycoproteins in HCV entry (Wakita et al., 2005), the hepatotropism of HCV (Lindenbach et al., 2005; Wakita et al., 2005) and the role of identified HCV host cell entry factors (Koutsoudakis et al., 2006; Lindenbach et al., 2005; Wakita et al., 2005; Zeisel et al., 2007; Zhong et al., 2005).

This model allowed also creating intra-genotypic virus chimeras to produce viral particles from different genotypes (Pietschmann et al., 2006). HCVcc development has been a breakthrough in the study of HCV infection, but this model is still limited since all the chimeras developed so far are based or derived from the non-structural proteins of the JFH-1 clone, a genotype 2a virus. As shown in Figure 3, this genotype is mostly found in Italy and Japan. Since non-structural proteins are also involved in HCV pathogenesis, this model does not allow the study of the replication of other genotypes in cell culture and some genotypes (such as the genotype 3) are known to have a different cytopathic effect (Rubbia-Brandt et al., 2000).

ii. *In vivo* models

1. Chimpanzees

Over the last 20 years, the study of HCV infection *in vivo* relied essentially on **chimpanzees (*Pan troglodytes*)**. Data obtained from these studies largely contributed to the current knowledge on HCV pathogenesis. The progression of the infection is similar to that observed in infected patients. The increase in viral RNA is detected few days following HCV infection, followed by an acute hepatitis characterized by an increase in the hepatic enzyme present in the blood stream (ALT) and development of an adaptive immune response. Furthermore, as observed in patients, chronic hepatitis is associated with histological injuries due to chronic hepatitis and with HCC (Bradley, 2000). However the use of this animal model is limited since some differences are observed compared to human pathology. The severity of the chronic infection is less than what observed in patients and the immune response of HCV infection is attenuated in chimpanzee compared to humans. In addition, the use of chimpanzees requires a special housing; they are expensive and difficult to handle (Barth et al., 2008). Furthermore, since 1988, the chimpanzee is listed as an endangered species which drastically limits the use of the chimpanzee for pre-clinical studies mostly. Thus, an alternative to study HCV infection *in vivo* was urgently needed.

2. Tupaïa

Tupaia belangeri is a tree shrew found in the South-East of Asia. It has been demonstrated that this animal is susceptible to a range of human viruses and thus has been proposed as an alternative HCV infection model system. Primary hepatocytes of this animal are susceptible to HCV infection and Thomas Baumert's team has shown that HCV infection of tupaïa hepatocytes was robust, since HCV could enter, replicate and produce infectious viral particles (Zhao et al., 2002). Recently, a study monitored HCV infection in tupaïas over a period of three years. Interestingly, the authors have shown that HCV RNA was detectable and animals showed mild inflammation during the acute phase of infection. This acute phase was then followed by the development of liver steatosis and formation of cirrhotic nodules and ended in tumorigenesis (Amako et al., 2010). It is worth noting that sera from infected tupaïa could infect naïve animals which showed similar pathology than the primary infected tupaïas demonstrating that the viruses produced were transmissible (Amako et al., 2010). Furthermore, it has been shown that the tupaïa homologues of the HCV entry factors CD81, SR-BI, CLDN1 and OCLN were able to support HCVpp or HCVcc infection in HCV entry resistant cells (Tong et al., 2011). These data show that *Tupaia belangeri* is an attractive small animal model for the study of HCV infection but the low and variable infection rates and HCV viremia in these animals are problematic. Nevertheless, since these animals have been demonstrated to support persistent infection, it makes these animals an interesting alternative to chimpanzees. However, further studies are required to better characterize this animal model for HCV infection.

3. Human liver-repopulated immunodeficient mice

A suitable model for HCV infection would be a mouse model supporting HCV infection. However, mice are naturally resistant to HCV due to blockades at several steps of the HCV life cycle. To overpass these restrictions, a first mouse model has been developed called the **Alb-uPA/SCID** (severe combined immunodeficiency) mouse model which allows to study HCV infection *in vivo*. These mice are the result of crossing two mice background the C:b-17/SCID/*bg* and the Alb-uPA mice (Mercer et al., 2001). Alb-uPA transgenic mice express a tandem of four murine “urokinase-

type-plasminogen activator" (uPA) genes under the control of an albumin (Alb) promoter which allows targeting the phenotype to the liver. Over-expression of the transgene in the liver accelerates murine hepatocytes death. Wood chuck, rat, mouse or human derived hepatocytes can then be transplanted into this mouse model and a repopulation is observed (Mercer et al., 2001). Mercer *et al.* have shown for the first time that Alb-uPA/SCID mice repopulated with human hepatocytes are able to support HCV infection *in vivo*. The human albumin level is monitored to assess human hepatocyte repopulation and once the mice stabilized, they can be infected with either HCV infected patient-derived sera or with HCVcc (Law et al., 2008; Mercer et al., 2001). Interestingly the viral load observed in these mice are similar to those observed in patients. Furthermore, HCV-infected Alb-uPA/SCID-derived serum can newly infect “naïve” mice rendering serial infections possible. It has been demonstrated that this model supports HCV infection over 4 months during which the function and structure of the liver are not altered (Barth et al., 2008). This mouse model allowed to confirm the role of the anti-receptor and neutralizing antibodies in the control of HCV infection (Lacek et al., 2012; Law et al., 2008; Meuleman et al., 2012; Meuleman et al., 2008; Vanwolleghem et al., 2008). The Alb-uPA/SCID mouse model is less expensive and its reproduction is faster than in chimpanzees but it requires an expertise for the isolation of human hepatocytes, the mice bleed easily because of the transgene, there is a high mortality in the production of the mice and they are immunodeficient. So altogether, these drawbacks limit the study of HCV infection to certain aspects.

Recently, Bissig *et al.* have developed another model where human hepatocyte repopulation in immunodeficient mice is regulated by oral administration of 2-(2-nitro-4-trifluoro-methylbenzoyl)-1.3-cyclohexanedione (NTBC). The transgenic mice used in this study have been immunosuppressed through the deletion of the fumaryl acetoacetate hydrolase (Fah), recombination activating gene 2 (Rag2) and the γ-chain of the receptor for IL-2 (Il-2r γ) (Bissig et al., 2010). The absence of the drug in the mouse blood leads to the death of mouse hepatocytes caused by toxic accumulation of tyrosine catabolites due to the absence of mouse Fah, while the presence of the human Fah homolog in human hepatocytes allows these cells to expand in the mouse liver (Bissig et al., 2010).

This *Fah-/-Rag2-/-IL2rg-/-* mouse model has valuable assets compare to the uPA-SCID mouse model, since the drug mediated control of mouse hepatocytes death render the *Fah-/-Rag2-/-IL2rg-/-* mouse model more handleable than the uPA-SCID mouse. Furthemore, the human hepatocytes repopulation of the *Fah-/-Rag2-/-IL2rg-/-* mouse model occurs at a higher rate (up to 95%) and can be done at any age of the mouse (Bissig et al., 2010). Nevertheless, both of these mice are a major drawback for the study of HCV infection *in vivo*, they both are immunodeficient.

4. Immunocompetent mice

These last years, several mouse models have been proposed to overpass species specificity of HCV. Recently, Washburn *et al.* used the Balb/C-Rag2-γC background mice where they expressed, under the control of an albumin promoter, a caspase 8 fused with FK506 binding domain (FKBP) transgene (AFC8) which has suicidal activity. They injected CD34+ hematopoetic stem cells in these mice and delivered a prodrug that leads to dimerization of the caspase 8 and murine hepatocyte death allowing further repopulation by foetal liver hepatocyte progenitor cells (Robinet and Baumert, ; Washburn et al., 2011). This model is the first mouse model which allows to study the immune response during HCV infection in a mouse. However some drawbacks limit the use of this mouse model for the study of HCV infection. HCV RNA was only detected at low level in the liver and could not be detected in the serum of infected animals. And the second major drawback is that no anti-HCV antibody could be detected in these mice due to an incomplete reconstitution of functional B cell population (Robinet and Baumert). This model is thus a step forward for the development of an immunocompetent mouse model but is not yet fully functional for the study of all aspects of HCV infection.

Another immunocompetent mouse model has been proposed recently for the study of HCV entry *in vivo*. Indeed, Dorner *et al.*, based on the results of Ploss *et al.* (Ploss et al., 2009) (detailed later in this manuscript), have injected adenoviruses encoding the human entry factors CD81 and OCLN and the murine homologues of CLDN1 and SR-BI in mice with a FVB.129S6(B6)–Gt(ROSA)26Sortm1(Luc)Kael background (called R26–LSL–FLuc).

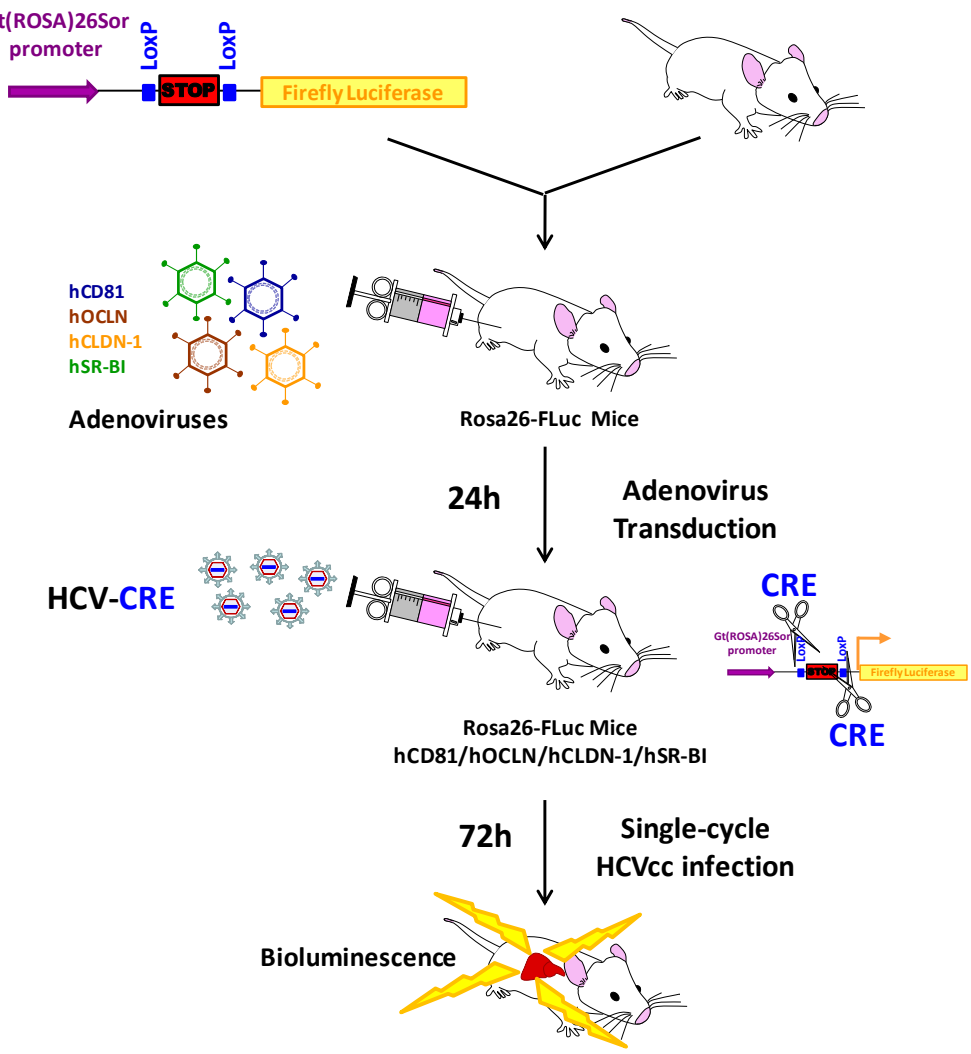


Figure 7: Immunocompetent mouse model to study single-cycle HCV infection. *Gt(ROSA)26Sor$^{tm1(Luc)\ Kaelin}$ mice (Rosa26-Fluc) contain in the Gt(ROSA)26Sor Locus a LoxP-flanked STOP codon restricting the firefly luciferase expression. Adenoviruses encoding hCD81, hOCLN, hCLDN-1 and hSR-BI were injected into Gt(ROSA)26Sor$^{tm1(Luc)Kaelin}$ mice 24h prior to challenge with recombinant bicistronic HCVcc expressing CRE protein (HCV-CRE). Upon HCV entry, CRE recombinase activates the luciferase reporter and bioluminescence into mouse hepatocytes. Signals can be monitored using a live animal bioluminescence imaging device. Alternatively, transgenic mice where CRE expression leads to activation of a nuclear-localized GFP/β-galactosidase reporter (Rosa26-GNZ) allows to assess HCV permissivity of murine hepatocytes by quantifying GFP+ murine hepatocytes using flow cytometry. (Figure and legend from (Zeisel et al., 2011a)).*

These mice contain loci where a stop codon is flanked by two lox-P sites, upstream of a firefly luciferase gene. Upon HCV-CRE infection, a bicistronic virus engineered to encode and express CRE recombinase, the stop codon is excised and firefly luciferase is then highly and constitutively expressed **(Figure 7)** (Dorner et al., 2011; Dorner et al., 2012; Zeisel et al., 2011a).

This mouse model is suitable to study HCV entry in mice but the system only allows to monitor single round infection as mouse hepatocytes are not able to support HCV

replication (Dorner et al., 2011). This mouse model shows that engineering mice to express factors important in the HCV life cycle is a valid strategy in order to develop a mouse model supporting the entire HCV life cycle. Such a model would be a suitable model and could be manipulated by the largest number without any pre-requirements and this would speed up the development of more tolerated antiviral treatments. The characterization of the factors involved in the species specifity and in the strict hepatotropism of HCV will help the development of such mouse model.

D. HCV tropism

HCV tropism is mainly hepatic. The liver is the primary target of HCV infection and carries billions of HCV RNA copies per gram of infected tissue. Nevertheless, HCV RNA and/or proteins have been detected in other organs of infected patients, suggesting that hepatitis C virus could have extra-hepatic reservoir(s).

i. Hepatotropism of HCV

So far, the factor(s) defining HCV hepatotropism is (are) unknown. HCV infection relies on many host cell factors at all steps of the viral life cycle (see section B. The viral life cycle), so it is likely that there is not only one but several host cell factors restricting HCV infection to hepatocytes. The different hypotheses that may explain the HCV liver tropism are discussed below.

HCV entry requires at least the four host cell factors CD81, OCLN, CLDN1 and SR-BI for an efficient entry, but none of these factors is liver specific. This suggests that either (i) these factors are differentially expressed in the liver and this would explain HCV tropism, or (ii) other factors participating to the HCV entry process but not as key players are liver-specific or (iii) finally a factor expressed in other tissues but not in the liver restricts HCV infection. It is also possible that all these suggestions explain the liver tropism of HCV.

Receptor expression levels at the surface of hepatocytes have been shown to be important for HCV entry. Indeed, CD81 expression can influence HCV entry in human hepatoma cells as enhanced expression correlated with enhanced HCV entry

(Akazawa et al., 2007; Koutsoudakis et al., 2007). Furthermore, the same observation has been made for SR-BI (Grove et al., 2007). The density of human hepatoma cells has been also shown to enhance some HCV entry factors expression, such as CDLN1 and SR-BI, leading to increased HCVpp and HCVcc internalization (Schwarz et al., 2009). Schwarz *et al.* have demonstrated that upon tight cell-cell contact, SR-BI and CLDN1 expression increased, leading to an increase in HCV receptor complex formation (Schwarz et al., 2009). Furthermore, studies have shown in liver biopsies from infected patients that HCV entry factor expression is modulated compared to non-infected patients (Mensa et al., 2011; Nakamuta et al., 2011; Reynolds et al., 2008). Altogether these data suggest that HCV entry factor expression is important for HCV entry into the liver, but since these factors are not liver specific, it does not explain the hepatotropism of HCV, but certainly contributes to it.

Among the molecules that have been shown to interact with HCV without being involved directly in the HCV entry process, the liver related **C-type lectin L-SIGN** has been proposed to be a candidate to explain HCV hepatotropism. Indeed C-type lectins are pathogen recognition receptors that bind unspecifically to mannose residues from pathogen-derived glycoproteins (Alvarez et al., 2002). L-SIGN is mainly expressed on liver sinusoid endothelial cells (LSEC) that compose the liver sinusoid. HCV circulates in the blood and to reach hepatocytes, HCV has to cross the liver sinusoid. It has been proposed that L-SIGN could be the first interaction point for HCV to reach its target cell. Indeed, DC-SIGN, another C-type lectin which is more broadly expressed, and L-SIGN are able to tightly bind HCV E2 glycoprotein (Gardner et al., 2003; Lozach et al., 2004; Lozach et al., 2003; Pohlmann et al., 2003). More interestingly, it has been shown that HeLa cells engineered to express either L-SIGN or DC-SIGN were able to bind HCVpp and HCVcc and to trans-infect Huh7.5 cells (Cormier et al., 2004). These results have also been obtained with human dendritic cells which naturally express DC-SIGN (Cormier et al., 2004). These results suggest that C-type lectins play an important role in the transmission of HCV to neighboring hepatocytes. However, these results have not been confirmed *in vivo* yet. The role of the LSEC in HCV transmission to hepatocytes remains to be clarified.

Once the sinusoid crossed, HCV reaches hepatocytes to bind to the putative HCV entry factors. It is not clear which receptor HCV binds first. Since the viral

particle is associated with lipoproteins it has been suggested that the natural lipoprotein receptors LDL-R and SR-BI would be the first HCV binding molecules (reviewed in (Zeisel et al., 2011b)). In this complex entry process, CD81 has been demonstrated to be an important or the most important factor. CD81 has a wide tissue expression profile, so this molecule does not define the hepatic tropism of HCV. However, CD81 binds to several partners in the tetraspanin web (Levy and Shoham, 2005). Recently, it has been shown that HCV infection was inhibited by a cleavage product of the CD81 binding partner **EWI-2**. Indeed, EWI-2 which naturally binds to CD81 in the tetraspanin web is further cleaved by a cellular protease, in a shorter product called **EWI-2wint** (EWI-2 without its N-terminus) which interacts with CD81 (Rocha-Perugini et al., 2008). Interestingly, in order to address the impact of this EWI-2wint mediated HCV infection inhibition; Rocha-Perugini *et al.* have assessed the expression of this molecule in different cell lines and found that HCV permissive cell lines did not contain EWI-2wint while resistant cell lines do have the cleavage product of EWI-2, EWI-2wint. These data suggest that EWI-2, which is further cleaved in non-permissive cells into EWI-2wint, can be a restriction factor for HCV entry and could participate in HCV tropism by restricting HCV entry into non-liver cells.

Following entry, the viral RNA is translated to produce structural and non-structural proteins, and then the replication of HCV RNA takes place involving several host factors. Among all factors involved in HCV replication, **miR-122** is the most important one. The mechanism by which this miRNA acts on HCV RNA replication remains to be defined but it is assumed now that miR-122 participates clearly in HCV hepatotropism. Indeed, miR-122 is a liver specific miRNA and represents 70% of the total miRNA population expressed in the liver (Lagos-Quintana et al., 2002) (See section B. ii. 2. a. microRNAs). It has been shown that miR-122 interaction with HCV RNA is crucial for HCV replication and inhibition of this interaction lead to drastic decrease of HCV replication both *in vitro* and *in vivo* (Jopling et al., 2005; Lanford et al., 2010). Cells lacking the expression of this miRNA are not able to support HCV replication while in opposite, the expression of this miRNA in HCV replication resistant cells allows a robust HCV replication (Kambara et al., 2012; Narbus et al., 2012). Furthermore, it has been recently shown that the expression of this miRNA in non-hepatic cells allows sustained HCV RNA replication of sub-genomic replicons

(Chang et al., 2008; Fukuhara et al., 2012). In addition, a study noticed that rodent non-hepatic cells expressing this miRNA were able to support HCV RNA replication, when IRF3, an interferon specific transcription factor, was knocked-down (Chang et al., 2008). Recently, an intriguing study has shown that stem cell-derived hepatocytes, which are chemically differentiated in absence of feeder cells, were able to support HCV replication (Wu et al., 2012). Wu *et al.* have demonstrated that during the differentiation process, cells become permissive 7 to 10 days after the differentiation started. Intriguingly, these authors have demonstrated that the differentiating stem cells became HCV permissive when they started to express miR-122 as they differentiated into hepatocytes like cells, and this differentiation was also accompanied with up-regulation of cellular host factors, such as EGFR, EphA2 and phosphatidylinositol 4-kinases type III alfa (PI4KIIIα) (Wu et al., 2012). These results suggest that miRNA-122 is an important factor for HCV replication and for defining HCV hepatotropism.

Furthermore, the assembly and release process relies also on factors involved in the VLDL secretion pathway, such as apoB, apoE and MTP. Hepatocytes are specialized cells in the production of lipoproteins and this fact is also likely to define HCV hepatotropism. The absence of the factors involved in the VLDL pathway in non-hepatic cells is probably a characteristic that restricts HCV assembly and release to hepatocytes. Fukuhara *et al.* were able to infect non-permissive cells by expressing miR-122 and missing key HCV entry factors in non-hepatic cells (Fukuhara et al., 2012). Interestingly, while the virus was able to replicate its RNA, the authors failed to detect infectious HCV particle release from their engineered cell lines. Lipid metabolism-associated protein expression analysis revealed that LDL-R and DGAT-1 was expressed in non-hepatic cells while apoE, apoB and MTP were expressed at very low levels or even not expressed at all, and this would explain the incapability of these cells to support HCV assembly and release (Fukuhara et al., 2012). Since apoE is a central factor involved in HCV assembly and release, and since this factor seems to not be expressed or at low level (i. e. in neuron-derived cell line) in non-hepatic cells, this fact could participate in explaining HCV hepatotropism.

ii. Extra-hepatic tropism

Although HCV mainly infects the liver, it has been reported that some non-hepatic cells could support HCV infection but at lower levels than in hepatocytes of infected patients. The potential extra-hepatic resevoirs of HCV are discussed below.

- **Neurotropism of HCV**

HCV infection is associated to extra-hepatic symptoms such as fatigue and cognitive dysfunction or mixed cryoglobulinemia and non-hodgkin lymphoma (Kramer et al., 2002; Marukian et al., 2008). These symptoms suggested that HCV infection can occur outside its primary target organ and reach neuronal cells which could explain these symptoms. Evidence of HCV RNA presence in the brain came early in the field with the development of highly sensitive techniques for HCV RNA detection such as RT-PCR (Bolay et al., 1996; Maggi et al., 1999; Radkowski et al., 2002). The presence of HCV positive and negative strand RNA in brain biopsies suggests that HCV is able to infect cells from the brain. However, the type of cells that are infected and the underlying mechanisms remained to be determined. The discovery of the HCV host entry factors allowed determining which cells in the brain express these receptors and are permissive to HCV infection. A couple of studies have been published recently showing that HCV is able to infect neuroepithelioma cells (Burgel et al., 2011; Fletcher et al., 2010). The authors have assessed HCV entry factor expression in a large variety of brain-derived cell lines and identified neuro-endothelial cells as expressing the main HCV host entry factors CD81, OCLN, CLDN1 and SR-BI. They have assessed HCVpp permissivity of the tested brain-derived cell lines and identified few cell lines permissive for HCV entry. Then they assessed the ability of these cells to support HCV infection using HCVcc: while Fletcher *et al.* succeeded in infecting HCV entry permissive neuroepithelioma cell lines, Burgel *et al.* failed to find a cell line replicating the virus, even by transfecting viral RNA to overpass HCV entry (Burgel et al., 2011; Fletcher et al., 2010). In their study, Fletcher *et al.* have shown that the two neuroepithelial cell lines were able to support HCV entry and replication but failed to detect infectious HCV assembly and release from these cells (Fletcher et al., 2010). It is worth noting that using anti-HCV entry factor antibodies, the authors demonstrated that HCV follows the same entry

process in neuroepithelial cells as what observed in hepatocytes. This report is the first one showing that HCV is able to infect brain derived cells. More recently, the same team assessed the relevance of their *in vitro* findings by studying brain biopsies of infected individuals (Fletcher et al., 2012). Out of 10 subjects tested, 4 presented HCV RNA in the brain tissue samples. The authors have shown by immunohistochemistry that human brain endothelium expresses the four main HCV entry factors. They further characterized the cells expressing the receptors and identified the microvascular endothelial cells as a potential reservoir for HCV. To assess this hypothesis, they used two independently derived microvascular endothelial cell lines to assess their permissiveness to HCV entry, replication and assembly/release. Fletcher *et al.* have demonstrated that microvascular endothelium cell lines were able to support HCV entry and replication, and they could also detect infectious HCV particle assembly and release. Furthermore, Fletcher *et al.* have shown that HCV infection of microvascular endothelial cells lead to an increase in endothelium permeability and apoptosis, which could explain the neuropathic symptoms. The most fascinating part in this study is the fact that these cells do not express miR-122, suggesting that HCV replication in microvascular endothelial cells is miR-122 independent (Fletcher et al., 2012). While this observation should be confirmed, it has to be taken into account for the development of new therapeutics targeting miR-122, as these latter will not affect HCV replication in the blood brain barrier.

- **Lymphotropism of HCV**

PBMCs have been proposed as another potential reservoir for HCV. While viral RNA has been detected in PBMCs, the role of PBMCs as potential reservoir for HCV infection has been a real matter of discussion. It is not yet clear whether these cell subtypes replicate or not HCV RNA. Viral proteins or RNA have been detected in monocytes, dendritic cells, B cells and T cells (Caussin-Schwemling et al., 2001; Fournillier et al., 2004; Goutagny et al., 2003; Laporte et al., 2003; Navas et al., 2002; Rodrigue-Gervais et al., 2007). While these studies could find evidence of PBMC infection by HCV, other studies argue that HCV infection in these cells is due to artifacts of the techniques used to detect the virus (proteins or RNA) (Boisvert et al., 2001; Marukian et al., 2008; Zehender et al., 1997). If PBMCs do replicate HCV

RNA, it is at very low levels rendering difficult the detection of such replicative activity in PBMCs. But the presence of viral RNA in these cells can also be due to their natural ability to uptake pathogens, as a study has shown that PBMCs from healthy patients became HCV RNA positive upon contact with HCVcc, but as simple HCV carriers (Fujiwara et al., 2012). This study also suggests that, upon treatment, patients who cured HCV from plasma cleared also HCV RNA from PBMCs arguing against PBMCs as a reservoir for HCV. The discrepancy between studies arguing for and against a potential HCV infection of PBMCs reflects that the techniques used to detect potential PBMC infection by HCV are not sensitive enough. Stamataki *et al.* have shown that primary and immortalized B-cells express some HCV binding/entry factors such as DC-SIGN, CD81 or SR-BI (Stamataki et al., 2009). The authors have shown that B-cells enhance HCV infection by delivering HCV particles to hepatoma cells. Interestingly, antibodies directed against SR-BI or DC-SIGN decreased B-cell mediated HCV infection, suggesting that these molecules are involved in this process (Stamataki et al., 2009).

HCV is a liver adapted pathogen and currently JFH1 is the only strain capable of replicating and producing infectious viral particles *in vitro* rendering difficult the study of non-hepatic cells infection. Stamataki *et al.* have used the JFH1 strain to realize their study. It is difficult to discriminate whether HCV could not replicate in B-cells because it has not the capacity to do so or because the strain used cannot do so. HCV is a hypervariable virus; it is conceivable that this variability allows the virus to adapt in other tissues. Recent evidence shows that B-cells derived from HCV infected patients carry HCV IRES containing mutations that differ from those found in patient sera (Durand et al., 2012). Furthermore, the mutations in B-cells-derived HCV IRES lead to a decreased HCV RNA translation activity in human hepatoma cell lines compared to HCV IRES derived from patient sera, suggesting that these IRES are derived from extra-hepatic replication sites (Durand et al., 2012). These results argue for adaptive mutations of HCV in extra-hepatic replication sites. Marukian *et al.* used HCVcc to study the ability of B- or T-cells, monocytes macrophages and dendritic cells in supporting HCV infection and replication. Since they could not make the virus replicate in such cells, they conclude that HCV was not able to infect blood cells (Marukian et al., 2008). Since HCV is a liver-adapted virus, if HCV infects at lower rates blood cells, it is likely that it acquires adaptive mutations to adapt to this

unconventional environment, so using hepatoma cell derived HCVcc is not the best model to assess HCV infection of blood cells. Thus, B-cell line has been developed to support HCV RNA replication. Indeed, Sung *et al.* isolated B-cells from a HCV infected patient suffering of a mixed-cryoglobulinemia and monocytoid B-lymphoma and isolated B-cells continuously replicating HCV RNA (Sung et al., 2003). Interestingly, this cell line, called SB cells, could also sustain HCV assembly and release since HCV particle release was continuously detected in these cells (Sung et al., 2003). The released particles were demonstrated to be infectious for primary human hepatocytes and PBMCs, so they called SB cell-derived HCVcc the SB strain (Sung et al., 2003). Further characterization of this SB strain, a lymphotropic HCV strain, showed that it can infect human primary $CD4^+$ T cells and influence their proliferation (Kondo et al., 2010). Furthermore it has been shown that HCV SB strain affects IFNγ signaling and production (Kondo et al., 2007; Kondo et al., 2010). Apart from this specific strain, no other lymphotropic strain has been isolated, suggesting that this is a particular case of sustained HCV infection of lymphoid cells. It is not clear yet if HCV can infect PBMCs in a general way, if all patients present HCV B-cell infection or if this is more patient-dependent and rare. Further studies are required to assess this unexpected alternative tissue infection and to determine how robust B-cell infection is. Should HCV infects PBMCs, it would be at very low levels that should be sensitive to standard of care treatment. Furthermore, the replication would be miR-122-independent since PBMCs do not express miR-122 (for review see (Zignego et al., 2012))

iii. Species-specificity of HCV

In addition to the hepatotropism, HCV also presents a species tropism. Indeed, as mentioned before (in the section C. ii.), few *in vivo* model systems support the full HCV life cycle highlighting the rigorous species-specificity of HCV.

The development of a convenient immunocompetent small animal model to study HCV infection *in vivo* has been slowed down by this strict species-specificity (see section C. ii. *In vivo* models). A better comprehension of the factors restricting

HCV infection to human hepatocytes should help over passing the species-specific barrier in order to develop a transgenic mouse supporting the full HCV life cycle.

The discovery of the four main HCV entry factors allowed deciphering those factors that are species-specific and uncovered the combination of HCV entry factors allowing HCV entry into mouse cells. Ploss *et al.* have assessed a large range of combination possibilities using the human and the mouse homologues of HCV entry factors and found that the minimum human entry factors required to overpass the species restriction are human CD81 (hCD81) and human OCLN (hOCLN) (Ploss et al., 2009). Ploss *et al.* have identified the ECL2 of hOCLN as being important for HCV entry since mouse OCLN (mOCLN) chimeras bearing the hOCLN ECL2 are as efficient as cells expressing hOCLN to support HCV entry (Ploss et al., 2009). Recently, Michta *et al.* have assessed a wide range of OCLN species for their ability to support HCV entry in 786-O cell line, a human cell line lacking the expression of OCLN (Michta et al., 2010). The authors have demonstrated that OCLN from non-human primates were as efficient as hOCLN to support HCVpp entry. Futhermore, while canine OCLN had reduced ability for HCV entry but is still able to support it, rodent OCLN had very reduced capability to support HCVpp entry into 786-O cells (Michta et al., 2010). By alanin scanning mutagenesis, the authors identified a region of the ECL2 domain of hOCLN that is important for HCV entry and identified the two residues G223 and G224 in mOCLN that restrict HCV entry in mouse cells (Michta et al., 2010). Mutating these two residues into human residues restored the ability of mOCLN to support HCVpp entry. More recently, Ciesek *et al.* have shown in a rescue experiment using Huh7.5 cells constitutively expressing a short hairpin targeting OCLN that mOCLN was able to support HCVcc infection but less efficiently than its human homologue (Ciesek et al., 2011). Furthermore, they have identified the same two residues, G223 and G224, in mOCLN that were previously identified by Michta *et al.* Mutating these two residues into hOCLN residues restored mOCLN efficiency to support HCV infection at similar levels compared to hOCLN (Ciesek et al., 2011). In contrast, mutating hOCLN residues into mouse residues reduced the ability of hOCLN to support HCV infection (Ciesek et al., 2011).

CD81 is also a species-specific factor. It has been previously shown that CD81 from different species were able to support HCV infection of human HepG2 CD81 expression-defective cells. The non-human CD81 mediated infection of HepG2

cells was however lower than in hCD81 expressing cells (Flint et al., 2006). In a context of non-hepatic mouse cell infection, Ploss *et al.* have shown that expression of mCD81 in combination with hOCLN, CLDN1 and SR-BI does not allow HCVpp entry into mouse cells (Ploss et al., 2009). More recently, Bitzegeio *et al.* have been able to adapt HCVcc to use mCD81. To do so, they expressed mCD81 in Lunet Huh7 cells where endogenous CD81 expression is low and transfected these cells with HCV RNA. Through several passages of the cells and reinfection of the mCD81 Huh7 Lunet cells, they have been able to force the virus to use mCD81 for infection. Sequence analysis of the mCD81-adapted virus revealed four adaptive mutations in E1, E2 and p7 of the virus. It is worth noting that the two mutations inside the E2 sequence were present in the HVR1 region of the glycoprotein (Bitzegeio et al., 2010). Interestingly, the mCD81 adaptive mutations did not affect drastically hCD81 usage by the virus. Furthermore, the fourth mutation in the p7 sequence does not seem to contribute much in mCD81 usage of HCV, since the virus carrying the 3 mutations in E1 and E2 was as efficient to infect mCD81/ Lunet Hu7 cells as the virus carrying the four mutations (Bitzegeio et al., 2010). In addition, these authors have demonstrated that the mCD81-adapted virus binds hCD81 with more efficiency and is less dependent on OCLN and SR-BI usage as assessed by neutralizing anti-receptor sensitivity (Bitzegeio et al., 2010). Interestingly, they have demonstrated that mCD81-adapted virus was able to infect non-hepatic mouse cells, engineered to express the four mouse homologues of HCV entry factors, as efficiently as in cells expressing the four human HCV entry factors (Bitzegeio et al., 2010). Together these data show that few adaptive mutations in HCV glycoproteins are sufficient to overpass the species-specific barrier and suggest that HCV could be adapted to infect mouse hepatocytes.

Ploss *et al.* have shown that the mouse homologues of hCLDN1 and hSR-BI were efficient in supporting HCV entry when co-expressed with hCD81 and hOCLN (Ploss et al., 2009). It is likely that mCLDN1 and mSR-BI do not function as well as human homologues to support HCV entry, but the differences in their activities are less drastic than the differences observed between mouse and hCD81 and hOCLN. Recently, Haid *et al.* have identified residues in mCLDN1 which limit HCV infection of a human hepatoma cell line naturally expressing low levels of hCLDN1 (Haid et al., 2010). These three mouse specific residues lie within the second ECL and in the C-terminal transmembrane domain of mCLDN1 and are responsible for the moderate

reduction in HCV entry (Haid et al., 2010). These results suggest that mCLDN1 is not as efficient as the human receptor to support HCV entry.

Mouse SR-BI, another receptor suggested by Ploss *et al.* to be as efficient as its human homolog to support HCV entry, has been shown recently to support HCV infection at half the level of hSR-BI (Catanese et al., 2010). The residues involved in this discrepancy are located within a domain close to the N-terminal transmembrane domain of SR-BI (from aa 70 to 87) and a single amino acid E210 was able to abolish soluble E2 binding to SR-BI. This results reveal that mSR-BI is not as efficient as hSR-BI to support HCV infection in opposition to what was previously observed by Ploss *et al*. More recently, Dorner *et al.*, using a recently developed mouse model for the study of single-cycle HCV infection, have shown that mSR-BI is as efficient as hSR-BI to support HCV infection (Dorner et al., 2011). The authors have crossed their mouse model with a SR-BI$^{-/-}$ mouse to reach either heterozygosity or homozygosity and found that, after expression of hCD81, hOCLN and hCLDN1 in these crossed mice through adenoviral transduction, HCV infection correlated with the amount of SR-BI expressed in the mouse. The homozygous SR-BI$^{-/-}$ mice were less susceptible to HCV infection than the heterozygous mice. But more interestingly, upon complementation of hCD81, hOCLN and hCLDN1 and either hSR-BI or mSR-BI in these SR-BI$^{-/-}$ crossed mice, they demonstrated that mSR-BI was as efficient as hSR-BI to support HCV infection *in vivo*. These discrepancies can be explained by the recent study showing the multiple function of SR-BI in HCV entry (Dao Thi et al., 2012). These results should be taken into account for the development of mouse cells supporting HCV infection.

Furthermore, recently the NPC1L1 cholesterol receptor has been shown to be important for HCV entry (Sainz et al., 2011). Its precise role in HCV entry is not yet defined and it is not yet known whether NPC1L1 binds directly HCV particles or if particle binding occurs through an indirect process. However, it has been proposed that this entry factor could remove cholesterol from the HCV associated lipoproteins thus revealing important domains for E1E2 interaction with host cell surface factors (Sainz et al., 2011). Interestingly, the authors have noticed that NPC1L1 is a human and non-human primate specific cholesterol receptor, suggesting that NPC1L1 could be a species-specific factor which restricts HCV infection to human hepatocytes, probably by impeding HCV glycoproteins to interact properly with other HCV entry

factors due to lipoprotein-mediated steric hindrance. The role of NPC1L1 in HCV entry into mouse cells requires further studies to reveal its precise role and to understand its potential involvement in HCV species-specificity.

Beyond the entry step, the species-specificity at the HCV replication step is likely to rely on differences between mouse and human factors involved in HCV replication. The key factor for HCV replication, miR-122, is unlikely to explain the inability of mouse cells to support HCV replication since the sequence between mouse and human mature miR-122 is identical and miR-122 expression in primary mouse hepatocytes has been shown to be as high as in Huh7 cells (Jopling et al., 2005). This suggests that sequence differences of other HCV replication factors are likely to explain the inability of mouse cells to support HCV infection or that mouse cells express factor(s) that restrict HCV replication. The study of HCV replication in mouse cells began with the use of sub-genomic replicons. Zhu *et al.* have been able to replicate sub-genomic replicons in a mouse hepatoma cell line, but this replication has been possible only after acquiring specific adaptive mutations (Zhu et al., 2003). Later, Uprichard *et al.* have been able to establish a sustained HCV replication using sub-genomic replicons in multiple mouse cell lines without any specific adaptive mutations (Uprichard et al., 2006). So far, no factor has been identified to explain the species-specificity of HCV replication.

So it has been hypothesized that mouse cells express factors which can restrict HCV replication. Evidence of such mechanisms have been shown previously, where mouse embryonic fibroblasts (MEF) deleted of factors of the interferon response, such as protein kinase K (PKR) (Chang et al., 2006) or IRF-3 (Lin et al., 2010), were able to replicate sub-genomic replicons, suggesting the importance of the mouse innate immune response in controlling HCV RNA replication. IRF-3 deletion was associated with miR-122 expression to allow sustained HCV RNA replication (Lin et al., 2010). Recently, immortalized mouse hepatocytes derived from interferon alfa receptor (IFNAR) and IPS-1 knock-out mice showed sustained HCV infection upon hCD81 expression, as assessed by RNA and viral protein detection (Aly et al., 2011). However, a recent study has used the heterokaryon formation technique to assess whether mouse hepatoma cell lines could express dominant negative restriction factors (Frentzen et al., 2011). The authors fused HCV replicating Huh7.5 cells with HCV structural protein-expressing mouse hepatoma cells and then

assessed HCV replication in newly formed heterokaryons. They have shown through this technique that mouse cells do not express dominant negative factors blocking HCV replication in mouse hepatocytes (Frentzen et al., 2011). But in this context, all the HCV replication factors are available for HCV replication and one cannot exclude that in a mouse hepatocytes context even recessive negative factors would be sufficient to block HCV replication. More recently, it has been reported that MEFs expressing HCV entry factors, miR-122 and HCV NS3-4A protease were able to support HCV RNA replication when type I and III interferon was impaired in these cells (Presentation from Alexander Vogt, EASL, Barcelona, April, 2012). This team has also shown that the mouse orthologs of MAVS and TRIF were efficiently cleaved in MEFs by the HCV NS3-4A protease, but this was not sufficient for sustained HCV RNA replication in these cells. These results suggest that innate immune response plays an important role in controlling HCV RNA replication in mouse hepatocytes.

Assembly and release of infectious HCV particle, the last step of the HCV lifecycle, has recently been shown to be supported by mouse hepatoma cells. Indeed, Long *et al.* have demonstrated that several mouse hepatoma cell lines do not express apoE, an important factor for the assembly and release of infectious HCV particles, while primary mouse hepatocytes showed a strong expression of apoE (Long et al., 2011). Using sub-genomic replicons, they developed mouse cells replicating HCV RNA and they have been able to assess HCV assembly and release in mouse hepatoma cells by trans-complementation of the HCV structural proteins (Long et al., 2011). Upon human or mouse apoE expression, infectious HCV particle release was detected from the HCV replicating mouse cells. By density gradient fractionation, the authors have also shown that mouse cells derived HCV particles have similar biophysical properties as human hepatoma cell-derived HCV.

Altogether, these results suggest that, to date, HCV RNA replication in mouse hepatocytes is the last step that needs to be over passed in order to reconstitute the entire HCV life cycle *in vitro*. The characterization of factors that restrict HCV RNA replication in mouse hepatocytes is probably the last step to develop an immunocompetent mouse model to study HCV infection *in vivo*.

AIM OF THE STUDY

HCV infection is a global health burden affecting more than 170 million worldwide. An estimated 10 to 25 % of the HCV infected patients will naturally clear the virus, meaning that 75 to 90% of the remaining individuals will develop a chronic hepatitis. Among those individuals, 20 to 30% will develop cirrhosis for which 1 to 4 % of the cases will develop HCC. The sole therapeutic option for HCC is liver transplantation but the liver graft will be systematically re-infected. The standard of care against HCV infection is currently based on pegylated IFN alfa (PEG-IFN-α) and ribavirin, which is not prescribed to all patients due to strong side effects, and is not efficient in all patients receiving the treatment.

Recently, two direct acting antivirals (DAAs), Boceprevir® and Telaprevir®, both targeting the HCV NS3 protease, have been approved for genotype 1-infected patients in combination with PEG-IFN-α and ribavirin and this new standard of care will definitely change the health care of HCV patients. These drugs are expensive and because they are DAAs, viral resistance has been observed, rendering their administration necessarily associated to IFN and ribavirin in a triple therapy. It is expected that among previously untreated patients, an estimated 30% will not respond to triple therapy and will fail to clear the virus. And the same percentage of treatment failure is expected to be observed for previous treated patient who are non-responders to standard of care and retreated with triple therapy (for a review, see (Pearlman, 2012)). The development of more tolerated and efficient molecules are thus warranted.

The development of such drugs has been hampered by the lack of small animal models easily handleable and supporting the entire HCV life cycle. Indeed, HCV is a rigorous hepatotropic virus and HCV infection is strictly limited to human beings and non-human primates. The characterization of the factors restricting HCV infection to human hepatocytes would definitely help in the development of a transgenic mouse supporting the entire HCV life cycle. Such a mouse will be a valuable tool in the comprehension of *in vivo* HCV infection and would speed up preclinical studies for the treatment of HCV.

In the first part of my PhD, we focused in the comprehension of the factors restricting HCV infection to hepatocytes. We aimed to use the current knowledge on HCV infection to assess the reconstitution of HCV life cycle in non-hepatic cells, by expressing key HCV factors for entry, replication and assembly/release. Through this strategy, we have been able to develop a highly HCV entry permissive cell line upon HCV entry factor expression, which supports robust HCV replication upon a single key HCV replication factor expression, and we have been able to detect HCV assembly and release in the engineered cell line after expression of a key HCV assembly and release factor.

In the second part of my PhD, since HCV has a strict species-specificity, we aimed to develop, mouse hepatoma cell lines which express the main known factors important for the HCV life cycle. Thus, we translated the previously established strategy used for human non-hepatic cells to reconstitute the HCV life cycle in mouse hepatoma cells. We expressed defined human entry, replication and assembly factors in three different mouse hepatoma cell lines and were able to overpass HCV entry and assembly restriction in these cell lines. Viral replication remains the last step within the HCV life cycle that needs to be overcome to allow robust HCV infection of mouse cells.

RESULTS

Part I:

Reconstitution of the entire HCV life cycle in non-hepatic cells

The first goal of my PhD was to develop a non-hepatic cell line supporting the entire HCV life cycle. We used the human embryonic kidney cell line HEK-293T to highlight those factors that restrict HCV infection to hepatocytes. Evans *et al.* have shown that expression of claudin-1 renders 293T cells permissive to HCVpp (Evans et al., 2007). We have shown that overexpression of CD81 and OCLN in 293T cells did not restore HCVpp permissiveness while single expression of CLDN1 rendered 293T cells permissive to HCVpp, confirming the crucial role of CLDN1 in HCV entry. Furthermore, we have shown that expression of CLDN1 with CD81 and OCLN rendered 293T cells as permissive as Huh7.5.1 and additional expression of SR-BI in this cell line (293T-4R) lead to high HCVpp permissivity of 293T cells. Next, we have shown that the 293T-4R cell line was refractory to HCV RNA replication, indicating that HCV entry is not the only step restricted in non-hepatic cells. Studies have shown that miR-122 enhances HCV replication in non-hepatic cells (Chang et al., 2008; Fukuhara et al., 2012), since we have shown that miR-122 is not expressed in 293T cells, we next expressed this miRNA in the 293T-4R cell line. Expression of miR-122 in the 293T-4R cells allowed robust HCV RNA replication and genuine HCV infection of this 293T-4R/miR122. HCV RNA replication kinetics in this engineered 293T-4R/miR122 cells was similar to those observed in Huh7.5.1 cells. Nevertheless, we could not detect infectious HCV particle release from infected 293T-4R/miR122, suggesting that assembly and release of infectious HCV particles is the last restricted step in non-hepatic cells for a productive HCV infection. Our laboratory and others have shown the primordial role of apoE in HCV assembly and release process (Benga et al., 2010; Chang et al., 2007; Jiang and Luo, 2009). We have assessed the expression of this factor in 293T cells and shown that apoE expression is lacking in the 293T-4R/miR122. When expressing apoE in the 293T-4R/miR122, we could detect infectious HCV release from 293T cells. With the assembly and release of viral particles detected in 293T cells, we demonstrate the reconstitution of the entire HCV life cycle in non-hepatic cells.

These results highlight a set of host cell factors important for the infection of hepatitis C virus in non-hepatic cells. The results of this study are presented as an original article that has been accepted for publication in Journal of Virology. Additional results from this study are presented in the “supplementary data” section following this article.

Short-Form Paper

Reconstitution of the entire hepatitis C virus life cycle in non-hepatic cells

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Abbreviations:

HCV, hepatitis C virus; HCVpp, retroviral HCV pseudo-particle; HCVcc, cell culture-derived HCV; OCLN, occludin; CLDN1, claudin-1; SR-BI, scavenger receptor class B type I; miR-122, microRNA-122; apoE, apolipoprotein E; EGFR, epidermal growth factor receptor; VLDL, very-low-density lipoprotein.

Abstract

Hepatitis C virus (HCV) is a human hepatotropic virus, yet the relevant host factors restricting HCV infection to hepatocytes are only partially understood. We demonstrate that exogenous expression of defined host factors reconstituted the entire HCV life cycle in human non-hepatic 293T cells. This study shows robust HCV entry, RNA replication, and production of infectious virus in human non-hepatic cells, and highlights key host factors required for liver tropism of HCV.

Main Text

Virus-host interactions that determine and restrict specific tissue and host tropisms display complex evolutionary history and have significant consequences on the pathogenesis of viral infection and human disease. Viral hepatitis is a major disease burden. Indeed, infection of hepatocytes by a variety of hepatotropic viruses from different orders and families can lead to tissue inflammation, fibrosis, and hepatocellular carcinoma. Hepatitis C virus (HCV), a member of the family *Flaviviridae,* is a prime example of a virus that causes chronic hepatitis worldwide. While HCV primarily infects hepatocytes of humans and chimpanzees, the virus has been shown to enter neuronal and endothelial cells of the blood-brain barrier. However, infection of these cells occurs at a low level and production of infectious viruses is greatly diminished relative to hepatically derived cells (9, 10). Unlike HCV, other members of the family *Flaviviridae* have a much broader tissue and species tropism. For example Dengue virus infects and replicates both in the midgut epithelia of Aedes aegypti mosquitoes, and in human monocytes and hepatocytes (20, 25, 39). Moreover, a virus closely related to HCV was recently identified from dogs' respiratory samples (18). A large panel of host factors required for HCV has been identified so far (36). However, the key host factors mediating liver tropism of the virus and allowing reconstitution of the viral life cycle in human cells is still only partially understood.

Taking advantage of our current knowledge of host factors involved in HCV infection, we sought to engineer a human kidney cell line (293T) to be capable of sustaining the entire HCV life cycle. The aim was to define those host factors that are necessary and sufficient for allowing the HCV life cycle, in order to understand the liver tissue-specificity of HCV.

293T cells were obtained from ATCC and their identity was verified by genomic profile comparison to the LGC Standards database by short tandem repeat profiling as described (1) (Fig. 1A). In order to render them infectable by HCV, we used lentiviral vectors to express the four principal HCV host entry factors: claudin-1 (CLDN1), CD81, occludin (OCLN), and scavenger receptor class B type I (SR-BI) (2, 7, 34, 35) by using previously described expression constructs and methods (3, 24). Four 293T stable cell lines were selected to express either CLDN1 alone, CD81/OCLN with or without CLDN1, or CLDN1/CD81/OCLN together with SR-BI (293T-4R). After verifying stable expression of these proteins using receptor-specific antibodies (Fig. 1B), we infected these cells with HCV pseudoparticles expressing the envelope glycoproteins of HCV genotype 1b (HCVpp; HCV-J strain, described in (31)). While CLDN1 expression alone conferred limited permissiveness for HCV infection as previously described (7), expression of all four factors enhances HCV entry to a level that was around four-fold higher than Huh7.5.1 cells, which is the liver-derived model hepatoma cell line for studying HCV infection (Fig. 1C).

Genuine cell culture infection of HCV (HCVcc) was then investigated in 293T-4R cells using a chimeric virus composed of two genotype 2a isolates (designated Jc1 (19, 32)) and engineered for Renilla luciferase expression (JcR2a; (38)). However, as shown in Fig. 2A, overcoming the HCV entry block was not sufficient for robust viral RNA replication in 293T cells.

Several studies have shown that microRNA (miR)-122 is a liver-specific host factor critical for HCV replication (5, 16, 17, 28). Since Northern blot analyses demonstrated non-detectable miR-122 expression in 293T-4R cells (Fig. 2C), we investigated whether exogenous miR-122 expression reconstituted viral RNA replication. Indeed, stable expression of this factor, by using miR-122 encoding

lentiviruses in the 293T-4R line, conferred the cells permissive for bona fide HCVcc infection, with replication to comparable levels as Huh7.5.1 cells as assessed by luciferase reporter activity (Fig. 2B). Further confirmation of genuine infection was garnered by observing similar infectivity ($TCID_{50}$) with HCVcc (Jc1) without a reporter gene, by detecting expression of viral protein NS5A (Fig. 2B). We verified expression of miR-122 in transduced 293T-4R/miR122 cells, and the level was comparable to that of Huh7.5.1 cells as assessed by Northern blot (Fig. 2C), and the cell proliferation rate of the different cell lines was similar (data not shown). Kinetics of HCV replication in 293T-4R/miR122 cells matched those of Huh7.5.1 cells, suggesting that aside from miR-122, cell factors present in human liver- and kidney-derived cells are equally efficient for replication as assayed by luciferase reporter gene expression (Fig. 2D). Expression of viral proteins in infected cells was further confirmed using HCV core-specific immunofluorescence (Fig. 2E) and flow cytometry (data not shown).

To further confirm whether viral entry and replication in stably transduced 293T cells is mediated by the same host and virus factors as in human Huh7.5.1 hepatoma cells, we used well-characterized entry and replication inhibitors. Antibodies directed against the HCV entry factors CD81, CLDN1, and SR-BI (JS-81, BD Biosciences, (11), Zahid et al., unpublished, respectively) were effective in inhibiting infection (Fig. 2F). Moreover, both a polyclonal serum recognizing apolipoprotein E (apoE) (29), and a monoclonal antibody recognizing the LDL receptor binding domain of apoE (37) effectively neutralized HCV infection of 293T-4R/miR122 cells (Fig. 2F). The same was true for the recently identified HCV entry inhibitor, erlotinib, which targets the kinase activity of the host entry regulatory protein, epidermal growth factor receptor (EGFR) (Fig. 2F) (24). Likewise, well

characterized inhibitors of HCV NS3 protease or polymerase, telaprevir (VX950) and mericitabine (R7128), impaired HCV replication in 293T-4R/miR122 cells (Fig. 2F). These data demonstrate that HCVcc RNA replication in kidney-derived 293T-4R/miR122 cells is efficient, and dependent on similar mechanisms as in liver-derived Huh7.5.1 cells.

Despite efficient entry and RNA replication of 293T-4R/miR122 cells infected with recombinant HCVcc, these cells did not release infectious virions, suggesting that kidney-derived cells lack factors required for viral assembly and release. Therefore, we aimed to reconstitute virus production by expression of HCV assembly factors. HCV production shares factors involved in very-low-density lipoprotein (VLDL) assembly, a process that occurs exclusively in hepatocytes (13, 14, 27). While the necessity of apolipoprotein B (apoB) in HCV production is controversial (15), apoE is known to be critical, and is incorporated into the virion (26). We therefore expressed the most common isoform of apoE (apoE3) in 293T-4R/miR122 cells by using a lentiviral vector encoding human apoE3 as described previously (23), and confirmed its expression by flow cytometry using an apoE-specific antibody (Fig. 3A). We then infected 293T-4R/miR122/apoE cells. Subsequently, the production and release of viral particles was assessed by incubating naïve Huh7.5.1 cells with the supernatants from these cells. Indeed, 293T-4R/miR122/apoE released infectious HCV particles as shown by a marked and highly significant increase in infectivity (as assessed by luciferase activity of JcR2a virus and $TCID_{50}$ of Jc1 virus without a reporter gene) of the supernatant compared to the supernatant of 293T-4R/miR122 cells without apoE expression (Fig. 3B). Although the production of infectious particles was lower than in Huh7.5.1 cells studied in side-by-side experiments, these data indicate that apoE is a key factor for virus production in reconstituting the viral

life cycle in non-hepatic cells. This diminished HCV production was not due to diminished replication levels as apoE transduced cells had similar HCV replication levels to 293T-4R/miR122 cells prior to apoE expression (data not shown). To test if HCV produced by these cells is reliant only on human apoE3 isoform or could use other forms of apoE, we similarly transduced human apoE2 and apoE4 isoforms, as well as murine apoE (Fig. 3C). Viruses produced from 293T cells expressing these apoE isoforms and the mouse ortholog had similar infectivity compared to human apoE3 isoform (Fig. 3D).

Focusing on the most common apoE isoform (apoE3), we further characterized the kinetics and attributes of these viruses. First, we confirmed that HCV particles from engineered 293T cells could establish infection by monitoring the increase in HCV genomes over time in Huh7.5.1 target cells after exposure to the supernatant of HCVcc-infected 293T-4R/miR122/apoE cells (Fig. 4A). Next, we characterized the kinetics of HCV RNA production from infected 293T-4R/miR122/apoE cells by measuring HCV RNA in the media at serial time points following infection (Fig. 4B). Interestingly, the levels of HCV RNA released into the culture media of 293T-4R/miR122/apoE cells was similar to levels of HCV RNA in the media of Huh7.5.1 cells after 72h, whereas cells that were not transduced with apoE released minimal amounts of HCV RNA, likely due to previously reported non-specific release of HCV RNA during replication (Fig. 4B)(33). These data suggest that the specific infectivity differs between virus produced from Huh7.5.1 cells and 293T cells engineered to express essential host factors. An estimation of the specific infectivity of the released viruses ($TCID_{50}$/HCV RNA genomes) revealed approximately a 30-fold difference between the differently derived viruses (1/900 for Huh7.5.1-derived virus and 1/26,000 for 293T-4R/miR122/apoE-derived virus). It should be noted that

HCV particles produced from 293T-4R/miR122/apoE cells proved to have a similar route of infection to hepatically-derived HCVcc, in that entry into Huh7.5.1 cells was neutralized by well-characterized HCV entry inhibitors including CD81-, SR-BI-, CLDN1-, apoE-specific antibodies, and erlotinib (Fig. 4C). Fractionating the virus by iodixanol density gradients revealed that the infectious virions produced from 293T-4R/miR122/apoE cells have similar buoyant density as those from Huh7.5.1 cells (Fig. 4D).

The data presented here demonstrate that trans-expression of OCLN, CD81, CLDN1, SR-BI, miR-122, and apoE endow 293T human kidney-derived cells with the capacity to support the complete HCV life cycle. Expression of four principal entry factors and miR-122 generated cells with higher entry and similar replication kinetics as the extensively optimized Huh7.5.1 cells (4, 41). It should be noted in this context, that the recently identified entry factor EGFR is also expressed in 293T cells (data not shown, 24, 40). We confirmed that expression of CLDN1 alone appears to be sufficient for infection of 293T cells (7), and expand these findings in that high-level expression of the four canonical HCV entry factors make previously impenetrable cells four-fold more permissive than Huh7.5.1 cells. These observations were confirmed by HCVcc infection of 293T cells engineered to express miR-122 in addition to variable sets of entry factors (data not shown). While the present study focused on engineering a human cell line for infection, it has been demonstrated that concomitant high level expression of the four human entry factors is required for robust entry of mouse hepatocytes *in vivo* (6). Since none of the identified entry factors are exclusively expressed in the liver, it is likely that the combined expression of these host factors at substantial levels allows the virus to productively infect the human liver, rather than a single liver-specific entry factor restricting HCV infection.

Investigators have shown that miR-122 expression increases HCV replication in mouse embryonic fibroblasts and other hepatoma cell lines such as HepG2 cells (21, 17, 28). Furthermore, HEK-293 cells modified to express miR-122 are capable of sustaining selectable HCV subgenomic replicons, although expression of mutated miR-122, at sites required for HCV RNA binding, can also sustain these replicons (5). We demonstrate here *de novo* replication following an infection event of a non-hepatic cell line engineered to express HCV host factors. Our data also demonstrate that there is no restrictive factor of HCV entry and viral RNA replication that is present in 293T cells. HCV entry and replication in human blood brain barrier endothelial and neuronal cells have been described (9, 10). In contrast to the kidney-derived cells described here, HCV replication in blood brain barrier endothelial cells occurred via a miR-122 independent mechanism, yet at a diminished level (9). Thus, the cell lines developed in this study may be useful as a tool to further understand the molecular mechanisms of extra-hepatic infection.

The production of HCV from 293T-4R/miR122/apoE cells was diminished relative to Huh7.5.1 cells, but markedly and significantly higher than in cells without apoE expression. This demonstrates that apart from apoE, all the other factors necessary for the production of infectious particles are present in 293T cells, yet additional host factors may increase efficient production levels. The cell line generated in this study is likely to allow further discovery of the minimal set of host factors required for robust viral production. Additional relevant factors enhancing viral production may be apoB (27), DGAT1 (13), or microsomal triglyceride transfer protein (MTP) (12, 14). Notably, apoE has recently been demonstrated to be essential for virus production; apoE-deficient mouse hepatocytes with trans-expression of HCV

RNA and proteins along with apoE are able to produce high levels of infectious virions (23).

In summary, this study demonstrates that a small set of defined host factors is sufficient to reconstitute the complete viral life cycle in non-hepatic cells. These results advance our knowledge on tissue-specific factors for HCV infection and provide novel tools to elucidate host and restriction factors for the HCV life cycle.

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Figure Legends

Figure 1. Expression of four HCV entry factors renders 293T cells highly permissive to HCVpp entry. (A) Short tandem repeat (STR) profile of the 293T cells used in this study (Cell line authentication, LGC Standards) was performed as described previously (1). The names of tested loci are indicated in bold and peak positions from STR profile of 293T cells were compared to LGC Standards database. (B) 293T cells (cultured in DMEM high glucose, Life Tech) were transduced with lentiviruses (as described in (3)) to express given HCV entry factors. After transduction, cells were selected with blasticidin (12 µg/ml) for 2 weeks. Blasticidin-resistant cells were assessed by flow cytometry using monoclonal antibodies (CLDN1 (11), OCLN (Cat.# 33-1500 Invitrogen), SR-BI (Zahid et al., submitted manuscript)) recognizing indicated entry factors. Entry factor transduced cells (dark grey histograms) were compared to naïve 293T cells (light grey histograms) and isotype control antibody (Cat.# 10400C, Life Technologies, white dashed histograms). X axis: fluorescence intensity, Y axis: number of events. (C) Transduced 293T cells were assessed for HCVpp (genotype 1b; HCV-J strain; produced as described in (31)) entry by determining luciferase activity 72h post-infection as previously described (35). Results were first normalized to vesicular stomatitis virus pseudoparticle entry (VSV-Gpp; produced as described in (8)), and then compared to Huh7.5.1 cells (cultured as described in (41)). Results are expressed as means +/- SD of percentage HCVpp entry relative to entry into Huh7.5.1 cells from three independent experiments performed in triplicate, and 100% relative infectivity is represented by a solid line. Statistical analysis for entry factor expressing cells relative to naïve 293T cells was performed using the Student's *t* test, *P<0.05.

Figure 2. 293T-4R cells support robust HCV infection upon miR-122 expression. (A) Stable 293T-4R cells described in Fig. 1 were challenged with HCVcc (JcR2a; produced as described in (38)) or were mock infected and luciferase activity was assessed 72h post-infection as described previously (38). Results are expressed as means +/- SD of relative light units (RLU) from three independent experiments performed in triplicate. (B) 293T-4R cells were stably transduced using miR-122 encoding lentiviruses (Cat.# mh15049, ABM Good) and puromycin (2,5 µg/ml) resistant cells were selected over 2 weeks. 293T-4R/miR122 cells and Huh7.5.1 cells were then infected with HCVcc or mock infected for 6h. Infection was assayed by monitoring luciferase activity 72h post-infection. Results are expressed as means +/- SD of relative light units (RLU) from three independent experiments performed in triplicate. Jc1, an HCVcc without a luciferase reporter (32) was likewise used to infect Huh7.5.1 and 293T-4R/miR122 cells and its infectivity was assessed by limiting-dilution assay ($TCID_{50}$) by detecting viral protein NS5A using immunohistochemistry, represented as grey bars (22). Results are expressed as means +/- SD of $TCID_{50}$/ml from three independent experiments. (C) Northern blots of miR-122 and miR-16, and U6 RNA as a loading control, extracted from 293T-4R, 293T-4R stably expressing miR-122, and Huh7.5.1 cells as positive control. Northern blots using a miR-122-specific probe were performed as described previously (30). Oligonucleotide lengths (nt) are indicated on the left of each blot. (D) 293T-4R, 293T-4R/miR122 and Huh7.5.1 cells were incubated side-by-side with HCVcc (JcR2a) and luciferase activity was monitored every 24h over a 72 h period. Results are expressed as means +/- SD of relative light units (RLU) of three independent experiments performed in triplicate. (E) Huh7.5.1, 293T-4R, and 293T-4R/miR122 cells were infected for 72 h and HCV core protein (core antibody C7-50, Thermo Scientific,), or

non-specific IgG, as a control (Cat.# 10400C, Life Technologies) were observed by immunofluorescence; nuclei were stained using DAPI. (F) 293T-4R/miR122 cells were pre-incubated for 1h at 37°C with the indicated entry inhibitors, antivirals or controls (monoclonal antibodies (mAb), anti-CD81 (JS81, BD Biosciences), anti-CLDN1 (11), anti-SR-BI (Zahid et al. submitted manuscript), polyclonal (pAb) anti-apoE (Cat #178479, Calbiochem), anti-apoE mAb was described in (37), 20 µg/ml, erlotinib: 10 µM (Cat.# E-4997, LC Laboratories), protease inhibitor telaprevir VX950: 1 µM; polymerase inhibitor mericitabine R7128: 1 µM; both synthesized by Acme Bioscience Inc. , DMSO: 0.7%, and then infected with HCVcc (JcR2a) in the presence of given entry inhibitors or antivirals. Cell lysates were assessed for luciferase activity 72h post-infection. Results are expressed as means +/- SEM of percentage HCVcc infection compared to controls, from three independent experiments performed in triplicate, and 100% relative infectivity is represented by a solid line. In panels A, B, and D, detection limits are represented by dashed lines. Statistical analysis relative to control was performed using the Student's *t* test, *P<0.05.

Figure 3. Infectious HCV particles are released from 293T-4R/miR122 cells upon apoE expression. (A) 293T-4R/miR122 cells were transduced with an apoE3 encoding lentiviral vector described in (23). 72h post-transduction, cells that were or were not transduced were stained for flow cytometry analysis. ApoE expression was analyzed using a specific apoE antibody (clone D6E10, Cat.# ab1906, Abcam, untransduced cells are represented as light grey histogram and transduced cells are shown as dark grey histogram) and an isotype antibody (Cat.# 10400C, Life Technologies) was used as control (white dashed histograms). Huh7.5.1 cells were

used for control of apoE expression and PBS is presented as control of the isotype antibody (thick black histogram). (B) Transduced 293T-4R/miR122/apoE cells were infected with HCVcc (JcR2a, or Jc1). 6h post-infection, cells were washed three times with PBS, and fresh culture medium was added. 72h post-infection, media from infected cells was passaged onto naïve Huh7.5.1 cells. Cell lysates of JcR2a infected cells were assessed for luciferase activity 72h post-infection. Results are expressed as means +/- SD of relative light units (RLU) of three independent experiments performed in triplicate. The detection limit is represented by a dashed line. The infectivities of Jc1 derived from Huh7.5.1 or 293T-4R/miR122/apoE infected cells were assessed by limiting-dilution assay ($TCID_{50}$) by detecting NS5A by immunohistochemistry, represented as grey bars. Results are expressed as means +/- SD of $TCID_{50}$/ml from three independent experiments. # represents below detectable levels. Statistical analysis relative to the control was performed using the Student's *t* test, *P<0.05. (C) 293T-4R/miR122 cells were transduced with indicated apoE isoform-encoding lentiviral vectors (24), or mock transduced (Control). 72h post-transduction, cells were either lysed or seeded for HCVcc infection. Cell lysates were assessed for apoE expression by Western blot either by using apoE antibody (clone D6E10, Cat.# ab1906, Abcam) for human apoE (h-apoE) expression or using a mouse apoE specific antibody for mouse apoE (m-apoE) expression (Cat# ab20874, Abcam). Huh7.5.1 and primary mouse hepatocytes (PMH) were used as controls for human and mouse apoE expression, respectively. (D) The different apoE isoform-expressing 293T-derived cells were assessed for their capacity to produce infectious virus by infecting them with HCVcc (JcR2a) and 72h post-infection, supernatants of infected 293T-derived cells were passaged onto naïve Huh7.5.1 cells. 72h after initiating this infection, Huh7.5.1 cells were lysed and luciferase

activity assessed. Results are expressed as means +/- SD of relative light units (RLU) from a representative experiment performed in triplicate. The dashed line represents the detection limit.

Figure 4. Characterization of HCVcc derived from 293T-4R/miR122/apoE cells (A) Culture media from Jc1-infected 293T-4R/miR122, 293T-4R/miR122/apoE, and Huh7.5.1 cells were passaged onto naïve Huh7.5.1 target cells. Total RNA from these Huh7.5.1 target cells was extracted at indicated time points and HCV RNA was quantitated by RT-qPCR as described (11). Values were normalized to the internal control gene GAPDH and are represented as HCV RNA to GAPDH RNA ratio. Results are expressed as means +/- SD from an experiment performed in quadruplicate. (B) HCV RNA production was measured by infecting 293T-4R/miR122, 293T-4R/miR122/apoE and Huh7.5.1 cells side-by-side with HCVcc (Jc1). RNA from supernatants of infected cells was extracted at indicated time points and HCV RNA quantitated by RT-qPCR. Results are expressed as means +/- SD of copies/ml from an experiment performed in triplicate. (C) Culture media of infected 293T-4R/miR122/apoE cells were harvested 72h post-infection and passaged onto naïve Huh7.5.1 cells that were pre-incubated with either control IgG, DMSO, or with indicated entry inhibitors. Results represent mean percentages of HCV infection (as assessed by luciferase activity) relative to control +/- SD from a representative of two independent experiments performed in triplicate, and 100% relative infectivity is represented by a solid line. Virus used was JcR2a with a $TCID_{50}$ of 10^5 to 10^6/ml. (D) Density distributions of infectious 293T-4R/miR122/apoE- and Huh7.5.1-derived HCVcc (Jc1) were determined by overlaying 0.5 ml of culture media on a 5 ml, 4-40% iodixanol step gradient, and ultracentrifuging samples for 16h at 40,000 rpm on a

SW-55 rotor (Beckman Coulter). Fractions were carefully harvested from the top of each tube, and density was determined by weighing 0.5 ml of each fraction. Each fraction was assayed for infectivity by $TCID_{50}$ by detecting NS5A as described (22).

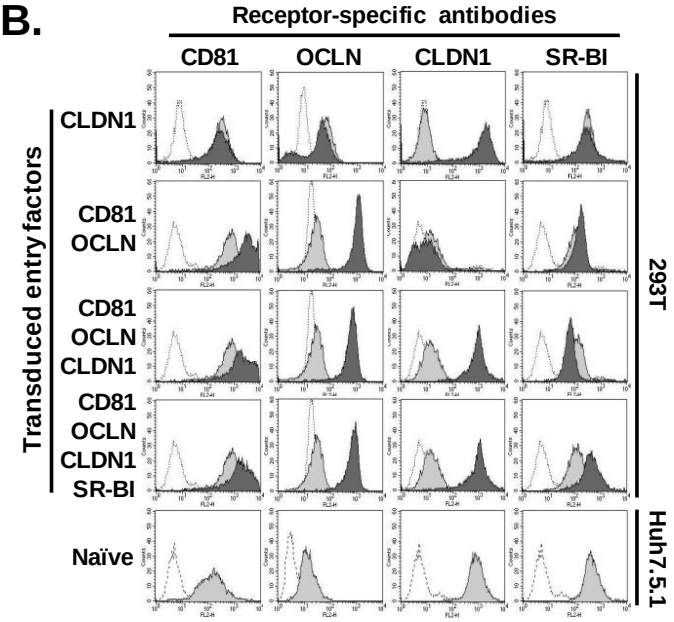
Figure 1

A.

Loci Tested	AMELO	THO1	D5	D13	D7
ATCC Reference: CRL-1573 (HEK293)	*X, X*	*7, 9.3*	*8, 9*	*12, 14*	*11, 12*
293T cells	X, X	9.3, 9.3	8, 9	12, 13, 14	11, 12

Loci Tested	D16	CSF	VWA	TPOX
ATCC Reference: CRL-1573 (HEK293)	*9, 13*	*11, 12*	*16, 19*	*11, 11*
293T cells	9, 13	11, 12	16, 19	11, 11

B.



C.

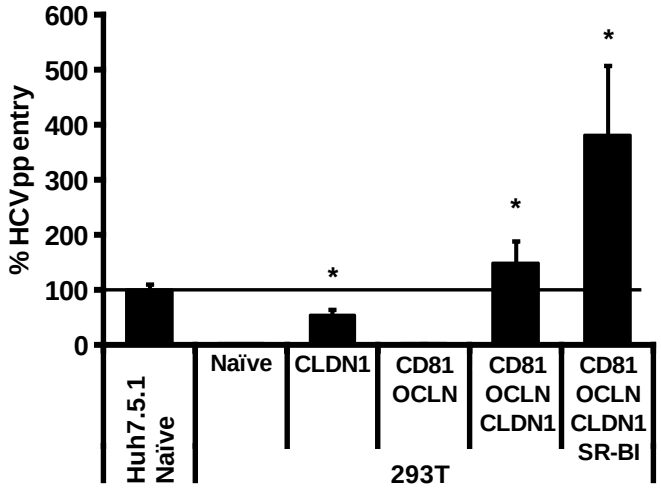


Figure 2

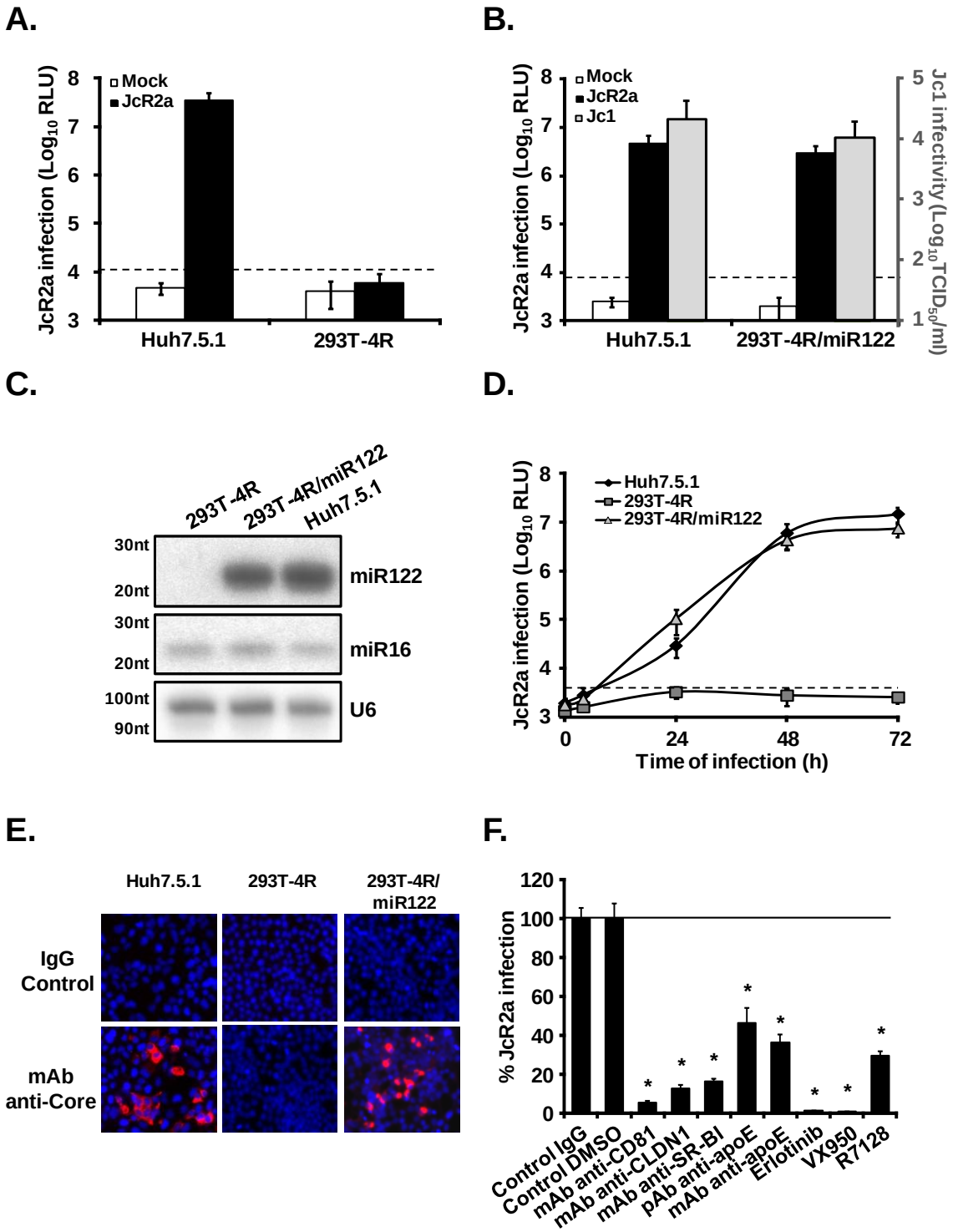

A.
Mock
JcR2a
JcR2a infection (Log10 RLU)
Huh7.5.1
293T-4R
B.
Mock
JcR2a
Jc1
JcR2a infection (Log10 RLU)
Jc1 infectivity (Log10 TCID50/ml)
Huh7.5.1
293T-4R/miR122
C.
293T-4R
293T-4R/miR122
Huh7.5.1
30nt
20nt
miR122
30nt
20nt
miR16
100nt
90nt
U6
D.
Huh7.5.1
293T-4R
293T-4R/miR122
JcR2a infection (Log10 RLU)
Time of infection (h)
E.
Huh7.5.1
293T-4R
293T-4R/ miR122
IgG Control
mAb anti-Core
F.
% JcR2a infection
Control IgG
Control DMSO
mAb anti-CD81
mAb anti-CLDN1
mAb anti-SR-BI
pAb anti-apoE
mAb anti-apoE
Erlotinib
VX950
R7128

Figure 3

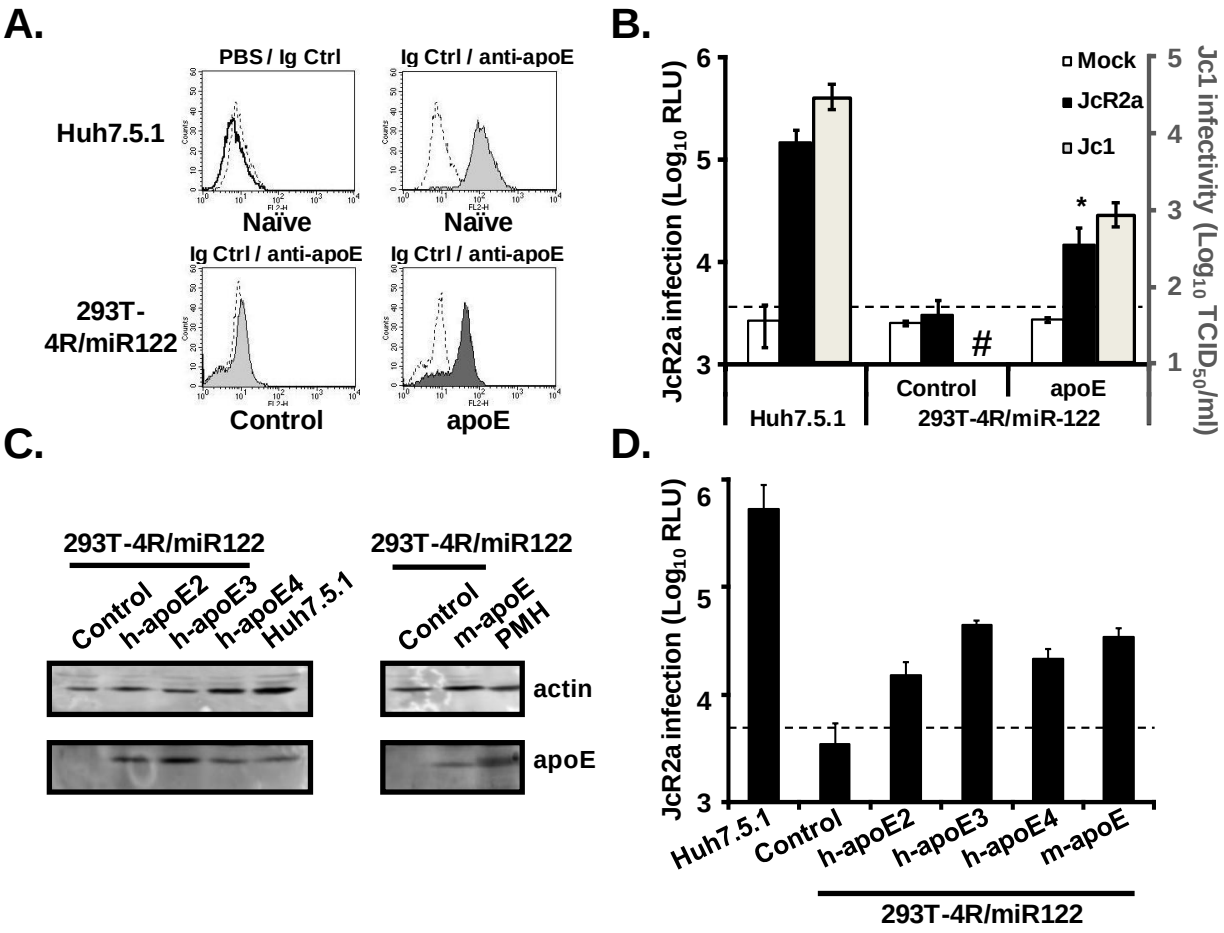
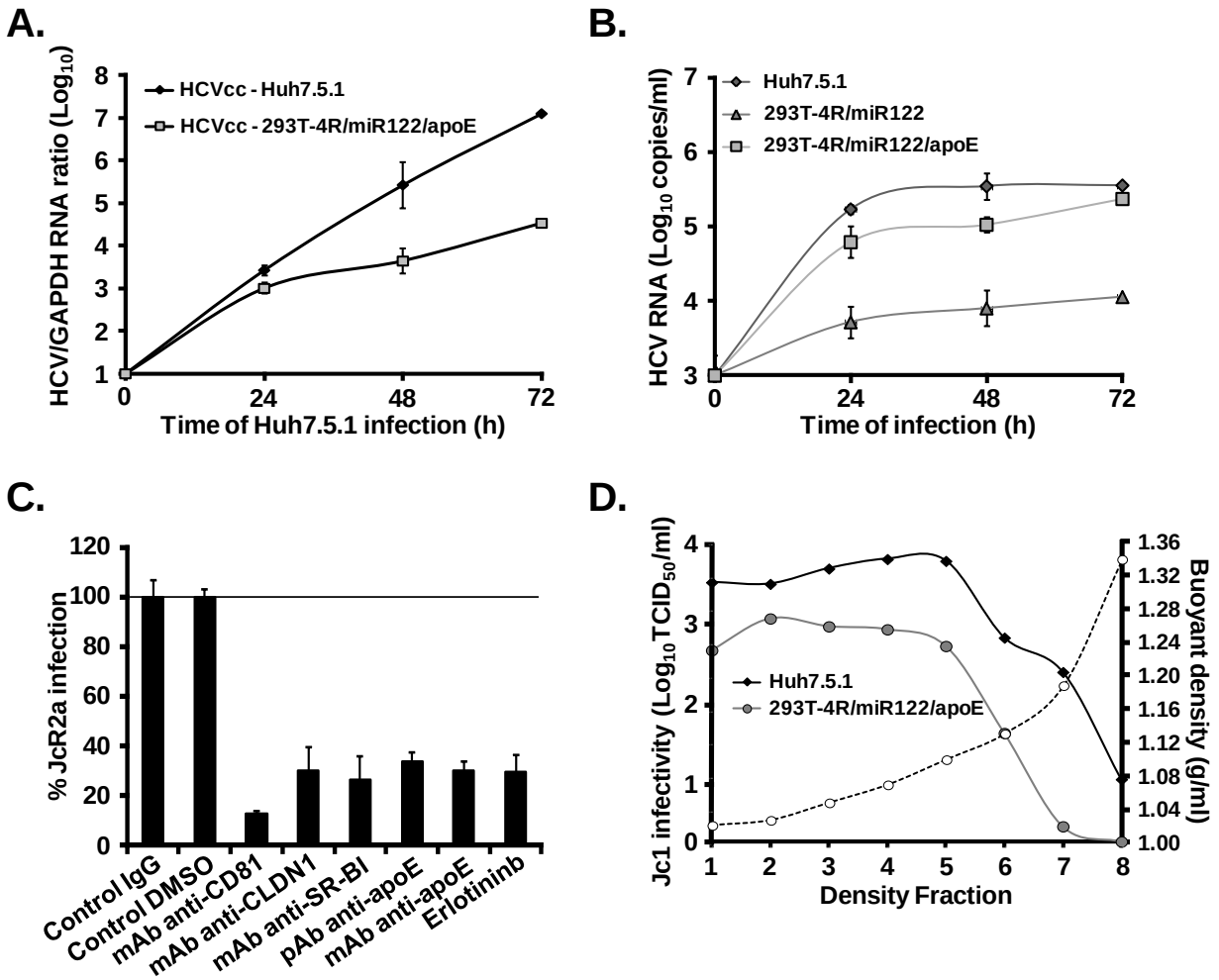

A.
PBS / Ig Ctrl
Ig Ctrl / anti-apoE
Huh7.5.1
Naïve
Naïve
293T-4R/miR122
Ig Ctrl / anti-apoE
Ig Ctrl / anti-apoE
Control
apoE
B.
Mock
JcR2a
Jc1
JcR2a infection (Log10 RLU)
Jc1 infectivity (Log10 TCID50/ml)
Huh7.5.1
Control
apoE
293T-4R/miR-122
C.
293T-4R/miR122
293T-4R/miR122
Control
h-apoE2
h-apoE3
h-apoE4
Huh7.5.1
Control
m-apoE
PMH
actin
apoE
D.
JcR2a infection (Log10 RLU)
Huh7.5.1
Control
h-apoE2
h-apoE3
h-apoE4
m-apoE
293T-4R/miR122

Figure 4


A.
HCV/GAPDH RNA ratio (Log10)
HCVcc - Huh7.5.1
HCVcc - 293T-4R/miR122/apoE
Time of Huh7.5.1 infection (h)
B.
HCV RNA (Log10 copies/ml)
Huh7.5.1
293T-4R/miR122
293T-4R/miR122/apoE
Time of infection (h)
C.
% JcR2a infection
Control IgG
Control DMSO
mAb anti-CD81
mAb anti-CLDN1
mAb anti-SR-BI
pAb anti-apoE
mAb anti-apoE
Erlotininb
D.
Jc1 infectivity (Log10 TCID50/ml)
Buoyant density (g/ml)
Huh7.5.1
293T-4R/miR122/apoE
Density Fraction

Supplementary data

1) EGFR expression does not explain the high permissiveness of the 293T cells to HCVpp

The 293T cells have been engineered to express the indicated entry factors using lentiviral vectors **(Figure 1B of the manuscript)**. During the selection process, antibiotic-resistant cell clones expressing the highest level of entry factors have been selected. Our laboratory has demonstrated that EGFR plays an important role in the regulation of HCV entry into primary hepatocytes and human hepatoma cells. It has been shown that EGFR regulates the formation of the complex CD81-CLDN1 which is important for HCV entry. Furthermore, expression of EGFR in mouse cells engineered to express the four human entry factors renders these cells drastically more permissive to HCVpp entry (Lupberger et al., 2011). EGFR is known to regulate cell growth and has been implicated in several cancers. To preclude the possibility that we have selected a single cell clone overexpressing EGFR and contributing to the high permissiveness of the 293T-4R cells, we assessed the expression of this entry factor in the developed 293T cell lines by flow cytometry. EGFR expression into engineered 293T cells was compared to naïve 293T cells.

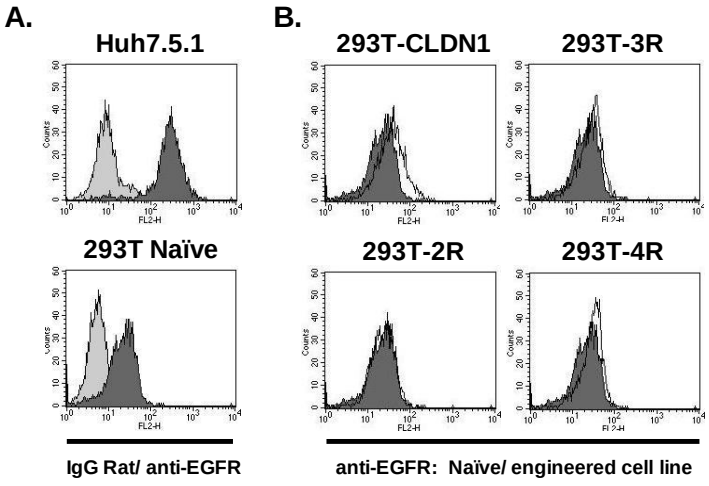


Figure 8: EGFR is not overexpressed during the selection process. ***A.*** *EGFR expression in naïve cells. Naïve 293T and Huh7.5.1 cells were stained with a monoclonal anti-EGFR antibody (dark grey histograms) or with a rat isotype control antibody (light grey histograms). B. EGFR expression in engineered 293T cell lines. Indicated engineered 293T cells were stained with a monoclonal anti-EGFR antibody (white histograms) and expression compared to naïve 293T cells (dark grey histograms).*

Parental 293T cells do have a basal expression of EGFR which is lower than EGFR expression in Huh7.5.1 cells **(Figure 8A)**. Engineered 293T cell lines did not show overexpression of EGFR compared to naïve cells **(Figure 8B)**. These data demonstrate that the high HCVpp permissivity of the 293T-4R cells is due to the overexpression of the HCV entry factors and cannot be an artifact of EGFR overexpression in the engineered 293T cells which could have occurred during the selection process.

2) Expression of miR-122 in engineered 293T cells allows HCV replication in HCVpp permissive cells.

HCV infection has been shown to rely on several host cell factors. We have shown in this study that overexpression of the HCV entry factors in the non-hepatic cell line 293T allows rendering these cells highly permissive to HCV entry. We wondered whether, in the context of non-hepatic cells, HCV entry was a limiting step for HCV infection. We took advantage of the cell lines developed in this study to express miR-122 in the 293T cells engineered to express different combinations of HCV entry factors. Using these cells, we assessed whether we could confirm the results obtained with HCVpp in a context of a true HCV infection. HCV entry factor-expressing cells were transduced with lentiviral vectors encoding miR-122 and puromycin-resistant gene. Puromycin-resistant cells were plated for HCVcc infection using a *Gaussia* luciferase reporter virus (Marukian et al., 2008). We could correlate the data obtained in the HCV entry assay using HCVpp **(Figure 1C of the manuscript)** with HCV infection assay using HCVcc **(Figure 9)**. Indeed, 293T-2R cells that were resistant to HCV entry due to a lack of CLDN1 expression did not show luciferase activity when expressing miR-122 and infected with HCVcc. 293T-3R/miR122 were able to support HCV replication but to a lesser extent than 293T-4R/miR122 **(Figure 9)**. These data suggest that HCV entry is a limiting step for HCV infection in non-hepatic cells. Thus, efficient HCV entry in non-hepatic cells is important to allow a robust HCV infection.

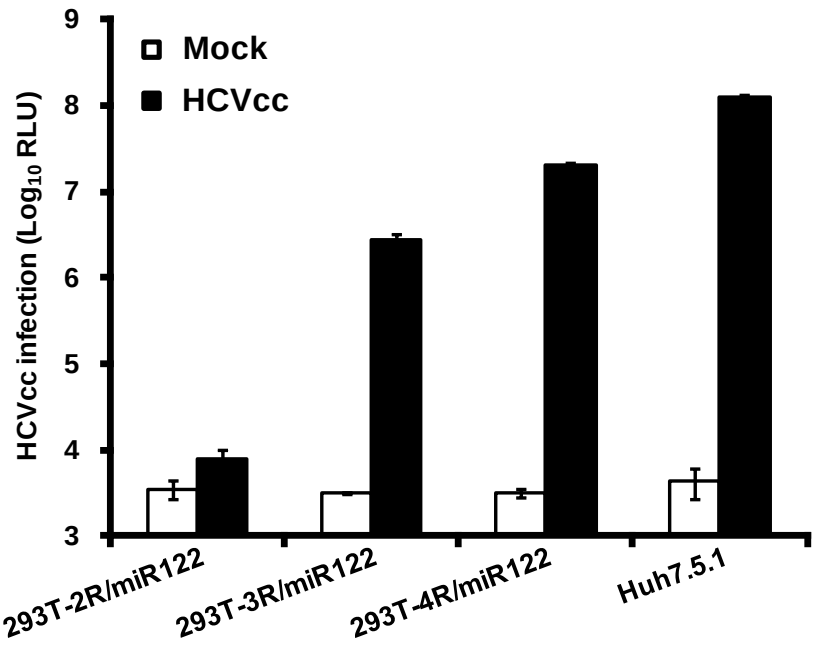


Figure 9: 293T cells engineered to express different sets of HCV entry factors and miR-122 are permissive for HCVcc entry and replication. ***293T cells engineered to express HCV entry factors (2R:CD81,OCLN, 3R:CD81,OCLN,CLDN1, 4R:CD81,OCLN,CLDN1,SR-BI) were transduced with lentiviral vectors encoding miR-122 and under selection for two weeks. Selected cells were then plated and either mock (empty bars) or HCVcc carrying a Gaussia luciferase reporter gene (Marukian et al., 2008)(black bars) infected. Luciferase activity was assessed 72h post-infection. Results are from one representative experiment performed in triplicate and error bars represent standard deviation.***

3) Viral antigen detection in 293T-4R/miR122cells

The analysis of the reconstitution of the HCV life cycle in the non-hepatic cell line 293T has been mainly performed using the cell-culture derived HCVcc (JcR2a), which contains a *Renilla* luciferase reporter embedded between HCV core protein and the glycoprotein E1 (Reiss et al., 2011). To ascertain that the monitored luciferase activity is associated with sustained viral protein expression, we assessed the expression of HCV core protein in 293T-4R, 293T-4R/miR122 and Huh7.5.1 cells over a period of 72h every 24h using flow cytometry. We could correlate the luciferase activity obtained in the assessment of HCV replication in the 293T-4R/miR122 **(Figure 2D of the manuscript)** with the expression of HCV core protein **(Figure 10)**. It is worth noting that the expression of core protein followed the same kinetics as observed for the luciferase activity. Indeed at 24h post-infection, 293T-4R/miR122 cells show a stronger luciferase activity **(Figure 2D of the manuscript)** and higher number of core-positive cells **(Figure 10)** compared to Huh7.5.1 cells.

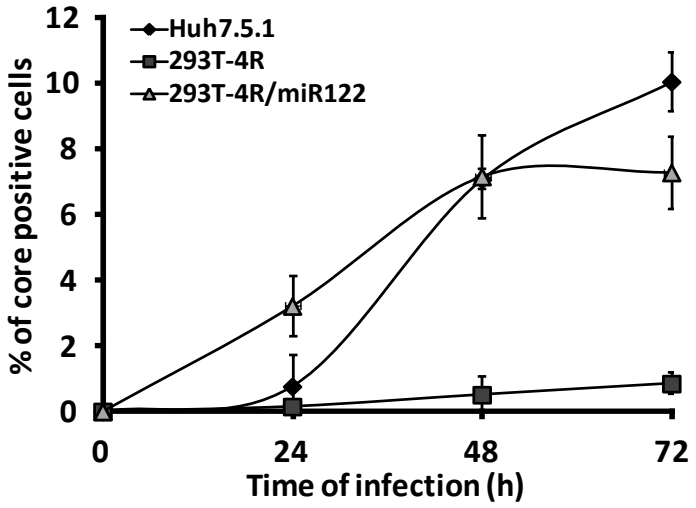


Figure 10: HCV replication in 293T-4R/miR122 is associated with sustained viral protein expression. *293T-4R, 293T-4R/miR122 and Huh7.5.1 cells were infected side-by-side with HCVcc JcR2a. Every 24h and during a period of 72h, cells were trypsinized, washed and fixed with paraformaldehyde. Core protein expression was assessed in fixed cells using a monoclonal anti-core protein. Results are expressed as means of percentage core protein positive cells from two independent experiments performed in triplicates. Error bars represent standard deviation.*

At 48h post-infection, the two cell lines have similar luciferase values and number of core protein positive cells. At 72h, Huh7.5.1 cells show a stronger luciferase activity and number of core protein positive cells compared to 293T-4R/miR122. Together these data demonstrate the robustness of the HCV replication in 293T-4R/miR122 assessed both by a reporter gene (luciferase) and by detecting directly viral antigen (core protein).

4) Intracellular amounts of miR-122 do not influence HCV replication in 293T-4R cells

We have shown that 293T-4R/miR122 cells support a robust HCV infection. We wondered whether modulating the amount of intracellular miR-122 would influence HCV replication in the highly HCV entry permissive 293T-4R cells. To answer this question, we transfected increasing amounts of a microRNA-122 mimic (transfected amount vary between 20 to 40 pmol) into the 293T-4R cells and infected these cells with HCVcc (JcR2a). Interestingly, the amount of intracellular miR-122 did not influence HCV RNA replication **(Figure 11)**. These results suggest that the effect of

miR-122 on HCV replication is independent on the amount of intracellular miR-122 available. These results are in line with previously published data on HepG2 cells (Narbus et al., 2012). Authors have shown that even low amount of miR-122 in HepG2 cells allow sustained HCV replication in these cells (Narbus et al., 2012).

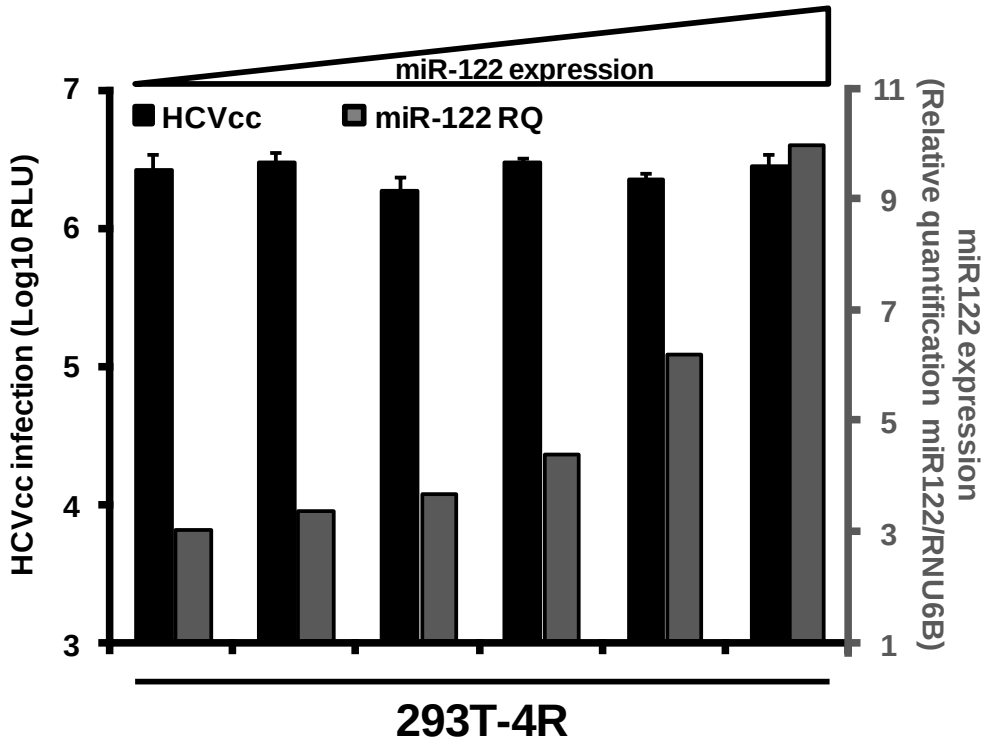


Figure 11: The amount of intracellular miR-122 does not influence HCV replication in 293T-4R cells. *293T-4R cells were transfected, using Lipofectamin 2000®, with increasing amounts of a microRNA-122 mimic. 24h post-transfection, 293T-4R cells were washed and infected with HCVcc (JcR2a). 72h post-infection, luciferase activity was assessed and total RNA extracted for determining relative quantification of intracellular miR-122.*

Part II

Investigation of factors responsible for the HCV species specificity

The second goal of my PhD was to develop mouse cellular models expressing previously highlighted factors for the study of HCV infection in mouse hepatoma cells. Ploss *et al.* have shown that expression of the human factors CD81 and OCLN is the minimum to render mouse cells permissive to HCVpp (Ploss et al., 2009). During this study, I have used three different mouse cell lines, AML12, BNL-1 and Hepa1.6 to study the impact of the expression of different sets of receptors on HCV entry. I have shown that co-expression of the human factors CD81 and OCLN do not allow a robust HCVpp entry in any of the three studied mouse cell line. Moreover, I have also shown that expression of the three human factors CD81, OCLN and CLDN1 allows rendering mouse hepatoma cells permissive to HCVpp, fact which is not observed when CD81 and OCLN are co-expressed with SR-BI. Furthermore, the expression of SR-BI in the cells expressing the three other main HCV entry factors increases the permissivity of the mouse cells, but HCV entry remains relatively limited compared to Huh7.5.1 cells. We have shown that these permissive mouse cells do not allow HCV RNA replication when these cells are infected with HCVcc. The translation analyses of HCV RNA in mouse cells have shown that it was properly recognized and translated in the developed mouse cells, thus excluding any species specific restriction at this step of the viral life cycle. As Lang et al. have recently shown that mouse apoE is able to support HCV assembly; these results suggest that viral RNA replication is most likely the limiting step of the HCV life cycle in HCVpp entry permissive mouse cells.

1) HCV entry factor expression in mouse hepatoma cell lines

Mouse cells are naturally resistant to HCV infection. The first restricted step in mouse cells is HCV entry. The identification of host cell factors involved in the HCV entry process allowed identifying those factors that are necessary to render mouse cells permissive to HCV entry. Ploss *et al.* identified OCLN as a HCV entry factor restricting HCV entry into human hepatocytes (Ploss et al., 2009). The authors have assessed several combinations of mouse and human HCV entry factors and identified human CD81 and human OCLN as the minimum set of human entry factors to support HCV entry in mouse cells (Ploss et al., 2009). With the goal to develop mouse hepatoma cells that reconstitute the entire HCV life cycle, we decided to work on three different mouse hepatoma cells which are the AML12, BNL-1 and Hepa1.6 cells. AML12 and Hepa1.6 cells have been previously shown to be permissive to HCVpp upon expression of human HCV entry factors (Ploss et al., 2009). We first assessed the expression of endogenous mouse homologues of HCV entry factors by flow cytometry. We used primary mouse hepatocytes (PMH) and mouse fibroblasts expressing the four mouse homologues of HCV entry factors (NIH3T3 4xM) (Bitzegeio et al., 2010) as positive controls. **Figure 12** shows the basal expression of the mouse homologues of HCV entry factors. Except Hepa1.6 cells, all the cells assessed expresses high levels of mCD81 **(Figure 12)**. While PMH and Hepa1.6 cells express little amounts of mOCLN, AML12 and BNL-1 do not express this important HCV entry factor **(Figure 12)**. PMH, AML12 and BNL-1 do not express mCLDN1 while Hepa1.6 cells do express this mouse factor. Furthermore, PMH and Hepa1.6 cells do express mSR-BI while AML12 and BNL-1 do not express mSR-BI (**Figure 12)**. These data show that most of the important HCV entry factors are not expressed at significant levels in our mouse hepatoma cell lines suggesting that these receptors will have to be overexpressed to detect HCV entry in line with the study from Ploss *et al.* who expressed hCD81 and hOCLN in combination with mCLDN1 and mSR-BI to detect HCVpp entry into mouse cells (Ploss et al., 2009)

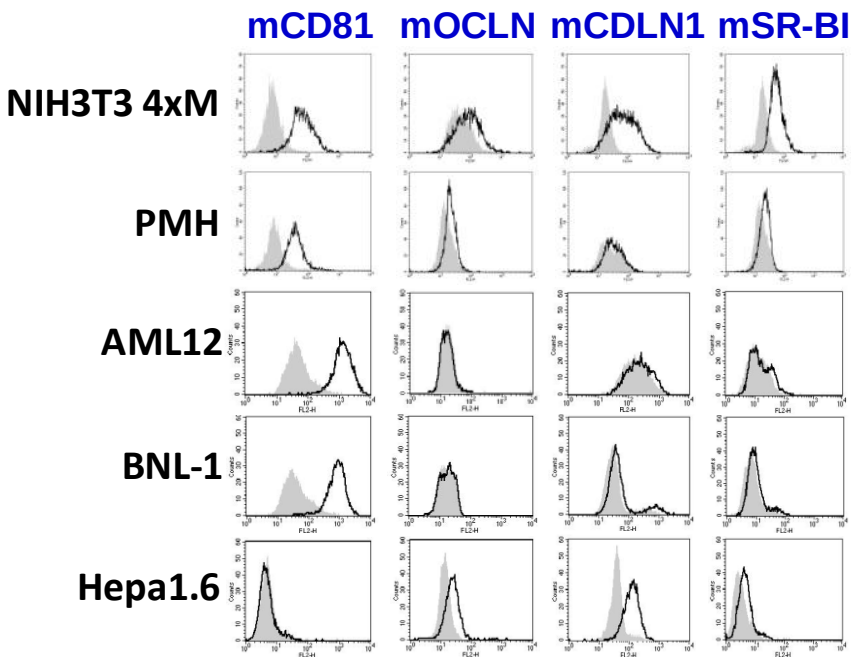


Figure 12: Endogenous expression of mouse homologue of HCV entry factors on primary and mouse hepatoma cells. *The basal expression of the murine homologue of HCV entry factors was assessed by flow cytometry in primary mouse hepatocytes, AML12, BNL-1 and Hepa1.6 cells. Cells were stained with a hamster monoclonal (CD81), mouse monoclonal (OCLN) or rat polyclonal (CLDN1, SR-BI) primary antibodies and stained later with an appropriate secondary PE antibody. Control isotype antibodies are presented as grey histograms and murine entry factors are presented by white histograms.*

2) Expression of the human HCV entry factors in mouse hepatoma cells using lentiviral vectors.

In order to render mouse hepatoma cells permissive to HCV entry, we expressed different combinations of the human HCV entry factors in these cells. Based on the previously published results from Ploss *et al.*, we first expressed the 2 species-specific entry factors hCD81 and hOCLN (2R). Using previously developed lentiviral vectors encoding the human HCV entry factors (Bitzegeio et al., 2010), we transduced the mouse hepatoma cells AML12, BNL-1 and Hepa1.6 to express hCD81 and hOCLN. 72h post-transduction, we selected transduced cells with appropriate antibiotics and assessed the expression of the human entry factors by flow cytometry **(Figure 13)**. We screened several cell clones for the clone expressing the highest level of the transduced receptors. As shown in **Figure 13**, expression of hCD81 and hOCLN was as high as, or even higher, than the expression observed in Huh7.5.1 cells. We assessed the permissivity of these mouse hepatoma cells

expressing the 2 receptors (2R) for HCVpp. As shown in **Figure 14**, the three mouse cell lines engineered to express hCD81 and hOCLN are not permissive to HCVpp entry in line with our assumption that the low endogenous mCLDN1 and mSR-BI expression in these cells **(Figure 12)** may not be sufficient to support robust HCV entry. We thus expressed the remaining human factors to detect HCV entry into the mouse hepatoma cells. Since HCV entry seems to be a limiting step for HCV infection, we aimed to have the highest HCV entry rate into the mouse hepatoma cells. Using the previously developed mouse hepatoma cells 2R, we first tranduced these cells to express hCLDN1 to develop mouse hepatoma cells expressing hCD81, hOCLN and hCLDN1 (3R). We screened several clones to get the highest expression of hCLDN1**(Figure 13)**. We then developed mouse hepatoma cells expressing the four human HCV entry factors (4R) by transducing the engineered mouse 3R cells with lentiviral vectors encoding for hSR-BI. To preclude any down-regulation of the first transduced receptors through promoter competition effect, we assessed by flow cytometry the expression of the four human HCV entry factors in mouse hepatoma cells 4R. As shown in **Figure 13**, none of the previously transduced human receptors had been down-regulated during the selection process. The three newly developed mouse hepatoma cell lines all express high levels of the four main human HCV entry factors **(Figure 13)**.

3) Expression of human HCV entry factors renders mouse hepatoma cells permissive to HCVpp but is not sufficient for HCVcc infection

Using the engineered mouse cells expressing 2R, 3R or 4R, we assessed their capability in supporting HCV entry using HCVpp. Mouse cells expressing only 2R were not permissive to HCVpp entry **(Figure 14)**, most likely due to the low level of endogenous expression of the two other HCV entry factors, CDLN1 and SR-BI **(Figure 12)**.

Interestingly, expression of hCLDN1 in the mouse hepatoma cells engineered to express hCD81 and hOCLN, was sufficient to detect HCVpp entry into mouse hepatoma cells. And these results were confirmed in the three different mouse cell lines **(Figure 14)**.

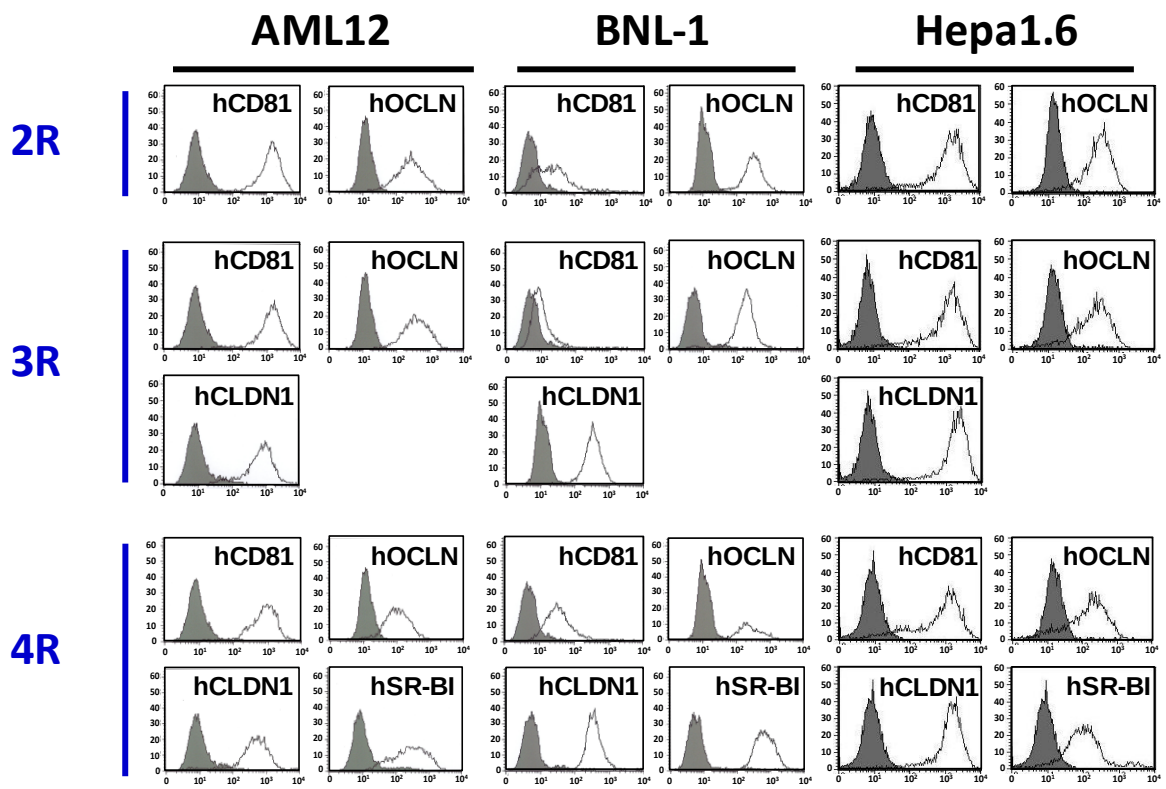


Figure 13: Expression of human HCV entry factors in engineered mouse hepatoma cell lines AML12, BNL-1 and Hepa 1.6. *Mouse hepatoma cell lines were transduced with 2 (hCD81, hOCLN), 3 (hCD81, hOCLN and hCLDN1) or 4 (hCD81, hOCLN, hCLDN1 and hSR-BI) lentiviral vectors carrying the HCV entry factors as indicated on the left. Transduced cells were selected and analysed by flow cytometry for entry factor expression. Histograms corresponding to expression of the respective human entry factors (white) are overlaid with histograms of naïve cells (grey) incubated with anti-human entry factor antibody. (h: human, X axis: counts, Y axis: FL2-H).*

It seems that expression of the three human receptors hCD81, hOCLN and hCLDN1 (3R) is sufficient to allow HCV entry into mouse hepatoma cells, since further expression of hSR-BI in the mouse 3R cells only modestly enhance HCV entry **(Figure 14)**. It is worth noting that HCV entry is about 20% of the Huh7.5.1 permissivity in AML12-4R and BNL-1-4R cells while Hepa1.6-4R were permissive about 50% of the permissivity observed in Huh7.5.1 cells. The reason of this discrepancy is unknown and cannot be explained by the expression level of the human receptors in the engineered mouse cells, since the expression level is similar between the three different mouse cell lines **(Figure 13)**. Thus, other host factors that might be differentially expressed between these different cell lines may account for the higher permissivity of Hepa1.6-4R cells.

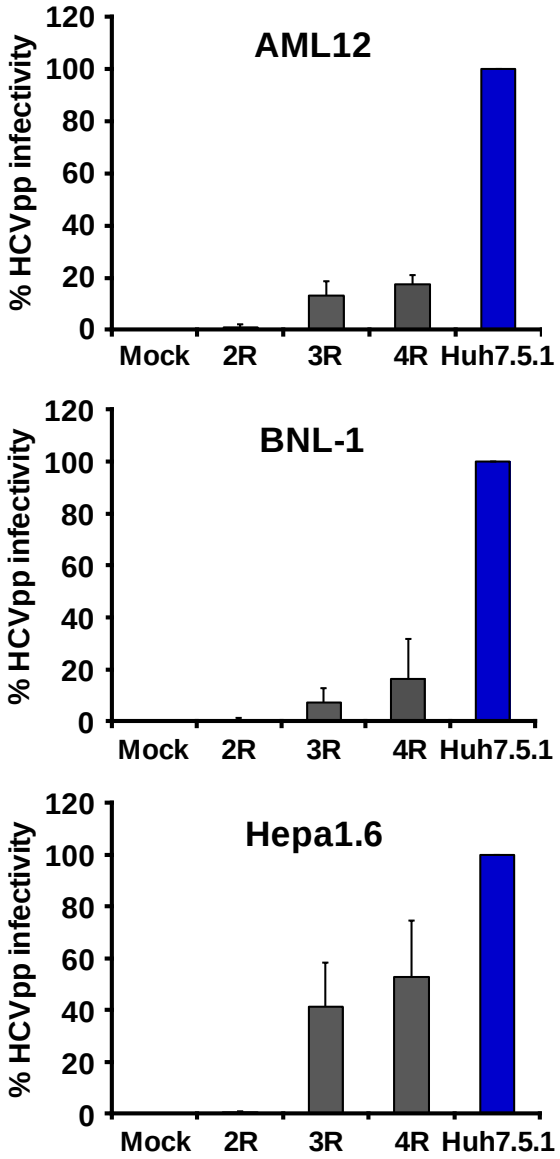


Figure 14: Expression of the four human HCV entry factors renders mouse hepatoma cells permissive to HCVpp entry. *AML12, BNL-1 and Hepa1.6 cells were transduced for human HCV entry factor expression (Figure 13) and challenged with HCVpp (1b) or VSVpp. Luciferase activity was determined after 72 h. The background luciferase signal was substracted from HCVpp and VSVpp signals. The HCVpp signal was then normalized to VSVpp entry and then normalized to HCVpp entry in Huh7.5.1 cells to allow for cross-experimental comparison. Results are expressed as means +/- SD of three independent experiments performed in triplicate.*

We next assessed the ability of the HCV entry permissive mouse hepatoma cells to support HCV infection. Thus, we infected the mouse 4R cells with a sensitive *Renilla* luciferase reporter virus (JcR2a) and assessed luciferase activity 72h post-infection. As shown in **Figure 15**, mouse hepatoma cells expressing the human HCV entry factors do not support HCVcc infection.

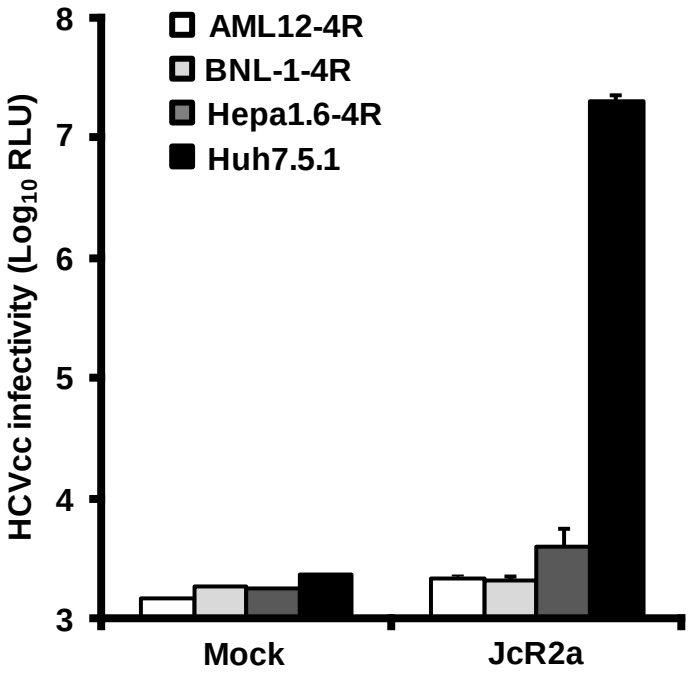


Figure 15: HCVpp entry-permissive mouse hepatoma cells are resistant to HCVcc infection. *AML12, BNL-1 and Hepa1.6 cells expressing the four human entry factors and naïve Huh7.5.1 cells were challenged with a HCVcc (JcR2a) virus. 72h post-infection, cells were lysed and luciferase activity assessed. Results are expressed as means of luciferase activity +/- SD of one experiment performed in triplicate.*

These results confirm that mouse hepatocytes are naturally resistant to HCV infection. Moreover, these data suggest that beyond the species-specific barrier of HCV entry, HCV infection of mouse cells are restricted at step(s) downstream of HCV entry.

4) HCV RNA translation is not restricted in mouse hepatoma cells

The following step downstream of HCV entry is the translation of the genomic RNA by host cell translational machinery. Among the studies assessing HCV life cycle in mouse cells, none has assessed the capability of mouse cells to translate the incoming viral RNA. To preclude that this step is limited in the HCV entry permissive mouse hepatoma cells, we used a plasmid previously developed in our laboratory, where a CMV promoter drives the transcription of HCV IRES upstream of the 10 first amino acid of core protein fused to a luciferase gene (pIV1132) (Wolf et al., 2008). In

this context, if the murine translational machinery properly recognizes HCV IRES, we should observe high luciferase activity upon transfection of the plasmid.

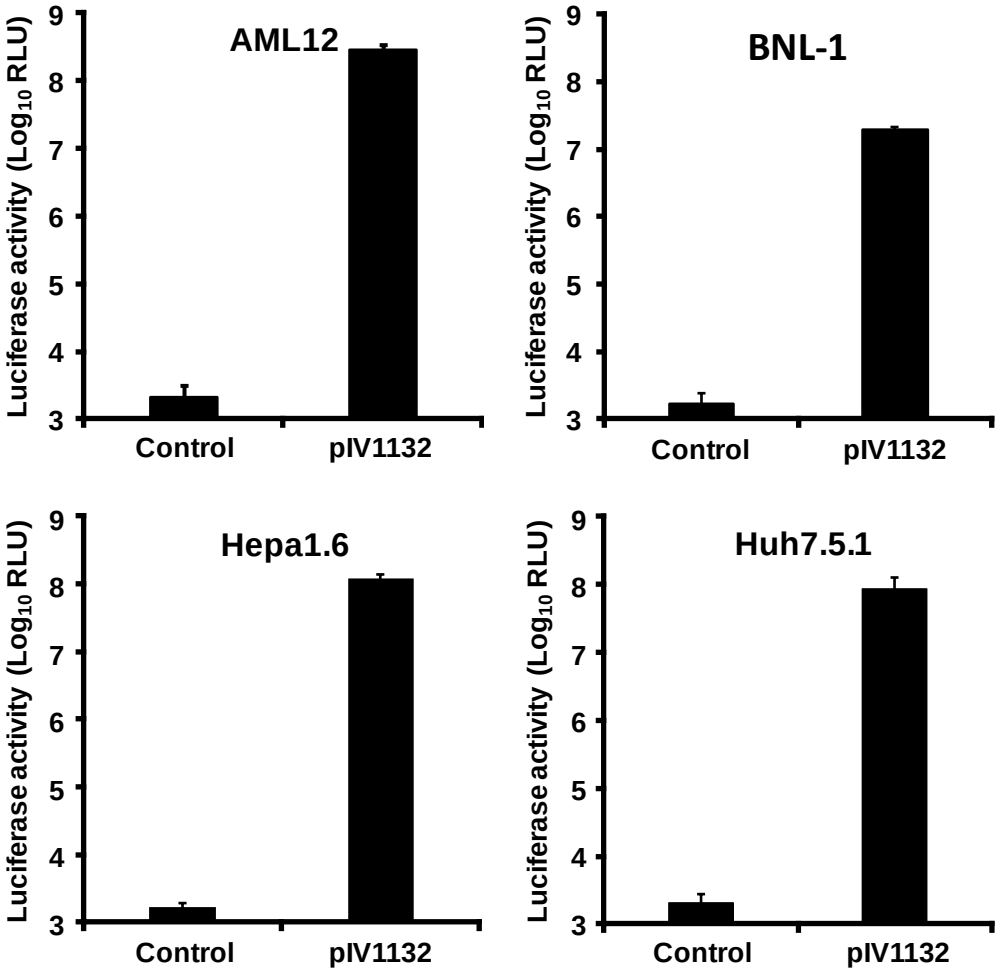


Figure 16: HCV IRES allows viral RNA translation in mouse hepatoma cells. *AML12, BNL-1, Hepa1.6 and Huh7.5.1 cells were transfected side-by-side with either a control or pIV1132 plasmid (Wolf et al., 2008). 72h post-transfection, cells were lysed and luciferase activity assessed. Results are means +/- SD of relative light units from three independent experiments performed in triplicates.*

We transfected side-by-side AML12, BNL-1, Hepa1.6 and Huh7.5.1 cells with the pIV1132 plasmid and 72h post-transfection, cells were lysed and assessed for luciferase activity.

As shown in the **Figure 16,** the three different mouse hepatoma cells showed similar proficiency in recognizing HCV IRES and translating downstream viral RNA. Indeed, the luciferase activity in mouse cells is as high as the luciferase activity in

Huh7.5.1 cells, while transfection of control plasmid showed no luciferase activity. These results demonstrate that mouse hepatoma cells are able to recognize and translate HCV viral RNA suggesting that the limited step of HCV infection in mouse cells is most likely HCV RNA replication.

5) MiR-122 expression is not sufficient to allow mouse hepatoma cells supporting robust HCV RNA replication.

Like for entry, HCV replication relies on several host factors. So far, miR-122 seems to be the most unexpected and the most important HCV replication factor. It is known that miR-122 interacts with HCV RNA and this interaction is crucial for HCV RNA replication. Drug mediated inhibition or insertion of specific mutations interrupting this interaction lead to a drastic decrease in HCV RNA replication *in vitro* and *in vivo* (Jopling et al., 2005; Lanford et al., 2010). It has been demonstrated that human hepatoma cells lacking the expression of this microRNA do not support HCV RNA replication, but restoring miR-122 expression in these cells restore as well HCV RNA replication (Kambara et al., 2012; Narbus et al., 2012). Several studies have assessed the capability of miR-122 to allow HCV RNA replication in non-hepatic cells as well as in mouse fibroblasts (Chang et al., 2008; Fukuhara et al., 2012; Lin et al., 2010). We have shown in the first part of this manuscript that expression of the human HCV entry factors and miR-122 in 293T cells allows a robust HCV infection. Thus, we next expressed microRNA-122 in the engineered mouse hepatoma 4R cells using a lentiviral vector encoding microRNA-122 and puromycin resistance gene. 72h post-transduction, puromycin was incorporated into the culture medium and puromycin-resistant cells were expanded and analyzed for miR-122 expression, by RT-qPCR and expression was compared to untransduced cells. As shown in the **Figure 17A**, miR-122 expression was readily detected in the HCV entry permissive mouse hepatoma cells and the range of miR-122 expression was comparable to the range of expression observed in Huh7.5.1 cells. MiR-122 expressing mouse hepatoma cells were then challenged with HCVcc encoding a *Gaussia* luciferase reporter virus or with a mutated virus defective for replication (GNN).

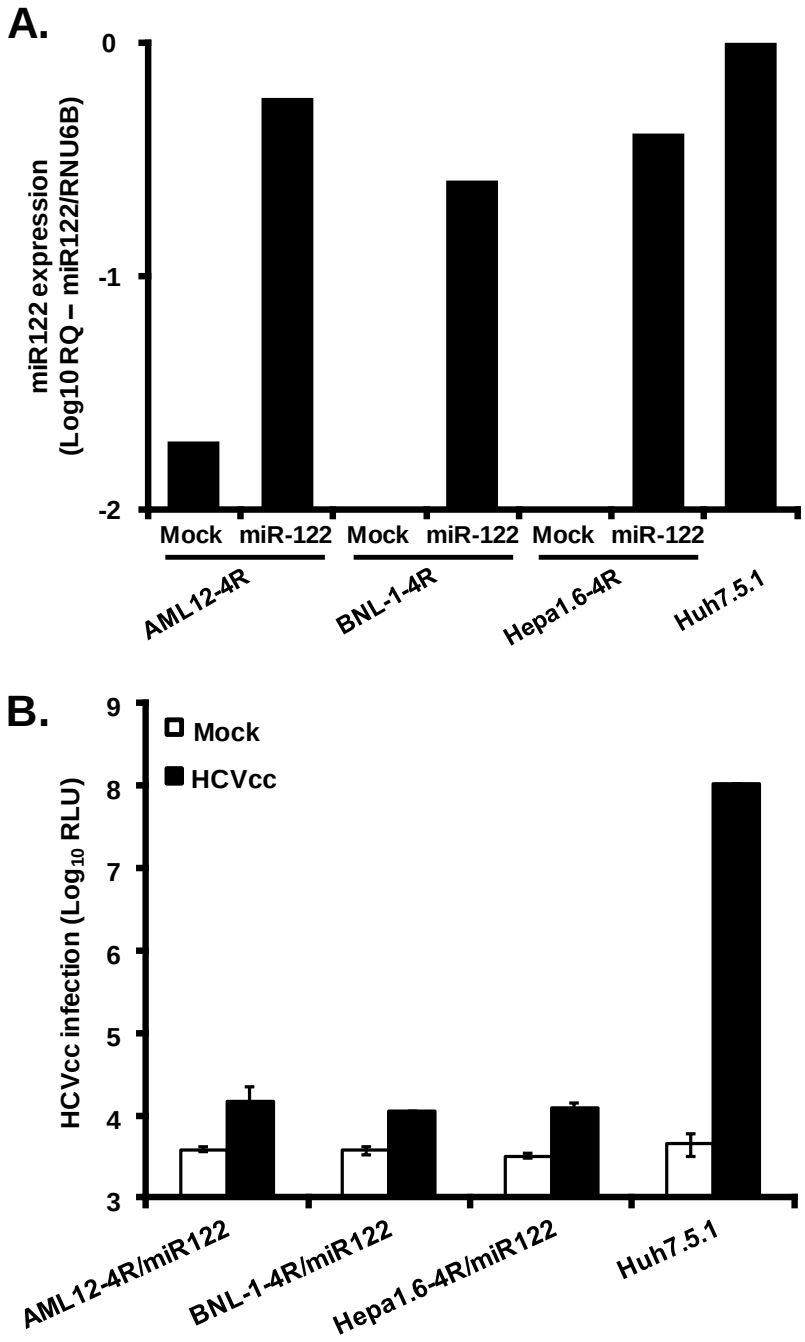


Figure 17: MiR-122 expression is not sufficient for robust HCV RNA replication in HCV entry permissive mouse hepatoma cells. ***A. Expression of miR-122 in mouse hepatoma cells. AML12-4R, BNL-1-4R and Hepa1.6-4R were transduced with a lentiviral vector encoding miR-122 and a puromycin-resitance gene. 72h post-transduction, cells were cultured in puromycin containing medium and puromycin-resistant cells were analysed by RT-qPCR for miR-122 expression and plated for HCVcc infection. Results are expressed as the relative quantification (RQ) of miR-122 in mouse hepatoma cells compared to Huh7.5.1 cells which have been plotted at 1 as reference. RNU6B was used as an internal RNA control. B. HCVcc infection of miR-122 expressing mouse hepatoma cells. HCV entry permissive mouse hepatoma cells expressing miR-122 were challenged with HCVcc containing a Gaussia luciferase reporter or with a replication defective HCVcc (GNN). 72h post-infection, luciferase activity was assessed. Results are expressed as means +/- SD of relative light units from one representative experiment performed in triplicate.***

As shown in **Figure 17B**, luciferase activity in infected mouse hepatoma cells expressing the HCV entry factors and miR-122 is low compared to the luciferase activity detected in Huh7.5.1 cells. Nevertheless a slight increase is observed when compared to the mouse hepatoma cells infected with the replication defective virus, suggesting that mouse hepatoma cells expressing miR-122 are able to replicate HCV RNA but to a lesser extent than the replication rate observed in Huh7.5.1 cells **(Figure 17B)**.

Since mouse hepatoma cells seem to replicate HCV RNA only at very low levels, we wondered whether the lower level of miR-122 expression in mouse cells compared to Huh7.5.1 cells could not explain in part, this low level of HCV RNA replication in mouse cells. To test this hypothesis, we transfected a miR-122 mimic or a microRNA control into mouse hepatoma cells expressing miR-122. Transfected cells were then either analyzed to determine miR-122 expression by RT-qPCR or plated to challenge them with a HCVcc containing the *Gaussia* luciferase reporter gene. Transfection of the miR-122 mimic in mouse hepatoma cells was very efficient since relative quantification of intracellular miR-122 showed high intracellular miR-122 levels **(Figure 18)** that exceeded the endogenous level quantified in miR-Control transfected Huh7.5.1 cells.

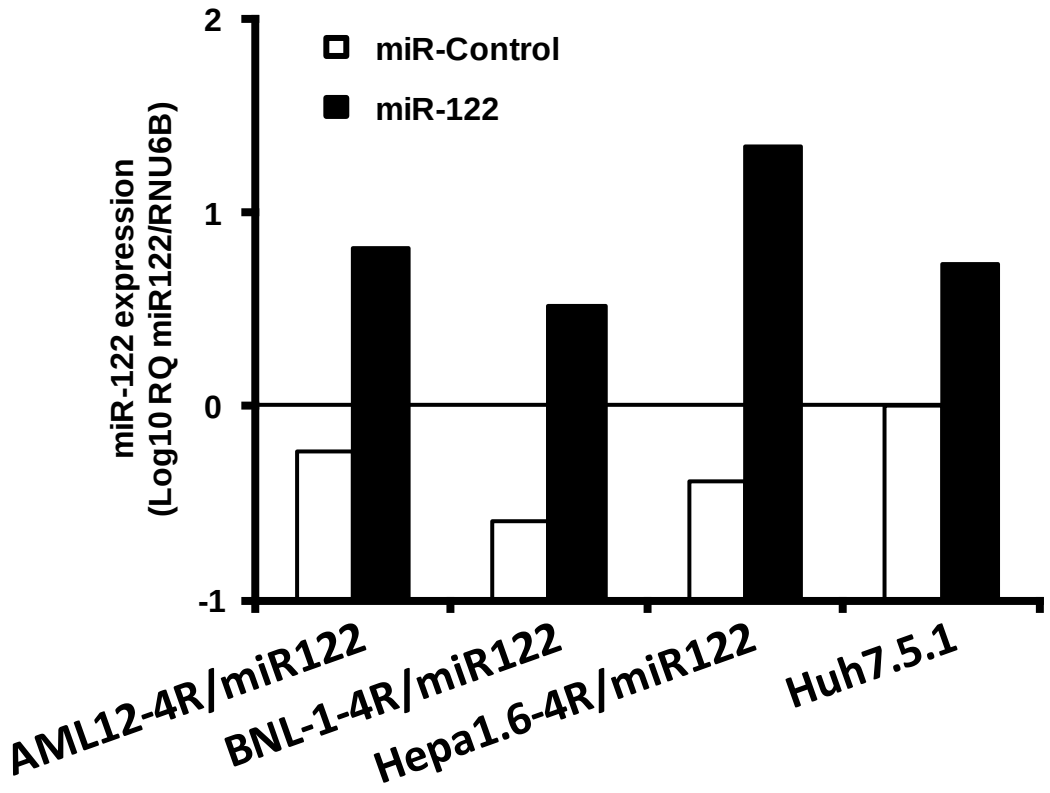


Figure 18: Mouse hepatoma cells constitutively expressing miR-122 are transfected with a miR-122 mimic or a miR-Control. *AML12-4R/miR122, BNL-1-4R/miR122, Hepa1.6-4R/miR122 or Huh7.5.1 cells were transfected with either a miR-122 or a miR-Control mimic using Lipfectamine 2000®. 24h post-transfection, cells were washed 3 times with PBS and lysed for total RNA extraction and miR-122 level analyzed by RT-qPCR. Results are shown as relative quantification (RQ) of miR-122 compared to Huh7.5.1 miR-122 levels. RNU6B was used as internal RNA control. Solid line represents the level of miR-122 in Huh7.5.1 cells which have been plotted at 1 as reference.*

As observed in **Figure 19,** HCV replication slightly increased upon additional transfection of miR-122 in mouse hepatoma cells that stably express miR122 compared to miR-Control transfection. Interestingly, this slight increase was also observed in miR-122 transfected Huh7.5.1 cells. Nevertheless, the considerable increase in intracellular miR-122 levels **(Figure 18)** did not drastically affected HCV replication of HCV entry mouse hepatoma cells. It is worth noting that HCV infection in miR122-transfected Huh7.5.1 cells did not significantly increase as compared to miR-Control-transfected cells, suggesting that high miR-122 levels do not influence drastically HCV RNA levels

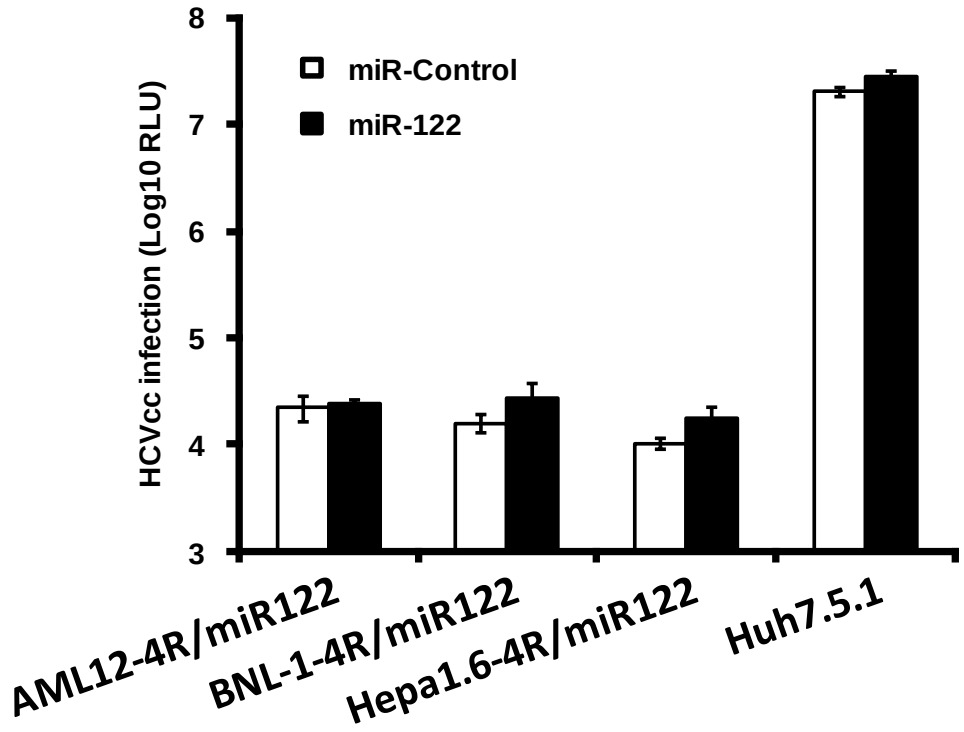


Figure 19: HCVcc infection of mouse hepatoma cells upon miR-122 mimic transfection. *AML12-4R/miR122, BNL-1-4R/miR122, Hepa1.6-4R/miR122 or Huh7.5.1 cells were transfected with either a miR-122 or a miR-Control mimic using Lipfectamine 2000®. 24h post-transfection, cells were challenged with HCVcc containing a Gaussia luciferase. 72h post-infection cells were lysed and assessed for luciferase activity. Results are expressed as mean of relative light unit from one representative experiment performed in duplicate.*

Altogether, these data suggest that, apart from miR-122 expression, other factors involved in HCV replication are lacking in mouse cells to allow a robust HCV RNA replication in these cells. However, it is also conceivable that mouse factors expressed in mouse hepatoma cells restrict HCV replication. Further studies are required to better understand the limitations for a robust HCV RNA replication in mouse cells. The mouse cells developed in this study are valuable tools to screen for such factors.

6) Restoring mouse apoE expression in mouse hepatoma cells

ApoE has been shown by our laboratory to be an essential cell host factor involved in HCV assembly and release, by interacting with the HCV NS5A protein (Benga et al., 2010). Furthermore, it has been recently shown, using mouse hepatoma cells harboring a sub-genomic replicon and trans-complemented with HCV structural

proteins, that mouse hepatoma cells expressing mouse apoE (m-apoE) allow robust HCV assembly and release (Long et al., 2011). These results suggest that apart from apoE, mouse hepatoma cells express all the factors necessary for HCV assembly and release. This suggests that HCV RNA replication is the last limiting step to reconstitute the HCV life cycle in mouse hepatocytes. In order to establish cell lines that would be useful to screen for factors involved in HCV RNA replication in mouse cells, we expressed mouse apoE in our engineered mouse hepatoma cells expressing the HCV entry factors and miR-122 using lentiviral vectors encoding m-apoE harboring a HA-tag (m-apoE-HA) and subsequently assessed mouse apoE expression.

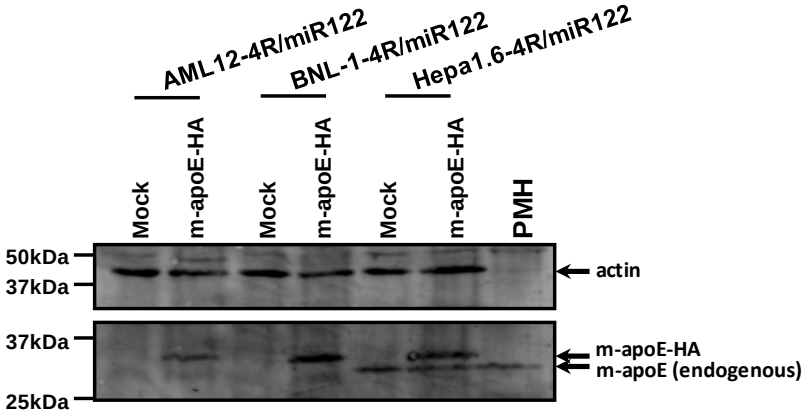


Figure 20: Transduction of mouse apoE in engineered mouse hepatoma cells. *AML12-4R/miR122, BNL-1-4R/miR122 and Hepa1.6-4R/miR122 cells were transduced with lentiviral vectors encoding HA-tagged mouse apoE (m-apoE-HA) (Long et al., 2011). 72h post-transduction, mock transduced or m-apoE transduced cells were analyzed for m-apoE expression by western blotting using a monoclonal anti-m-apoE. Primary mouse hepatocytes (PMH) were used as control for m-apoE expression.*

As shown in **Figure 20**, transduced mouse hepatoma cells express the HA-tagged mouse apoE that is easily distinguishable from endogenous mapoE due to its slower migration on SDS-PAGE. Interestingly, in contrast to AML12 and BNL1 cells, Hepa1.6 cells express endogenous mouse apoE, since a band running at the same level as the mouse apoE detected in PMH also appears in both transduced and untransduced Hepa1.6 cells.

The engineered mouse hepatoma cells from this study represent valuable tools to identify host factors required for HCV replication and missing in mouse cells or host factors restricting HCV replication in mouse cells.

DISCUSSION

AND

PERSPECTIVES

Acute HCV infection is most often asymptomatic and in the majority of individuals leads to the development of chronic hepatitis. HCV infected patients, apart from the hepatic activity failures, show extra-hepatic symptoms such as cognitive dysfunction, fatigue, mixed cryoglobulinemia and non-Hodgkin lymphoma suggesting that HCV has side-sites of infection constituting potential reservoirs (Kramer et al., 2002; Marukian et al., 2008). Furthermore, HCV infection is restricted to human and some non-human primates indicating that HCV has a species tropism. This species specificity has slowed down the development of novel therapeutics for the treatment of HCV. Current small animal models are not easily handleable and require specific techniques that restrict their use for the large scientific community. Furthermore, they do not allow the study of HCV infection in extra-hepatic tissues, since the current animal models are based on the transplantation of human hepatocytes in immunodeficient mice. Thus, the characterization of the factors allowing HCV infection in non-hepatic cells and in mouse hepatocytes will bring valuable information for the design of new therapeutics which will target potential HCV reservoirs and for the elaboration of a transgenic mouse model supporting the entire HCV life cycle.

1) HCV entry into human non-hepatic cells and mouse hepatoma cells

HCV entry is a complex process and, so far, the most characterized step of HCV life cycle (Zeisel et al., 2011b). It involves several host cell factors among which CD81, OCLN, CLDN1 and SR-BI. At present, only CD81 and SR-BI have been demonstrated to bind E2 glycoprotein (Pileri et al., 1998; Scarselli et al., 2002; Zeisel et al., 2007). Although they are crucial for HCV entry, CLDN1 does not appear to directly interact with HCV while the role of OCLN remains to be clarified (Evans et al., 2007; Ploss et al., 2009). Furthermore, while expression of the four HCV entry factors renders HCV entry resistant cells permissive to HCVpp, Ploss *et al.* have identified the minimum set of human HCV entry factors allowing HCVpp entry in mouse cells. Indeed, they demonstrated that the expression of hCD81 and hOCLN in combination with mCLDN1 and mSR-BI is sufficient to support HCV entry in mouse cells (Ploss et al., 2009). These results have recently been confirmed *in vivo* by delivering this set of HCV entry factor using adenoviral vectors in mice (Dorner et al., 2011).

In our study, we used the non-hepatic human embryonic kidney-derived cell line 293T, to reconstitute the entire HCV life cycle in non-hepatic cells and the three different mouse hepatoma cells AML12, BNL-1 and Hepa1.6 to study HCV infection restriction in mouse hepatoma cells. As a first step, we assessed by flow cytometry, expression of HCV entry factors in 293T cells and in mouse hepatoma cells. We confirmed that apart CLDN1, 293T cells express the three other HCV entry factors. Furthermore assessment of the expression of mouse homologs of HCV entry factors revealed that our mouse hepatoma cells do not express significant levels of mCLDN1 and mSR-BI. Moreover, apart for Hepa1.6 cells, all mouse hepatoma cells expressed significant levels of mCD81 and did not express mOCLN. Thus we aimed to assess HCV entry in these different cell lines by developing 293T-derived and mouse hepatoma-derived cell lines expressing or overexpressing different sets of HCV entry factors.

A previous study has shown that 293T cells expressing CLDN1 are permissive to HCVpp entry (Evans et al., 2007). In our experiments, we could confirm these results and we also showed that overexpression of the entry factors CD81 and OCLN does not rescue HCV entry in CLDN1-lacking 293T cells, confirming the crucial role of CLDN1 in HCV entry. Furthermore expression of hCD81 and hOCLN in the three mouse hepatoma cells did not allow detecting HCV entry, which is consistent since mouse hepatoma cells did not express mCLDN1 and mSR-BI. Next, we expressed CLDN1 in the cells overexpressing CD81 and OCLN to develop 293T and mouse hepatoma cells expressing 3 receptors (3R). While 293T-3R cells reached 1.5 times the permissivity of Huh7.5.1 cells, AML12-3R and BNL-1-3R permissiveness was about 20% of Huh7.5.1 cells and this percentage reached 40% for Hepa1.6-3R cells. Since 293T cells already express CD81 and OCLN, over-expression of these receptors in combination with CLDN1 enhanced HCV entry in the 293T cells. Moreover, Ploss *et al*. have shown than expression of this set of factors allow HCV entry in mouse cells but at less extent than expressing the four HCV entry factors (Ploss et al., 2009). Finally, we expressed the last HCV entry factor, SR-BI in the engineered human non-hepatic and mouse hepatoma cell lines expressing the 3 receptors to develop cell lines expressing the four HCV entry factors (4R). While expression of SR-BI in 293T-3R cells increased permissivity to 4 times the permissivity of Huh7.5.1 cells, this was not observed in mouse hepatoma cells where

further expression of SR-BI in mouse cells expressing 3R only slightly increased their permissivity to HCVpp. These results have important implication for the understanding of HCV entry in non-hepatic cells and in mouse hepatoma cells.

It has been shown in the human hepatoma cell line Huh7.5, that CD81 expression levels influence HCV entry (Akazawa et al., 2007; Koutsoudakis et al., 2007), while overexpression of OCLN does not seem to enhance HCV entry (Ciesek et al., 2011). This suggest that the higher permissivity of the 293T cells expressing CD81, OCLN and CLDN1 compared to Huh7.5.1 cells is most likely due to the overexpression of CD81 and CLDN1 rather than the high level of OCLN. Since 293T cells already express OCLN, it could have been of interest to see whether overexpression of CD81 and CLDN1 could have produced the same effect as the one observed when expressing the three receptors.

Several studies have shown that CD81 and CLDN1 form a complex important for HCV entry (Harris et al., 2010; Harris et al., 2008; Krieger et al., 2010) and this complex is regulated by a yet unknown mechanism which involves host cell kinases recently identified by our laboratory, i. e. EGFR and EphA2 (Lupberger et al., 2011). In this context, it is worth noting that we have shown in our study that 293T cells express basal level of EGFR, and we have demonstrated that none of the cell lines developed shows an overexpression of EGFR, which could have happened during the selection process. It is very likely that the high HCV permissivity mediated by the overexpression of CD81 and CLDN1 in 293T cells is regulated by endogenous EGFR expression. In our study, overexpression of SR-BI in 293T-3R cells was four times higher than the basal expression of SR-BI in 293T cells, and HCVpp permissivity in 293T-4R cells reached 4 times the permissivity of Huh7.5.1 to HCVpp. These results are in line with previous studies which have shown that overexpression of SR-BI in Huh7.5 cells increased permissivity to HCVcc infection (Grove et al., 2007; Schwarz et al., 2009).

Altogether, these results indicate that expression or overexpression of the herein defined HCV entry factors render non-hepatic cells permissive and even highly permissive to HCV entry. These data have important implications since it has been shown that HCV infection induces expression modulation of certain HCV entry factors in human hepatoma cells and infected human livers (Liu et al., 2009; Nakamuta et al.,

2011; Reynolds et al., 2008; Tscherne et al., 2007). Tscherne *et al.* have shown that Huh7.5 cells replicating a sub-genomic replicon were less sensitive to further infection with HCVcc (Tscherne et al., 2007). This superinfection exclusion was due to down-regulation of CD81 expression in sub-genomic replicating cells (Tscherne et al., 2007). Later on, Liu *et al.* have shown that CLDN1 and OCLN were also down-regulated in Huh7.5.1 cells to prevent superinfection (Liu et al., 2009). Since *in vitro* data suggested HCV entry factor expression modulation, studies aimed to verify *in vivo* whether this phenomenon occurs. Reynolds *et al.* have shown that CLDN1 is overexpressed in HCV infected human livers but no such expression modulation was shown for CD81 and SR-BI (Reynolds et al., 2008). While Nakamuta *et al.* observed significant CLDN1 down-regulation; they observed an increase of OCLN in infected livers (Nakamuta et al., 2011). The reason of this discrepancy is not yet known but it is clear that HCV can modulate host cell factor expression during infection; additional studies are required to determine precisely, which HCV entry factor expression is modulated during infection. Furthermore, a study has shown that CLDN1 and OCLN are up-regulated when HCV re-infects the liver graft after liver transplantation (Mensa et al., 2011). In this context, it has been observed that following liver transplantation, glucocorticoïd-mediated immunosuppressed patients showed more severe HCV recurrence, increased mortality and graft loss (Fafi-Kremer et al., 2010; Roche and Samuel, 2009). The mechanism by which glucocorticoïds enhance HCV infection has been clarified recently: indeed it has been shown that Huh7.5 cells treated with glucocorticoïds are tenfold more permissive to HCV entry than naïve cells, despite a slight reduction in their ability to replicate HCV RNA upon glucocorticoïd treatment (Ciesek et al., 2010). The authors have shown that OCLN and SR-BI were up-regulated both at the messenger RNA and protein levels following glucocorticoïd treatment (Ciesek et al., 2010). These data show the importance of HCV entry and the HCV entry factors in the viral life cycle for human hepatocyte infection. It is most likely that infection of non-hepatic cells is also dependent of proper HCV entry factor expression to sustain an efficient HCV entry in those cells. In our study, we have shown that robust HCV RNA replication is dependent on robust HCV entry. Furthermore, we have shown that HCV infection of our engineered 293T cells is dependent of the HCV entry factors expressed since well characterized monoclonal antibodies directed against CD81, CLDN1 and SR-BI, could potently and significantly inhibit HCV infection, suggesting that HCV infection of non-hepatic cells relies on the

same entry process as observed in HCV infection of hepatocytes. Thus, assessing HCV entry factor expression in non-hepatic cells is the first step to determine potential reservoir(s) of HCV.

It has been demonstrated that some neuronal cells could support HCV entry indicating that HCV can enter non-hepatic cells (Burgel et al., 2011; Fletcher et al., 2010). The authors have shown that neuroepithelioma cell lines express HCV entry factors and these cells are permissive to HCVpp entry. Fletcher *et al.* demonstrated that HCV entry in these cells is dependent on the HCV entry factors since they could block HCV entry using specific entry factor inhibitors (Fletcher et al., 2010). Very recently, the same team has demonstrated the presence of HCV RNA in the brain of HCV infected patients but they quantified 1000 to 10000 times lower copies than in the liver (Fletcher et al., 2012). They demonstrated that human brain endothelium express HCV entry factors and, using human brain endothelial cells, they demonstrated that these cells were permissive to HCVpp entry (Fletcher et al., 2012). These data provide evidence that HCV can enter non-hepatic cells.

In the case of mouse hepatoma cells, we have shown that expression of the four HCV entry factors in the three different mouse hepatoma cells conferred low HCV entry permissivity. It is clear that mCD81 and mOCLN cannot support HCV entry (Michta et al., 2010; Ploss et al., 2009); however it is possible to force the virus to adapt HCV glycoproteins in using mCD81 (Bitzegeio et al., 2010). These data are important because they demonstrate that the high genetic variability of HCV allows the virus to adapt to unconventional environment. In the case of OCLN, the domain involved in HCV entry has been identified in the EL2 of the protein (Ploss et al., 2009). Additional mutagenesis analysis revealed that the two residues A223 and A224 in mOCLN are responsible for the species specificity (Ciesek et al., 2011; Michta et al., 2010). Reversing the mouse amino acids into human amino acids restored the ability of mOCLN in supporting HCV infection (Michta et al., 2010). While Ploss *et al.* and Dorner *et al.* suggest that mCLDN1 and mSR-BI are equivalently efficient in supporting HCV infection *in vitro* and *in vivo,* respectively (Dorner et al., 2011; Ploss et al., 2009), divergent results have been published recently. Indeed it has been shown that residues in mCLDN1 lead to a reduced efficiency of mCLDN1 to

support HCV entry, nevertheless authors confirm that mCLDN1 is still able to support HCV entry (Haid et al., 2010). In the case of SR-BI, it has been shown that mSR-BI supports HCV infection to a much less extent than hSR-BI does (Catanese et al., 2010). Ploss *et al.* have shown that Chinese Hamster Ovary (CHO) cells expressing hCD81, hOCLN and hCLDN1 were susceptible to HCVpp infection (Ploss et al., 2009). In our study, we obtained similar results using three different mouse hepatoma cell lines. By flow cytometry, we showed that the mouse hepatoma cell lines we used do not express significant levels of endogenous mSR-BI. Additional expression of hCD81, hOCLN and hCDLN1 was sufficient to detect HCVpp entry in these mouse hepatoma cell lines. Catanese *et al.* used shRNA to down-regulate hSR-BI in Huh7.5 cells and express shRNA resistant SR-BI constructs among those they have tested mSR-BI (Catanese et al., 2010). While mSR-BI is not able to bind HCV E2 glycoprotein, it seems that mSR-BI is still able to enhance HCV infection in these SR-BI knock-down Huh7.5 cells (Catanese et al., 2010). The reason of this discrepancy may be due to the fact that SR-BI plays multiple roles in HCV entry as recently suggested by Dao Thi et al. (Dao Thi et al., 2012). They have shown that SR-BI has an attachment function, an access function and an enhancement function (Dao Thi et al., 2012). They demonstrated that the attachment and access function does not require HCV E2 binding to SR-BI, thus mSR-BI which does not bind HCV E2 could still play a role in HCV infection (Dao Thi et al., 2012). Nevertheless, the results we obtained previously on non-hepatic cells show that efficient HCV infection requires absolutely efficient HCV entry. While Ploss *et al.* and Dorner *et al.* used the combination of hCD81, hOCLN, mCLDN1 and mSR-BI to render mouse cells and mice permissive to HCV entry (Dorner et al., 2011; Ploss et al., 2009), we decided to have the highest HCV entry efficiency in our mouse hepatoma cell lines and thus expressed the four human HCV entry factors. Using this strategy, we have been able to render the three mouse hepatoma cells permissive to HCVpp entry. It is worth noting that the rate of HCV entry observed in our hepatoma cells is in line with previously published results from Ploss *et al.* Indeed, AML12 cells expressing the four human HCV entry factors showed a permissiveness of about 20% of the Huh7.5 cells (Ploss et al., 2009). Interestingly, while the mouse fibroblast NIH3T3 cells are highly permissive to HCVpp upon expression of the human HCV entry factors, the three mouse hepatoma cell lines Ploss *et al.* have used in their study are still poorly permissive to HCV entry (Ploss et al., 2009). These results suggest that either a

factor is missing in mouse hepatoma cells to support efficient HCV entry or a mouse factor restricts HCV entry into mouse hepatoma cells and not in other mouse cell types.

EGFR and EphA2 have been demonstrated to regulate HCV entry by regulating the CD81-CLDN1 complex formation (Lupberger et al., 2011). Our laboratory has shown that expressing EGFR in the mouse hepatoma cell line AML12 expressing the four human HCV entry factors (AML12-4R) increased HCVpp permissivity (Lupberger et al., 2011). Furthermore, recently another putative HCV entry factor has been identified. NPC1L1 has been shown to play a role at post-binding steps but before or at membrane fusion (Sainz et al., 2011). The precise role of NPC1L1 is not defined yet but it has been proposed that NPC1L1 could uptake virus associated cholesterol revealing thus important HCV glycoprotein domains for proper HCV entry factor association, but this remains to be demonstrated (Sainz et al., 2011). Since NPC1L1 is expressed only in human and non-human primate hepatocytes, Sainz *et al.* have suggested that NPC1L1 could participate in HCV tropism and in the species specificity of HCV. It is worth noting that NPC1L1 factor cannot enhance HCVpp entry into mouse hepatoma cells since HCVpp are not associated with lipoproteins, thus NPC1L1 can only improve HCVcc infection. We do not have data on NPC1L1 expression in our mouse cell lines, but the cell lines developed in this study will be useful for investigating the role of NCP1L1 in mouse hepatoma cells.

None of the HCV entry factors identified so far is liver-specific, thus it is not clear why HCV can infect some cells and not others. A recent study has identified an inhibitor of HCV entry which is not present in hepatocytes but in other non-hepatic cells (Rocha-Perugini et al., 2008). This inhibitor is a cleavage product of the CD81 binding partner EWI-2 and is called EWI-2wint (EWI-2 without its N-terminal). Interestingly, the authors have assessed the expression of this cleavage product in different cell lines and demonstrated that 293T cells do not contain EWI-2wint but do express the uncleaved protein EWI-2 (Rocha-Perugini et al., 2008). It is likely that the high permissivity of the 293T-4R cells developed in our study is mediated, in part, by the absence of this inhibitor in our cell lines. Rocha-Perugini *et al.* showed that B cells, another suggested potential HCV reservoir, express EWI-2 and contain the

cleavage product EWI-2wint. Since, B lymphocytes cells do not express CLDN1 and OCLN and contain EWI-2wint, we can speculate that HCV B cell entry will not follow the same route of entry as the one in hepatocytes. Rocha-Perugini *et al.* did not assess EWI-2wint presence in neuroepithelioma cell lines. Since these cells are permissive to HCV infection, it would be of interest to know whether they express the protease responsible for the cleavage of EWI-2 into EWI-2wint. In our study, we did not assess the effect of EWI-2wint expression in our highly permissive 293T-4R cells, but such experiments would bring valuable information on the inhibition potential of EWI-2wint in non-hepatic cells.

Furthermore, beside the lack of potential entry factors in mouse hepatoma cells, it is conceivable that mouse cells express a factor restricting HCV entry. The great difference in terms of HCV permissivity between NIH3T3 and AML12 cells in the Ploss study suggests that several factors are responsible for this difference (Ploss et al., 2009). The presence of a potential restriction factor for HCV entry has been suggested recently (Hikosaka et al., 2011). Hikosaka *et al.* have developed a transgenic mouse model where the human HCV entry factors CD81, OCLN, CLDN1 and SR-BI were specifically expressed in the liver through an albumin promoter (Hikosaka et al., 2011). Authors have been able to express human entry factors in the livers of these mice and showed that soluble HCV E2 glycoprotein was able to bind to liver sections of these mice while liver sections of WT mice failed to do so (Hikosaka et al., 2011). Interestingly, HCVpp assays using primary mouse hepatocytes isolated from these transgenic mice did not allow the authors to detect HCVpp entry (Hikosaka et al., 2011). In this study, a technical limitation could explain these results. Indeed, the authors have used murine leukemia virus (MLV)-based HCVpp, and it is known that these pseudo-typed particles are poorly efficient in non-dividing cells such as primary hepatocytes. This could explain why the authors could not detect HCV entry in primary mouse hepatocytes expressing the four human HCV entry factors. Nevertheless, the authors investigated whether primary mouse hepatocytes express a restriction factor, and to assess this possibility they performed a fusion assay between Hep3B-HCVpp permissive cells and primary mouse hepatocytes expressing the four human HCV entry factors and observed a significant decrease of the heterokaryon permissivity to HCVpp (Hikosaka et al., 2011). In this experiment, the authors used unfused Hep3B cells as a positive control, and

concluded that the decrease in permissivity is significant (Hikosaka et al., 2011). In this context, it is difficult to conclude whether primary mouse hepatocytes express really a HCV entry restriction factor or if the decrease is mediated by the fusion itself, as a proper control would have been to fuse Hep3B cells with primary human hepatocytes. But these results suggest that a restriction factor for HCV entry into mouse hepatocytes is a possibility to take into account.

So far, the unique factor restricting HCV entry identified is EWI-2wint (Rocha-Perugini et al., 2008). This factor is able to interfere with HCV E2 glycoprotein and CD81 binding. The authors have shown that CHO cells are able to cleave EWI-2 into EWI-2wint molecule (Rocha-Perugini et al., 2008). These results suggest that CHO cells express the protease responsible for this cleavage, if we extrapolate these results, one can say that mouse cells do express as well the protease responsible for EWI-2 cleavage. CD81 binds several partners within the tetraspanin web, and CD81 is also a putative receptor for the parasites *Plasmodium falciparum* and *Plasmodium yoelii*. It has been recently shown that CD9P-1, another CD81 binding partner, can inhibit *Plasmodium yoelii* infection of Hepa1.6 cells (Charrin et al., 2009). However, unpublished data from Cocquerel's group demonstrated that CD9P-1 has no effect on HCV entry indicating that CD81 usage for efficient entry differs between *Plasmodium* parasites and HCV (Charrin et al., 2009). It is not known whether EWI-2 is expressed in mouse cells and whether mouse cells could cleave EWI-2 into EWI-2wint, but such HCV entry inhibition in mouse hepatocytes could explain in part the low level permissivity of mouse hepatoma cells expressing the four human HCV entry factors. Recently, a heterokaryon assay has been conducted to assess whether mouse cells express a dominant negative factor for HCV infection (Frentzen et al., 2011). The authors have used a special clone of Huh7 cells, called Huh7-Lunet cells, which expresses low level of CD81 and thus is less susceptible to HCV entry. Fusing these cells with cells expressing human CD81 allowed assessing whether the tested cells express a dominant negative factor for entry, replication and assembly/release. Using mouse cells as target cells, the authors showed that no dominant negative restriction factor is expressed in mouse cells to complete the HCV viral life cycle (Frentzen et al., 2011). It is not known how efficient this system is to assess HCV entry and the fusion of the membranes lead to a drastic rearrangement, thus one cannot exclude that the "dilution" of a potential HCV entry restriction factor after fusion of the membranes in this system fails to block HCVcc infection of

heterokaryon. The absence of a dominant negative restriction factor for HCV entry thus needs to be confirmed using a different method. Nevertheless, given that efficient entry is required for efficient HCV infection, it is crucial to increase HCV permissivity of mouse cells to further study HCV replication in mouse hepatoma cells.

Altogether these results indicate that, expression of the four human HCV entry factors allows HCV entry in non-hepatic cells and mouse hepatoma cells. Furthermore, not only one factor but most likely the expression of the four HCV entry factors at substantial level is responsible for a robust HCV entry. Efficient HCV entry in non-hepatic cells correlates with efficient HCV RNA replication, indicating that HCV entry is a limiting step for non-hepatic cell and probably also mouse hepatoma cell infection. Finally, the absence or presence of the HCV entry inhibitor EWI-2wint in non-hepatic cells is likely to participate in HCV hepatotropism. Nevertheless, it remains to be confirmed whether mouse hepatoma cells do or do not express a HCV entry restriction factor.

2) HCV replication in human non-hepatic cells and mouse hepatoma cells

The complex HCV entry process leads finally to fusion of the viral membrane with the endosomal membrane releasing the viral genome into cell host cytoplasm. The first step following HCV genome delivery is translation of the positive strand RNA. There is no evidence that HCV RNA translation is restricted in non-hepatic cells, suggesting that HCV RNA replication is most likely a restricted step in non-hepatic cells.

The highly HCV entry permissive 293T-4R developed in our study did not show ability to support HCV replication, suggesting that one or several host factors are missing in those cells for efficient HCV RNA replication. HCV replication is dependent on several host cell factors as well as on viral factors. Among those host factors involved in HCV RNA replication, miR-122 is the most intriguing and important for this step of HCV life cycle. The interaction of miR-122 with HCV RNA is a crucial prerequisite for efficient HCV replication both *in vitro* and *in vivo* (Jopling et al., 2005;

Lanford et al., 2010). Furthermore, hepatoma cell lines lacking the expression of this miRNA are not able to support HCV infection, but restoring miR-122 expression in these cells restores HCV infection (Kambara et al., 2012; Narbus et al., 2012). We have shown that expression of miR-122 in the 293T-4R renders these cells capable of *de novo* HCV RNA replication upon HCVcc infection. These results are in line with previously published data showing that miR-122 enhances HCV RNA replication in 293T cells (Chang et al., 2008; Fukuhara et al., 2012). Chang *et al.* have shown that miR-122 enhanced the ability of selectable sub-genomic replicons in forming antibiotic-resistant cell colonies (Chang et al., 2008). However, they also observed HCV replication, in 293T cells expressing a mutant of miR-122 defective in HCV RNA binding, to levels comparable to cells expressing the wild-type miR-122 (Chang et al., 2008). Fukuhara *et al.* have shown HCV RNA replication in several non-hepatic cells expressing exogenous miR-122 (Fukuhara et al., 2012). They have been able to assess several cell lines with different tissue origins and found that upon miR-122 expression most of the cell lines were able to significantly support HCV RNA replication (Fukuhara et al., 2012). These results suggest that miR-122 participates in the hepatotropism of HCV. In the Fukuhara study, 293T cells expressing exogenous CLDN1 failed in replicating HCV RNA since these cells do not express miR-122. In our study, the highly permissive 293T-4R cells were not able to support a robust HCV replication. Evans *et al.* observed HCV replication in 293T cells expressing exogenous CLDN1 (Evans et al., 2007). They assessed HCV viral proteins in 293T-CLDN1 cells 72h post-infection and observed HCV NS5A positive cells (Evans et al., 2007). It is difficult to say whether this is the result of robust replication or passive HCV RNA translation. Fukuhara *et al.* quantified HCV RNA copies in their study (Fukuhara et al., 2012). In our study, we used a virus carrying a *Renilla* luciferase reporter gene to assess HCV RNA replication and we confirmed the replication by assessing HCV core protein in the 293T-4R/miR122 cells by immunofluorescence imaging studies and flow cytometry. We failed to detect HCV core protein expression 72h post-infection in the highly permissive HCV entry 293T-4R cells in the absence of miR-122 expression, suggesting that without miR-122 expression, 293T cells do not support HCV RNA replication. It is worth noting that HCV RNA replication kinetics in 293T-4R/miR122 cells are comparable to those of Huh7.5.1 cells, suggesting that apart from miR-122, 293T cells express all the factors essential for efficient HCV replication. Furthermore, using previously characterized protease and polymerase

inhibitors, we showed that HCV RNA replication in the 293T-4R/miR122 cells is dependent on similar mechanisms than HCV replication in hepatocytes. These data suggest that miR-122 is a factor restricting HCV replication to hepatocytes and thus participating in the tropism of HCV.

However, even if miR-122 is a miRNA predominantly expressed in the liver, there is evidence that HCV is able to replicate within the brain (Fishman et al., 2008; Fletcher et al., 2012; Fletcher et al., 2010). Fletcher *et al.* have shown that two human brain microvascular endothelial cell lines were able to support HCV infection. Indeed they have shown, using two different HCVcc chimeras, that these cells express HCV entry factors and allow HCV RNA replication to a less extent compared to Huh7 cells (Fletcher et al., 2012). These results are interesting since these cells do not express miR-122, suggesting that HCV replication occur in a miR-122 independent manner (Fletcher et al., 2012). The role of miR-122 in HCV RNA replication is not very clear: it has been suggested that miR-122 could enhance HCV RNA translation, mask HCV IRES from innate immunity or modulate HCV RNA abundance (Henke et al., 2008; Machlin et al., 2010; Niepmann, 2009; Norman and Sarnow, 2010; Villanueva et al., 2010). Diaz-Toledano *et al.* have identified a double helical switch in the HCV IRES involving miR-122 (Diaz-Toledano et al., 2009). It was previously known that a large sequence within the core encoding sequence is able to perfectly base pair a sequence present within the IRES to prevent HCV RNA translation (Honda et al., 1999; Kim et al., 2003). Diaz-Toledano *et al.* have shown that binding of the miR-122 to its two 5' IRES sequences lead to the disruption of RNA-RNA interaction releasing the IRES and thus stimulating HCV RNA translation (Diaz-Toledano et al., 2009). This has been recently confirmed by the Niepmann's group *in vitro* and *in vivo* (Goergen and Niepmann, 2012). Furthermore, Narbus *et al.* have been shown that even a low amount of miR-122 is sufficient to support robust HCV replication (Narbus et al., 2012). It is possible that undetectable amounts of miR-122 in brain microvascular endothelial cells are sufficient to support low level HCV RNA replication or that high HCV genetic variability can lead to HCV adaptation to extra-hepatic tissues. Such genetic adaptations have been observed in HCV genomes derived from infected human brain tissue (Fishman et al., 2008). It is also possible that both of these mechanisms participate in extra-hepatic HCV infection. We have shown that miR-122 participate in efficient and robust HCV RNA replication

in non-hepatic cells and did not assess low level miR-122 expression on HCV RNA replication. One cannot exclude that low level replication of HCV in a miR122-dependent or -independent mechanism allows the virus to stay within the host without being detected by the immune system. Further experiments are required to determine how HCV is able to replicate in brain-derived microvascular endothelial cells and determine whether brain-derived HCV contains adaptive mutations. These results have important implications, since drugs blocking miR-122 are currently in clinical trial. These results suggest that potent inhibition of HCV infection through miR-122 activity inhibition cannot be achieved in a monotherapy, otherwise it is taking the risk of a HCV recurrence from miR122-independent HCV replication in a reservoir.

To date, the only way to replicate HCV RNA in naïve mouse cell is to use genomic or sub-genomic replicons under antibiotic selection pressure (Lin et al., 2010; Uprichard et al., 2006; Zhu et al., 2003). Our results confirmed that HCV entry permissive mouse hepatoma cells are resistant to HCV RNA replication. Thus, to ascertain that HCV RNA translation is not restricted in mouse hepatoma cells, we transfected a previously described plasmid encoding HCV IRES upstream of a luciferase gene where the transcription is driven by a CMV promoter (Wolf et al., 2008). Transfection of this plasmid into mouse hepatoma cells lead to high luciferase activity in the three mouse hepatoma cells developed in this study. These results demonstrate that HCV RNA translation is not restricted and strongly suggest that HCV RNA replication is likely to be the restricted step to infect mouse hepatoma cells.

Expression of miR-122 in non-hepatic cells enhances HCV RNA replication (Chang et al., 2008; Fukuhara et al., 2012; Zhu et al., 2003). While non-hepatic cells expressing HCV entry factor and miR-122 are able to support HCVcc infection, replication of HCV RNA in mouse cells requires the use of sub-genomic replicons with an antibiotic-selection pressure to force the virus to adapt to mouse cells (Lin et al., 2010; Uprichard et al., 2006; Zhu et al., 2003). Although these sub-genomic or genomic replicons allow to study HCV replication in mouse cells, the selection pressure has two major drawbacks. Either the virus will mutate and adapt to the host cell (Zhu et al., 2003) or selection pressure will select a cell clone that has the ability to replicate HCV RNA (Uprichard et al., 2006), but in any case these systems will

allow to mimic HCV infection of human hepatocytes. Thus determining the human factors lacking in mouse cells or mouse factors restricting HCV RNA replication in mouse hepatocytes will bring us closer to a genuine HCV infection of mouse hepatocytes. Expression of miR-122 in miR-122-lacking human hepatoma cells allows to restore HCV infection (Kambara et al., 2012; Narbus et al., 2012). Given the importance of this microRNA in HCV RNA replication, we verified whether it was expressed in our HCV entry permissive mouse hepatoma cell lines. MiR-122 was not expressed or at very low level in mouse hepatoma cell lines as quantified by RT-qPCR. Lentiviral vector-mediated miR-122 expression in mouse hepatoma cell lines was quantified in the transduced cells and miR-122 expression levels were lower than in Huh7.5.1 cells but at comparable range of expression. miR-122-expressing mouse hepatoma cells were challenged with HCVcc. Low luciferase activity was detected in HCVcc infected cells compared to a replication defective virus (HCVcc GNN). These results suggest that miR-122 expression is able to enhance HCV RNA replication in mouse hepatoma cells but at levels 1000 times lower than those observed in Huh7.5.1 cells. Since miR-122 expression rate was lower than in Huh7.5.1 cells, we decided to ascertain that this was not the reason of low HCV RNA replication in mouse hepatoma cells. MiR-122 mimic transfection in miR-122 expressing mouse hepatoma cells slightly increased luciferase activity in cells infected with HCV, compared to miR-Control infected cells. MiR-122 level in transfected cells was quantified and was much higher than in miR-Control transfected Huh7.5.1 cells. These results indicate that miR-122 expression rate does not influence HCV RNA replication in mouse hepatoma cells. Our HCV entry permissive mouse hepatoma cells expressing miR-122 contain lower level of miR-122 than in Huh7.5.1 cells, but we showed that high miR-122 expression rate does not overcome HCV RNA replication blockade in mouse hepatoma cells. Moreover, if we extrapolate the results from Narbus *et al.*, we can say that miR-122 expression levels in mouse hepatoma cells, although lower than in Huh7.5.1, are sufficient to support HCV RNA replication (Narbus et al., 2012). These results suggest that a factor important for HCV RNA replication is missing in mouse hepatoma cells for efficient HCV RNA replication, or a mouse factor is expressed and restricts HCV RNA replication in this context.

To infect human hepatocytes, HCV has the capability to control the innate immune defense by cleaving the key adaptor molecules MAVS (Li et al., 2005b) and

TRIF (Li et al., 2005a). This suggests that IFN response control is an important feature of an efficient HCV infection of human hepatocytes. Recently, Lin *et al.* assessed whether IFN response could inhibit HCV RNA replication in mouse cells and they used mouse embryonic fibroblasts (MEFs) from interferon regulatory factor 3 (IRF3) knock-out mice (Lin et al., 2010). Upon expression or miR-122 in IRF-$3^{-/-}$ MEFs, they have been able to detect HCV replication after electroporation of a sub-genomic replicon (Lin et al., 2010). Interestingly, the authors have shown that IRF-$3^{-/-}$ MEFs cells were able to significantly sustain HCV RNA replication but expressing miR-122 in these cells further enhanced HCV RNA replication (Lin et al., 2010). These results suggest that IFN response controls HCV replication in mouse cells. Another recent study confirmed the importance of HCV RNA replication control by innate immune response. Indeed, Aly *et al.* have developed mouse hepatoma cells supporting HCV infection by transducing hCD81 into hepatocytes derived from IPS-1 (also called MAVS, CARDIF and VISA) or IFN alfa receptor (IFNAR) knock-out (KO) mice (Aly et al., 2011). These results confirm the importance of the control of HCV RNA replication in mouse hepatocytes (Aly et al., 2011). Furthermore, the authors have demonstrated that knocking-out the TLR-3 adaptor molecule TRIF (also known as TICAM-1) does not allow HCV RNA replication suggesting that dsRNA detection by TLR-3 in mice is not the pathway restricting HCV RNA replication (Aly et al., 2011). Interestingly, the authors have been able to infect immortalized mouse hepatocytes derived from either IPS-1 or IFNAR KO mice, by expressing only hCD81 but at low level (Aly et al., 2011). Since, the development of an immunocompetent mouse model is crucial for the characterization of HCV infection *in vivo*, down-regulating or deleting key molecules involved in innate immune response such as IFNAR or IRF3 does not constitute a breakthrough for the development of such immunocompetent small animal model. The identification of the factor(s) involved in HCV control in mouse hepatocytes will allow the elaboration of mouse hepatocytes able to replicate HCV RNA. Since MAVS and TRIF are targets of HCV NS3-4A in human hepatocytes, one could speculate that genetic differences between mouse and human MAVS and TRIF could explain such control of HCV replication in mouse hepatocytes. While mouse MAVS has been clearly demonstrated to be cleaved by HCV NS3-4A (Ahlen et al., 2009), mouse TRIF has been shown to not be cleaved by this viral protease (Abe et al., 2007). More recently, a study from the Rice's group has shown that in MEFs HCV NS3-4A is able to cleave both mMAVS and mTRIF

suggesting that these molecules do not contribute to HCV replication control in mouse hepatocytes. Interestingly, the authors have shown that type I and III IFN response impairment allowed persistent HCV RNA replication in MEF cells expressing miR-122 (Alexander Vogt' presentation, EASL Meeting April 2012, Barcelona, Spain). These results suggest that innate immune response has an important role for HCV RNA replication control in mouse hepatocytes. Additional studies are required to characterize the precise factors responsible for this control. Furthermore, apart from restriction of HCV infection, it is also conceivable that other factors are lacking in mouse hepatoma cells to sustain a robust HCV RNA replication in mouse hepatocytes.

3) HCV assembly in human non-hepatic cells and mouse hepatoma cells

It is believed that HCV assembly takes place at the ER membrane in close proximity to lipid droplets. Assembly and release processes are not well characterized but these last years, effort has been done to understand the mechanisms allowing HCV assembly and release in hepatocytes. Since HCV is associated with low density lipoproteins in the bloodstream as LVPs, it has been suggested that HCV relies on the VLDL secretion pathway for its assembly and release (Huang et al., 2007). Thus, proteins such as MTP, apoB, DGAT-1 and apoE have been proposed to take part in the assembly and release of HCV particles (Benga et al., 2010; Diaz et al., 2006; Gastaminza et al., 2008; Herker et al., 2010). Among the host cell factors involved in HCV assembly and release, apoE is probably the most important, likely because this protein is found on the surface of the viral particles (Chang et al., 2007; Jiang and Luo, 2009) and because it has been shown to interact with the viral protein NS5A and to regulate HCV assembly (Benga et al., 2010). In our study, the 293T-4R/miR122 cells did not release infectious HCV particles, since we have shown that apoE is not expressed in 293T cells. We thus expressed apoE in order to assess whether non-hepatic cells, in presence of apoE, have the capability of supporting HCV assembly and release. We have been able to detect infectious HCV particle release from the 293T-4R/miR122/apoE cells. Viral assembly and release from these cells was low but significantly higher than in 293T-4R/miR122 cells. The low level of HCV release suggests that other factors are lacking in 293T cells to support robust HCV assembly and release.

The characterization of HCV particles released from 293T cells shows that particles are associated with apoE, since monoclonal and polyclonal anti-apoE antibodies potently inhibited 293T-derived HCVcc. These results suggest that 293T-derived HCVcc are assembled in the same way as in Huh7.5.1 cells but at lower rate. Furthermore, the 293T-derived HCVcc are able to re-infect Huh7.5.1 cells following the same route of entry as Huh7.5.1 derived HCVcc, since monoclonal antibodies against CD81, CLDN1 and SR-BI potently inhibited Huh7.5.1 infection by 293T-derived HCVcc. These results have important implications because it is believed that HCV rely on the VLDL pathway for assembly and release, although 293T cells do not contain the VLDL pathway, they are still able to support HCV assembly and release upon apoE expression. Fletcher *et al.* have shown that HCV is able to infect brain derived macrovascular endothelial cells; they detected HCV entry, replication, assembly and release of infectious HCV particles (Fletcher et al., 2012). They have been able to detect infectious HCV particle release from the HCV infected microvascular endothelial cells 8h post-infection, but failed to detect HCV particle release 12h post-infection indicating that low HCV particle release transiently occurs in these cells (Fletcher et al., 2012). The authors did not assess apoE expression in the cell lines they have used, but it is known that neuronal cells express high amounts of apoE (Xu et al., 2006). These results, along with the results we obtained in our study, support the idea that non-hepatic cells replicating HCV RNA and expressing apoE are able to assemble and release infectious HCV particles.

ApoE is expressed as different isoforms and apoE3 is the most common isoform. It has been suggested that HCV assembly and release is isoform-dependent, since apoE2 has been shown to be incapable of supporting HCV assembly and release (Hishiki et al., 2010). Our study shows that all the apoE isoforms tested are similarly capable in supporting HCV assembly and release in a non-hepatic context. These results are in line with a previous study which demonstrated that mouse cells replicating a sub-genomic replicon and transcomplemented with HCV structural proteins are able to efficiently support HCV assembly and release when different apoE isoforms are expressed (Long et al., 2011). Furthermore, the authors have demonstrated that in the context of mouse hepatoma cells replicating a sub-genomic replicon, mouse apoE was as efficient as human apoE to support HCV assembly and release (Long et al., 2011). In our study,

we confirmed these results in the context of HCV infection of non-hepatic cells and demonstrated that mouse apoE is as efficient as human apoE to support HCV assembly and release. Furthermore, it has been demonstrated using a heterokaryon assay that no dominant negative restriction factor is present in mouse cells to prevent HCV assembly and release (Frentzen et al., 2012; Frentzen et al., 2011). Since the mouse hepatoma cells we developed do not replicated HCV RNA, they are useful tools to identify factors involved in HCV replication. Thus to complete HCV life cycle in mouse hepatoma cells, we expressed mouse apoE in the engineered mouse cells. Further studies will allow assessing whether these engineered mouse hepatoma cells express all necessary host factors to allow efficient HCV assembly.

Finally, in our study, the low HCV particle release from 293T-4R/miR122/apoE infected cells strongly indicates that other factors are lacking in 293T cells for a robust HCV assembly and release. The 293T-derived cell line developed in this study is thus a valuable tool to assess the role of such factors in HCV assembly and release. Several factors have been involved in HCV assembly and most of them are part of the VLDL pathway. Given that 293T cells do not contain a functional VLDL pathway, the expression of the previously identified assembly/release factors in the engineered 293T cells would bring valuable information on their role in this process.

MATERIAL AND METHODS

I) Materials

a. Cells

All cells were maintained at 37°C with water saturated atmosphere and added of 5% CO_2.

Huh7.5.1 cells were grown in DMEM 4,5 g/l of glucose (PAA Laboratories) complemented with 10% of Fetal Bovine Serum (FBS) (PAN Biotech) and gentamycin (50 µg/ml, Gibco).

293T, BNL-1 (ATCC Catalog #: TIB-75) and **Hepa1.6** (ATCC Catalog #: CRL-1830) cells were grown in DMEM 4,5 g/l of glucose (GIBCO, Life Technologies) complemented with 10% of FBS (PAN Biotech) and gentamycin (50 µg/ml, GIBCO Life Technonologies).

AML12 (ATCC Catalog #: CRL-2254) cells were maintained in DMEM-HamF12 (50% DMEM High Glucose-50% HamF12 medium) complemented with 10% FBS, 5 ml ITS 100x (insulin, transferrin, selenium) (GIBCO, Life Technologies), dexamethazone (40 ng/ml).

For selection process of the cell lines expressing the different HCV life cycle factors, see the method section.

b. Reagents

➢ Plasmids

pIV1132 has been developed in the laboratory and previously described in (Wolf et al., 2008). This plasmid allows the transcription of the HCV IRES followed by the 10 first amino acids of the core protein fused to the firefly luciferase gene. This plasmid allows the study of the HCV IRES recognition and efficient HCV RNA translation.

pWPT-puromycin-miR-122 is a plasmid I have engineered to express miR-122 and a resistance gene to puromycine. This plasmid has been developed as follows. Due to restriction sites incompatibility, the expression cassette Puromycine-pri-miR-122 has been assembled into a pUC19 plasmid. A unique BstBI restriction site has been inserted by mutagenesis at position 339 in the pUC10 plasmid.

Puromycine and pre-miR-122 has been amplified from a pRTS plasmid and 293T genomic DNA, respectively, using the primers shown in Table 1.

Gene amplified	Sens	Sequence (5' - 3')
Puromycine	**Forward**	TGACGCGGATCCAGGCCTAAGCTTACGCGTCCTAG
		CGCTACCGGTCGCCACCATGGCCGAGTACAAGCCCA
	Reverse	ATAAAGCTTTCAGGCACCGGGCTTGCGGGT
Pri-miR-122	**Forward**	ATAGAATTCAGAGTGTTCAGCTCTTCCCATTGC
	Reverse	ATATTCGAAGTAACAACAGCATGTGAGAGGCAG

Table 1: Primers used to amplify puromycin and pri-miR-122 genes.

Puromycine resistance gene has been inserted into the pUC19 using BamHI and HindIII restriction sites present in the pUC19 plasmid. Pri-miR-122 sequence was inserted into the pUC19 plasmid through EcoRI and BstBI restrictions sites. pUC19 plasmid containing the engineered cassette has been digested with BamHI and BstBI to insert the cassette into the pWPT-GFP backbone previously digested with BamHI and BstBI. Puromycine and pri-miR-122 sequences were sequenced to preclude mutation insertion during the cloning. pWPT-GFP plasmid was obtained from Addagene, pRTS and pUC19 were kind gifts from S. Pfeffer (IBMC, University of Strasbourg, Strasbourg, France).

pWPI is a lentiviral vector allowing the simultaneous expression of two different proteins driven by an elongation factor alfa promoter and a encephalomyocarditis virus (ECMV) IRES. It has been used to develop the lentiviral vector for the expression of the HCV entry factors and apoE isoforms with either neomycin or blasticidin resistance gene. HCV entry factor-encoding plasmids were a generous gift from T. Pietschmann (Division of Experimental Virology, TWINCORE, Hannover, Germany) and they have been previously published (Bitzegeio et al., 2010). ApoE isoform-encoding plasmids were generously sent by R. Bartenschlager (Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany) and they have been previously published (Long et al., 2011).

The plasmids used for the production of HCVpp or VSVpp have been previously described (Bartosch et al., 2003a). Briefly, a plasmid encoding the proteins *gag* and *pol* of the human immunodeficiency virus (HIV) under a cytomegalovirus promoter, is

transfected together with a plasmid encoding the HCV glycoproteins E1-E2 (for the production of HCVpp) or the VSV G-protein (for the production of VSV-Gpp) under a CMV promoter. A third plasmid encoding the gene to be inserted, luciferase or GFP for HCV entry monitoring or HCV host factors and antibiotic resistance gene for lentiviral-mediated stable genomic insertion, is transfected with the two previous described plasmids. For pseudo-typed retroviral particles production, see the method section.

- **Viruses**

Jc1 is a chimeric virus where the structural protein encoding region of the J6 strain (genotype 2a) was fused to the non-structural protein encoding region of the JFH1 strain (genotype 2a) **(Figure 21)** (Pietschmann et al., 2006). This virus does not contain a reporter gene and infection is detected by assessing the presence of HCV RNA by RT-qPCR or HCV proteins by immunostaining.

JcR2a is a virus derived from the Jc1 virus. It encodes the Renilla luciferase gene embedded within the structural protein encoding region between the core and E1 proteins **(Figure 21)** (Reiss et al., 2011). Renilla luciferase is expressed in the cytoplasm of HCV infected cells. Plasmid encoding the JcR2a virus is a generous gift from R. Bartenschalger (Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany).

Jc1FLAG(p7-nsGluc2A) is a virus derived from the Jc1 virus. It contains a Flag epitope at the E2's N-terminus and a Gaussia luciferase gene embedded between the p7 and NS2 proteins **(Figure 21)** (Marukian et al., 2008). The replication defective virus Jc1FLAG(p7-nsGluc2A)/GNN contains a mutation within the RdRp NS5B encoding region, blocking HCV RNA replication. Gaussia luciferase has been engineered to be excreted in the extracellular medium from HCV infected cells. This system allows HCV infection monitoring overtime without lysing cells for luciferase release. Plasmids encoding the Jc1FLAG(p7-nsGluc2A) and Jc1FLAG(p7-nsGluc2A)/GNN viruses have been obtained from C. Rice (The Rockefeller University, New York, USA).

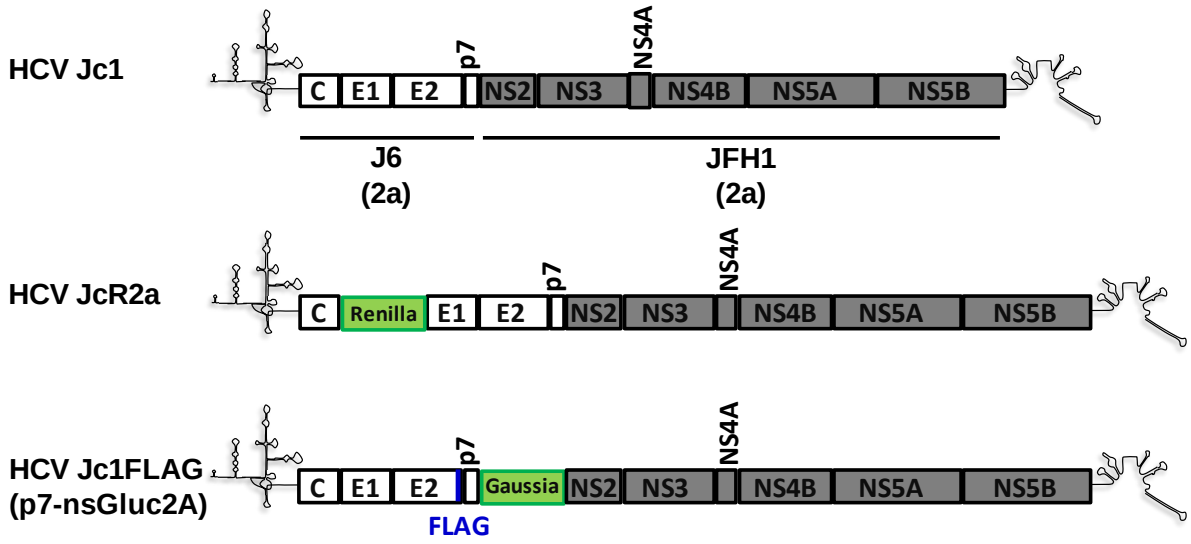


Figure 21: Chimeric HCV viruses used in the present study.

➢ Antibodies

Primary antibodies used in this study are described in the following table:

Antibodies	Clonality	Origin	Reference	Application
CD81	Monoclonal	Mouse	Cat.# 555675, BD Pharmingen	FACS, HCV entry inhibition
OCLN	Monoclonal	Mouse	Cat.# 33-1500 Invitrogen	FACS
CLDN1	Monoclonal	Rat	Fofana et al., 2010	FACS, HCV entry inhibition
SR-BI	Monoclonal	Rat	Zahid et al., submitted manuscript	FACS, HCV entry inhibition
EGFR	Monoclonal	Mouse	Cat.# sc-120, Santa-Cruz	FACS
h-apoE	Polyclonal Monoclonal Monoclonal	Goat Mouse Mouse	Cat.# 178479, Calbiochem Raffai et al. 1995 Cat.# ab1906, Abcam	HCV entry inhibition HCV entry inhibition FACS, WB
m-apoE	Polyclonal	Rabbit	Cat.# ab20874	WB
HCV Core	Monoclonal	Mouse	C7-50, Thermo Scientific	IF
Normal IgG	Polyclonal	Mouse	Cat.# 10400C, Life Technologies	FACS, IF, HCV entry inhibition
Normal IgG	Polyclonal	Rat	Cat.# 10700, Life Technologies	FACS, IF, HCV entry inhibition
Actin	Polyclonal	Rabbit	Cat.# A5060, Sigma	WB

Table 2: List of primary antibodies used in the present study.

➢ Other reagents

MiR-122 expressing cells were either developed using home-made miR-122 lentiviruses or commercial lentiviruses obtained from ABM Good (Cat.# mh15049,

ABM Good). These lentiviruses allow the simultaneous expression of pri-miR-122, puromycin resistance gene and GFP.

RT-qPCR for miR-122 expression analysis was performed using the miScript II RT for microRNA reverse transcription (Cat.# 218161, Qiagen) and the miScript SYBR Green PCR kit for miRNA quantification (Cat.# 218073, Qiagen). Quantification was performed using specific miR-122 primers (Cat.# MS00003416, Qiagen) and quantification was normalized to the internal small RNA RNU6B (Cat.# MS00033740, Qiagen).

MiR-122 mimic and miR-control were purchased from Dharmacon (Cat.# C-300591-05-0005 and CN-001000-01-05 respectively).

All the transfection performed in the present study were performed using Lipofectamin 2000® (Cat.# 11668-027, Life Technologies) following the manufacturer's instructions.

II) Methods

a. Pseudo-typed particle production

Pseudo-typed lentiviral particles were produced as previously described (Bartosch et al., 2003a). 293T cells were transfected with a combination of three plasmids using calcium phosphate as presented:

Plasmid 1	Plasmid 2	Plasmid 3	Product
Envelope	**Core**	**Inserted gene**	**Pseudo-particle**
HCV E1-E2	HIV-Gag/Pol	Luciferase	HCVpp
VSV-G	HIV-Gag/Pol	Luciferase	VSVpp
VSV-G	HIV-Gag/Pol	Host cell factors (CD81, OCLN, CLDN1, SR-BI, miR-122, apoE)	VSVpp

24h post-transfection, fresh medium was added and pseudo-particles harvested at 48h and 72h post-transfection. Pseudo-particles containing medium was filtered with a 0,45 µm filter to remove any cell fragments. Pseudo-particles were then used fresh or aliquoted and kept at -80°C.

b. Cell transduction

293T, AML12, BNL-1 and Hepa1.6 cells were transduced with host cell factors encoding VSVpp to express defined sets of host cell factors involved in HCV life cycle. To do so, VSVpp were produced as described above and concentrated. Concentrated VSVpp were deposited on cells in a 6 well plate ($2x10^5$ cells per well) for 6h. Following transduction, fresh medium was added for 72h. Sets of defined host cell factors are described in the following table:

293T cells	CD81	OCLN	CLDN1	SR-BI	miR-122	apoE
2R	CD81/Blast.	OCLN/Blast.	X	X	X	X
3R	CD81/Blast.	OCLN/Blast.	CLDN1/Blast.	X	X	X
4R	CD81/Blast.	OCLN/Blast.	CLDN1/Blast.	SR-BI/Blast.	X	X
4R/miR122	CD81/Blast.	OCLN/Blast.	CLDN1/Blast.	SR-BI/Blast.	miR-122/Puro	X
4R/miR122/apoE	CD81/Blast.	OCLN/Blast.	CLDN1/Blast.	SR-BI/Blast.	miR-122/Puro	apoE/Blast.
Blasticidine	12µg/ml	**Puromycine**	2,5µg/ml			

AML12 cells	CD81	OCLN	CLDN1	SR-BI	miR-122	apoE
2R	CD81/G418	OCLN/Blast.	X	X	X	X
3R	CD81/G418	OCLN/Blast.	CLDN1/G418	X	X	X
4R	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	X	X
4R/miR122	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	miR-122/Puro	X
4R/miR122/apoE	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	miR-122/Puro	apoE/Blast.
G418	500µg/ml	**Blasticidine**	24µg/ml	**Puromycine**	0,3µg/ml	

BNL-1 cells	CD81	OCLN	CLDN1	SR-BI	miR-122	apoE
2R	CD81/G418	OCLN/Blast.	X	X	X	X
3R	CD81/G418	OCLN/Blast.	CLDN1/G418	X	X	X
4R	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	X	X
4R/miR122	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	miR-122/Puro	X
4R/miR122/apoE	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	miR-122/Puro	apoE/Blast.
G418	1200µg/ml	**Blasticidine**	5µg/ml	**Puromycine**	2µg/ml	

Hepa1.6 cells	CD81	OCLN	CLDN1	SR-BI	miR-122	apoE
2R	CD81/G418	OCLN/Blast.	X	X	X	X
3R	CD81/G418	OCLN/Blast.	CLDN1/G418	X	X	X
4R	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	X	X
4R/miR122	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	miR-122/Puro	X
4R/miR122/apoE	CD81/G418	OCLN/Blast.	CLDN1/G418	SR-BI/Blast.	miR-122/Puro	apoE/Blast.
G418	1100µg/ml	**Blasticidine**	8µg/ml	**Puromycine**	1,8µg/ml	

Table 3: Summary of host cell factors expressed in the cell lines engineered in the present study.

Selection of transduced cells was started 72h post-transduction and maintained over a period of 2 weeks with the indicated concentration of the corresponding antibiotic(s). Antibiotic resistant cells were then assessed for host cell factor expression by FACS (HCV entry factors, apoE), RT-qPCR (miR-122) or western blot (apoE).

c. Flow cytometry

Flow cytometry analysis was used to assess HCV entry factors CD81, OCLN, CLDN1, SR-BI and EGFR, using the corresponding primary antbodies indicated in **table 2**. $2x10^5$ cells/well were washed with PBS and stained with primary antibody (10 µg/ml) or corresponding control antibody for 1h. Three PBS washes were performed on primary antibody treated cells to remove unbound antibody. Phycoerythrin (PE)-conjugated secondary antibodies were used to stain cells for 1h. Cells were then washed with PBS and fixed with PBS complemented of 2% paraformaldehyde (PFA).

In the case of OCLN, the commercial antibody recognizes an intracellular epitope. In this case a permeabilization step was performed before staining. After the initial PBS wash, cells were permeabilized using a permeabilization buffer (PBS, 1% FBS and 0,1% saponin) for 20 min on ice. Primary anti-OCLN antibody and secondary antibodies were diluted into permeabilization buffer.

d. miRNA detection by RT-qPCR

MiR-122 transduced cells were analysed for miR-122 expression using Qiagen kits (See I. b. Other reagents). Total RNA extraction from transduced cells was performed using Tri-reagent® (MRC, Cat.# TR 118) and following the manufacturer's instructions. 300 ng of total RNA was reverse transcribed using the miScript II RT kit. 2 µl of the reverse transcription reaction was used as a template for miR-122 expression analysis using the miScript SYBR Green PCR kit and the primers indicated in I. b. Other reagents section for miR-122 and RNU6B as an internal control. Relative quantification of the miR-122 was performed using the $2^{\Delta\Delta Ct}$ method. The calculation was done as following:

ΔCt = **Ct** (RNU6B-Sample) – **Ct** (miR-122-Sample)

ΔΔCt = **ΔCt** (Sample) – **ΔCt** (Reference cells (Huh7.5.1))

RQ= $2^{-\Delta\Delta Ct}$

e. HCVpp and VSVpp challenge

HCV entry host cell factor expressing cells were challenged with HCVpp and VSVpp to assess their permissivity to HCV entry. Cells were incubated either with medium, HCVpp or VSVpp for 4-6h at 37°C. Fresh medium was then added and cells were incubated during 72h at 37°C. 72h post-challenge, cells were lysed for luciferase activity. Percentage HCV entry was calculated as described in (Ploss et al., 2009). Briefly, the background luciferase signal was substracted from HCVpp and VSVpp signals. The HCVpp signal was then normalized to VSVpp entry and then normalized to HCVpp entry in Huh7.5.1 cells to allow for cross-experimental comparison. Results are expressed as means ± SD of three independent experiments performed in triplicate.

f. MiR-122 and miR-Control mimic transfection

MiR-122 stably expressing cells were transfected with a miR-122 mimic or miR-Control to assess the effect of miR-122 levels on HCV replication. To do so, cells were plated in 24 well plates and transfected as follows:

	Cells/well	miRNA (pmol)	Lipofectamine 2000 (µl)
293T	60000	20	1
AML12	50000	40	2
BNL-1	50000	40	1,5
Hepa1.6	60000	20	1

Table 4: Protocol for miR-122 mimic transfection.

Transfection was performed using Lipofectamine 2000® and following the manufacturer's instructions. 24h post-transfection, cells were either challenged with HCVcc or analyzed by RT-qPCR as described previously.

g. Western blot analysis

Cells transduced with an apoE encoding VSVpp were analysed by western blot. 50 µg of total protein lysate was loaded on a 12% bisacrylamide gel and run for 2h a 100V. Proteins were then transferred to an activated Polyvinylidenedifluoride (PVDF) membrane (Amersham). To activate the PVDF membrane, a methanol bath was performed for 30 s followed by an extensive wash with water. Transfer of proteins was realized using Bio-Rad tank transfer system for 1h15 at 300 mA. To avoid aspecific binding of antibody, PVDF membrane was blocked with PBS-Tween complemented with 5% of milk. Then, the membrane was first incubated with adequate primary antibody (See Table 2) followed by phosphatase alkaline conjugated secondary antibody. Signal was revealed using enhanced chemico fluorescence (ECF) and a Typhoon 9400 scanner (GE Healthcare).

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Study of cell host factors involved in Hepatitis C virus tropism

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Résumé

Le virus de l'hépatite C (HCV) est un problème majeur de santé publique. Le développement de nouveaux traitements pour lutter contre le HCV a été ralenti par l'absence de modèles d'études *in vitro* et *in vivo* convenables. Le but de mon travail de thèse a été, dans un premier temps, de caractériser les facteurs déterminant le tropisme hépatique du HCV. En exprimant des facteurs clés dans une lignée cellulaire humaine non-hépatocytaire, nous avons reconstitué *in fine* l'ensemble du cycle viral dans ces cellules. L'entrée du virus dans la cellule hôte fait intervenir différents récepteurs d'entrée dont CD81, occludin (OCLN), claudin-1 (CLDN1) et scavenger receptor class B type I (SR-BI). L'expression de ces quatre récepteurs sur cette lignée la rend hautement permissive à l'entrée du virus, mais ne permet pas de rétablir la réplication du virus. L'expression du micro-ARN 122, un micro-RNA important pour l'infection du HCV, dans les cellules exprimant les quatre récepteurs, restaure une forte réplication de l'ARN viral mais ne permet pas de détecter une production de particules infectieuses. L'expression de l'apolipoprotein E (apoE), jouant un rôle primordial dans l'assemblage et la sécrétion, rétablis cette dernière étape du cycle viral du HCV dans la lignée cellulaire humaine non-hépatocytaire. Dans un second temps, j'ai utilisé la stratégie, précédemment établie, pour étudier la spécificité d'espèce de l'infection du HCV dans plusieurs lignées hépatocytaires murines. Nous avons pu rendre ces cellules permissives à l'entrée du HCV et pu détecter une très faible réplication. L'ensemble de mes travaux apportent de nouvelles informations sur la compréhension des facteurs clés nécessaire au cycle viral du HCV dans des cellules murines et humaines.

Mots clés : Intéraction virus-hôte, Tropisme du HCV, spécificité d'espèce du HCV, entrée du HCV, replication du HCV, assemblage du HCV et modèle murin du HCV.

Summary

Hepatitis C virus (HCV) is a global health burden. The development of new therapeutics to treat HCV infection has been hampered by the lack of convenient *in vitro* and *in vivo* model systems. The goal of my PhD work was, in a first time, to characterize the factors determining the hepatotropism of HCV. By expressing key factors within a non-hepatic cell line, we reconstituted *in fine* the full HCV life cycle in those cells. Virus entry into the host cell requires different entry factors which are CD81, occludin (OCLN), claudin-1 (CLDN1) and the scavenger receptor class B type I (SR-BI). The expression of these four factors in this cell line renders it highly permissive to viral entry, but does not allow restoring replication of the virus. The expression of miR-122, a micro-RNA important for HCV infection, into the cell lines expressing the four HCV entry factors restore a strong replication of the HCV RNA but does not allow detecting infectious viral particle production. Further expression of the apolipoprotein E (apoE), which plays a critical role in the assembly and release process, restore the last step of the HCV life cycle in a non-hepatic cell line. In a second part of my PhD, I have used the previously developed strategy to study the species specificity of HCV infection using different mouse hepatoma cell lines. We have been able to render these cell lines permissive to HCV entry and have been able to detect a slight replication. Altogether, my results bring new information on the understanding of key factors important for HCV life cycle in mouse and human cells.

Key words: : Hepatitis C virus infection, virus-host interaction, HCV tropism, HCV species specificity, HCV entry, HCV replication, HCV assembly and HCV mouse model.