Acta virologica **65**: 279 – 287, 2021 doi: 10.4149/av_2021_307

Scavenger receptor class B type I is more conducive for genotype 1b hepatitis C virus internalization than low-density lipoprotein receptor

Xiangyi Cao[1,3#], Qiong Kang[2#], Jiang Deng[3#], Jun Xiao[4], Yanyu Zhang[3], Ping Ma[3], Xiaoang Yang[1*], Liping Lv[3*]

[1]BGI College & Henan Institute of Medical and Pharmaceutical Sciences in Academy of Medical Science, Zhengzhou University, P. R. China; [2]Dongcheng District Primary and Secondary School Health Care Centre, P. R. China; [3]Institute of Health Service and Transfusion Medicine, Beijing 100850, P. R. China; [4]Air Force Medical Center, PLA, P. R. China

Received December 2, 2020; revised March 23, 2021; accepted July 15, 2021

Summary. – The current limited understanding of HCV entry mechanisms hinders the development of specific antiviral drug screening techniques and vaccine assessment. HCV subtypes and cellular surface proteins both can affect virus tropism. Human factors such as low-density lipoprotein receptor (hLDLR), CD81 (hCD81), scavenger receptor class B type I (hSR-BI), claudin 1 (hCLDN1), and occludin (hOCLN) assist HCV entry into hepatocytes. Here, we studied the importance of five human proteins in the process of cell culture-derived (HCVcc) and serum-derived (HCV-sd) HCV entry using constructed humanized mouse hepatocytes and mouse models. We determined that unlike hLDLR, hSR-BI was an indispensable factor for 1b genotype HCV adsorption. Furthermore, this attachment can be completely prevented by treatment with a monoclonal antibody targeting hSR-BI. Our data support the idea that SR-BI is an essential factor in HCV infection, particularly during the initial HCV particle-binding step. This novel finding will facilitate the development of antiviral drugs and vaccines.

Keywords: hepatitis C virus; virus internalization; model construction; hSR-BI

Introduction

Hepatitis C virus (HCV) infection represents a global health problem. Approximately 130–170 million individuals are chronically infected with HCV, and the majority of those infected develop liver cirrhosis and/or hepatocellular carcinoma. The recently discovered direct-acting antivirals represent a promising treatment for the majority of patients (Li and Chung, 2019). However, the lack of vaccines and/or entry inhibitors remains a pressing issue with regard to the prevention of HCV infection. Corresponding small animal models are needed for drug screening and vaccine assessment, and tremendous efforts have been made to establish these models. Among them, the genetically humanized mouse model has become the most attractive. This model was first reported by Dorner *et al.* (2011) and was generated by expressing human HCV entry factors in the mouse liver.

Early HCV infection is a complex process that involves several events. Numerous studies have demonstrated that HCV particles are associated with very low-density lipoprotein (VLDL) or low-density lipoprotein (LDL) in the blood, and this leads to the formation of lipoviral particles (LVPs) (Nielsen *et al*., 2006). This interaction

*Corresponding authors. E-mails: lvlp2020@163.com (Liping Lv); xiaoangyang@163.com (Xiaoang Yang); phone: 8610-66932952 (Liping Lv), 86371-66659278 (Xiaoang Yang). #Xiangyi Cao, Qiong Kang and Jiang Deng contributed equally to this work.

Abbreviations: HCV = hepatitis C virus; hCD81 = human CD81; hCLDN1 = human claudin 1; HCVcc = cell culture-derived HCV; HCV-sd = serum-derived HCV; hLDLR = human low-density lipoprotein receptor; hOCLN = human occludin; hSR-BI = human scavenger receptor class B type I; p.i. = post infection

permits the virus to bind to target cells via lipoprotein receptors in addition to viral structural proteins. Several cellular factors reportedly mediate the HCV entry process, including low-density lipoprotein receptor (LDLR) (Agnello *et al*., 1999; Lv *et al*., 2009; Owen *et al*., 2009), scavenger receptor type B class I (SR-BI) (Scarselli *et al*., 2002), tetraspanin CD81 (Pileri *et al*., 1998), tight junction claudin 1 (CLDN1) (Evans *et al*., 2007), occludin (OCLN) (Liu *et al*., 2009; Ploss *et al*., 2009), and others (Barth *et al*., 2003; Koutsoudakis *et al*., 2006; Lupberger *et al*., 2011; Sainz *et al*., 2012; Martin and Uprichard, 2013). Recent evidence suggests that both human LDLR (hLDLR) and human SR-BI (hSR-BI) can bind native LDL and that they both play an important role in early viral infection. However, several unresolved questions remain regarding the functions of both factors. One of the unaddressed issues is whether they play equally important roles or whether one of them is predominant. To shed light on these questions, studies examining the interactions between HCV and target cells at the onset of early viral infection are suitable. In addition to serum-derived HCV (HCV-sd), a recently discovered cell-culture-derived infectious HCV (HCVcc) particles have been used in several experiments, including the study of initial HCV particle-binding steps (Owen *et al*., 2009; Sainz *et al*., 2010).

The development of an available cell model is a prerequisite to recapitulate the details of HCV natural infection *in vitro*. In the present study, we utilized lentivirus vectors expressing human hLDLR, hCD81, hSR-BI, hCLDN1, and hOCLN in tandem and obtained humanized murine hepatocytes by transfecting mouse hepatoma carcinoma Hepa1-6 cells with recombinant lentivirus. HCVcc and HCV-sd were used to test the HCV susceptibility of the cell models. Humanized murine hepatocytes stably expressing several human HCV entry factors were successfully established and were found to be susceptible to HCV infection. Based on these models, the function of hLDLR was assessed, and the proportion of hSR-BI-mediated HCV entry was determined. We also performed validation studies *in vivo*. In summary, we determined that hSR-BI played an indispensable role in HCV (genotype 1b) infection and that hLDLR could promote HCV-sd entry. Meanwhile, the function of hSR-BI in the context of early HCV (genotype 1b) infection is more important than that of hLDLR. Our findings support the idea that hSR-BI functions as an essential factor during HCV virus invasion.

Materials and Methods

Cells. Hepa1-6, Huh7.5.1, NIH3T3, HepG2, and HEK293FT cells were maintained in our laboratory. Cells were grown in DMEM with 10% FBS.

Plasmids. The packaging plasmids pMDLg/pRRE, pRSV-REV, and pCMV-VSV-G and the recombinant expression vectors pCDH-hLDLR-hSR-BI-hCD81-GFP, pCDH-hSR-BI-hCD81-GFP, and pCDH-hCLDN1-hOCLN-DsRed have been described previously (Lv *et al*., 2015). The linker between hLDLR and hSR-BI was TGGGG GGGGS GGGGS. The linker between hSR-BI and hCD81 was ASGGG GGGGS GGGGS. The hLDLR ORF was amplified from the pcDNA3-hLDLR vector using the primers: forward, 5'-CTA GTC TAG AGC CAC CAT GGG GCC CTG GGG CTG G-3', and reverse, 5'-CAT GAC CGG TCG CCA CGT CAT CCT CCA GAC TGA C-3'. hSR-BI ORF was amplified from human hepatoma HepG2 cells using the primers: forward, 5'-CTA GTC TAG AAC CGG TGG CGG TGG CGG TGG CGG AGG ATC CGG CGG AGG CGG CTC CGG CTG CTC CGC CAA AGC GCG-3', and reverse, 5'-CTA GCT AGC CAG TTT TGC TTC CTG CAG CA-3'. The hCD81 ORF was amplified from Huh7.5.1 cells using the primers: forward, 5'-CTA GTC TAG AGC TAG CGG CGG GGG CGG TGG CGG AGG CTC CGG A-3', and reverse, 5'-GGA GGG GGA TCC GGA GTG GAG GGC TGC ACC AA-3'. The fragments were digested and inserted into the pCDH-EF1-MCS-GFP vector, and the constructed vector was named pCDH-hLDLR-hSR-BI-hCD81-GFP/pCDH-hSR-BI-hCD81-GFP.

pCDH-hCLDN1-hOCLN-DsRed is a recombinant expression vector that expresses a fusion protein of hCLDN1-hOCLN. GGGGS GGGGS GGGAS was the linker between hCLDN1 and hOCLN that was used to maintain individual formation. Both hCLDN1 and hOCLN were amplified from human hepatocytes (QSN7701). The hCLDN1 ORF was amplified using the primers: forward, 5'-GCT CTA GAA TGG CCA ACG CGG GGC T-3', and reverse, 5'-CTA GCT AGC GCC ACC GCC GGA TCC TCC GCC ACC GGA GCC GCC TCC GCC CAC GTA GTC TTT CCC-3'. The hOCLN ORF was amplified using the primers: forward, 5'-CTA GCT AGC ATG TCA TCC AGG CCT CTT-3', and reverse, 5'-AAT GGG CCC GAG GGC CGG GAT TCT CCT CCA CGT CAC CGC ATG TTA GAA GAC TTC CTC TGC CCT CTG TTT TCT GTC TAT C-3'. The two fragments were digested and inserted into the pCDH-MCS-T2A -DsRed vector, and the constructed vector was named pCDH-hCLDN1-hOCLN-DsRed. The T2A sequences connecting gene fragments in all recombinant plasmids were 5'-AGG GCA GAG GAA GTC TTC TAA CAT GCG GTG ACG TGG AGG AGA ATC CCG GCC CT-3'.

Antibodies and reagents. The polyclonal anti-SR-BI antibody was obtained from BD Biosciences, and monoclonal anti-HCV NS3 and monoclonal anti-β-actin antibodies were obtained from Abcam. The EndoFree^{R} Plasmid Maxi Kit and the BCA Protein Assay Kit were purchased from Qiagen and CWbio Company, respectively. The luciferase assay system (10-Pack) was provided by Promega. TRIzol and RIPA (C1053) were purchased from Invitrogen and Millipore, respectively.

Virus production. Recombinant lentiviruses expressing hLDLR-hSR-BI-hCD81-GFP, hSR-BI-hCD81-GFP, and hCLDN1-hOCLN-DsRed were generated by co-transfection of HEK 293FT cells with vector RSV-REV, VSVG, and pMDLg/pRRE (Lv *et al*., 2009). The genotype 2a HCVcc was collected from the supernatants of Huh7.5.1 cells that were transfected with full-length JFH-1 RNA

transcribed from vector FL-J6/JFH-5'C19Rluc2AUbi (provided by C. Rice, Rockefeller University, NY) as previously described (Lindenbach *et al.*, 2005). Additionally, the HCVcc (genotype 1b) was a generous gift from Dr. Congyi Zheng (Guo *et al.*, 2009). Virus stocks were prepared by transfection of the plasmid pHCV-WHU-1 into Vero cells containing the recombinant vaccinia virus vTF7-3. HCVcc was collected in a serum-free medium. HCV-sd was acquired from the Beijing 302 hospital in compliance with the protocols approved by the institutional review boards (IRB).

Generation of humanized murine cell lines. Mouse hepatocytes (Hepa1-6) were seeded at 2×10^5 cells per well in a six-well plate. Subsequently, Hepa1-6 cells were infected with 400 µl of recombinant lentivirus expressing hCLDN1-hOCLN-DsRed (CO/Hepa1-6) in the presence of polybrene (8 µg/ml) at 37°C for 1 h. Then, complete medium (CM) was added, and another 72 h incubation was performed. The culture medium was replaced with CM containing 800 µg/ml of G418 on day 3 post infection (p.i.). Using flow cytometry and cell sorting, the first-generation transgenic cell line hCLDN1-hOCLN/Hepa1-6 with DsRed expression was obtained. An additional lentivirus expressing pCDH-hLDLR-hSR-BI-hCD81-GFP or pCDH-hSR-BI-hCD81-GFP was used to infect established CO/Hepa1-6 (DsRed) cells using the same procedure. Finally, the humanized murine cell lines hLDLR-hSR-BI-hCD81-hCLDN1-hOCLN/Hepa1-6 (LSCCO/Hepa1-6) and hSR-BI-hCD81-hCLDN1-hOCLN/Hepa1-6 (SCCO/Hepa1-6) that co-expressed DsRed and GFP were developed in the CM containing 800 µg/ml of G418 and 6 µg/ml of puromycin. Hepa1-6 cells that were transiently transfected with plasmids using Lipofectamine 2000 (Invitrogen) were used as a control.

Focus forming unit assay. LSCCO/Hepa1-6 and SCCO/Hepa1-6 cells were cultured overnight at 37°C and were then assessed using fluorescence microscopy. The cells were washed three times with PBS and fixed for 15 min in 4% paraformaldehyde, and this was followed by staining for 10 min with DAPI in the dark. The humanized murine cells were visualized and photographed using laser scanning confocal microscopy (Zeiss, Germany).

Western blot analysis. Humanized murine cells were collected and lysed in RIPA buffer containing proteinase inhibitors for 20 min at 0°C. Nuclei were removed by centrifugation (12,000 rpm, 20 min) at 4°C. The supernatants were then heated for 5 min at 100°C. Forty micrograms of proteins were loaded onto 10% SDS-PAGE and then transferred onto PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated with the monoclonal antibody anti-SR-BI overnight at 4°C. Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse antibody (1:2,000) for 1.5 h at room temperature (25°C). Finally, the chemiluminescent signal was detected using a chemiluminescence HRP substrate (Millipore Corporation, Billerica, MA 01821 USA).

Quantitative real-time PCR. hLDLR, hSR-BI, hCD81, hCLDN1, and hOCLN transcription levels in humanized murine cells were analyzed by qRT-PCR. Briefly, total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA synthesis was performed using a commercial reagent kit (ReverTra Ace qPCR RT Master Mix, TOYOBO). PCR was performed using a SYBR-PCR kit (SYBRR qPCR Mix, Toyobo). The parameters were as follows: 98°C for 2 min, 40 cycles of 95°C for 5 s, 56°C for 20 s, and 72°C for 15 s. The PCR assays were performed on a Bio-Rad CFX Manager machine, and the data were analyzed using the internal software package. Mouse GAPDH was used as the control.

The primers included:
hLDLR-forward: 5'-CGACGTTGCTGGCAGAGGAAATGA-3',
hLDLR-reverse: 5'-AAGACCCCCAGGCAAAGGAAGACG-3';
hSR-BI-forward: 5'-CGGATTTGGCAGATGACAGG-3',
hSR-BI-reverse: 5'-GGGGGAGACTCTTCACACATTCTAC-3';
hCD81-forward: 5'-TGTTCTTGAGCACTGAGGTGGTC-3',
hCD81-reverse: 5'-TGGTGGATGATGACGCCAAC-3';
hCLDN1-forward: 5'-CACCTCATCGTCTTCCAAGCAC-3',
hCLDN1-reverse: 5'-CCTGGGAGTGATAGCAATCTTTG-3';
hOCLN-forward: 5'-CGGCAATGAAACAAAAGGCAG-3',
hOCLN-reverse: 5'-GGCTATGGTTATGGCTATGGCTAC-3';
mGAPDH-forward: 5'-ACGGCCGCATCTTCTTGTGCA-3',
mGAPDH-reverse: 5'-ACGGCCAAATCCGTTCACACC-3'.

Virus infection and luciferase assay. For synchronized infection, 3×10^4 humanized murine cells were seeded into 48-well tissue culture plates overnight prior to infection. The cells were washed with cold DMEM without FBS, and HCVcc (1×10^5 pfu) or diluted patient serum (1:10) was added to the wells. After 2 h of incubation at 4°C, HCV binding of cells was assayed. Similarly, the internalization of HCV into target cells was analyzed after 6 h of incubation at 37°C. For assays using the *Renilla* luciferase (Rluc) reporter HCVcc, Rluc activity was measured with GloMax 96 luminometer to quantify HCVcc binding and internalization. Luminescence values were expressed as relative light units (RLU). HCVcc (genotype 1b) and HCV-sd binding and internalization were evaluated according to the copy numbers of viruses in RNA purified from cells as determined by qRT-PCR. The reaction parameters were as follows: 94°C for 2 min, followed by 50 cycles of 94°C for 30 s, 55°C for 20 s, and 72°C for 40 s. The primer sequences for the HCV genome included:

HCV-forward: 5'-CTGTGAGGAACTACTGTCTTC-3',
HCV-reverse: 5'-CCCTATCAGGCAGTACCACAA-3'.

Animal studies. BALB/c mice (female, 5–6 week-old) were purchased from Vital River (Beijing, China). The mice were housed under specific pathogen-free (SPF) conditions. The animal study was performed according to the protocols approved by the Ethics Committee of the Beijing Institute of Transfusion Medicine and the guidelines established by the Institutional Animal Committee. Mice were injected by hydrodynamic injection with lentivirus plasmids (1.25 mg/kg) that encoded human HCV receptors. At 72 h post-injection, mice were infected with

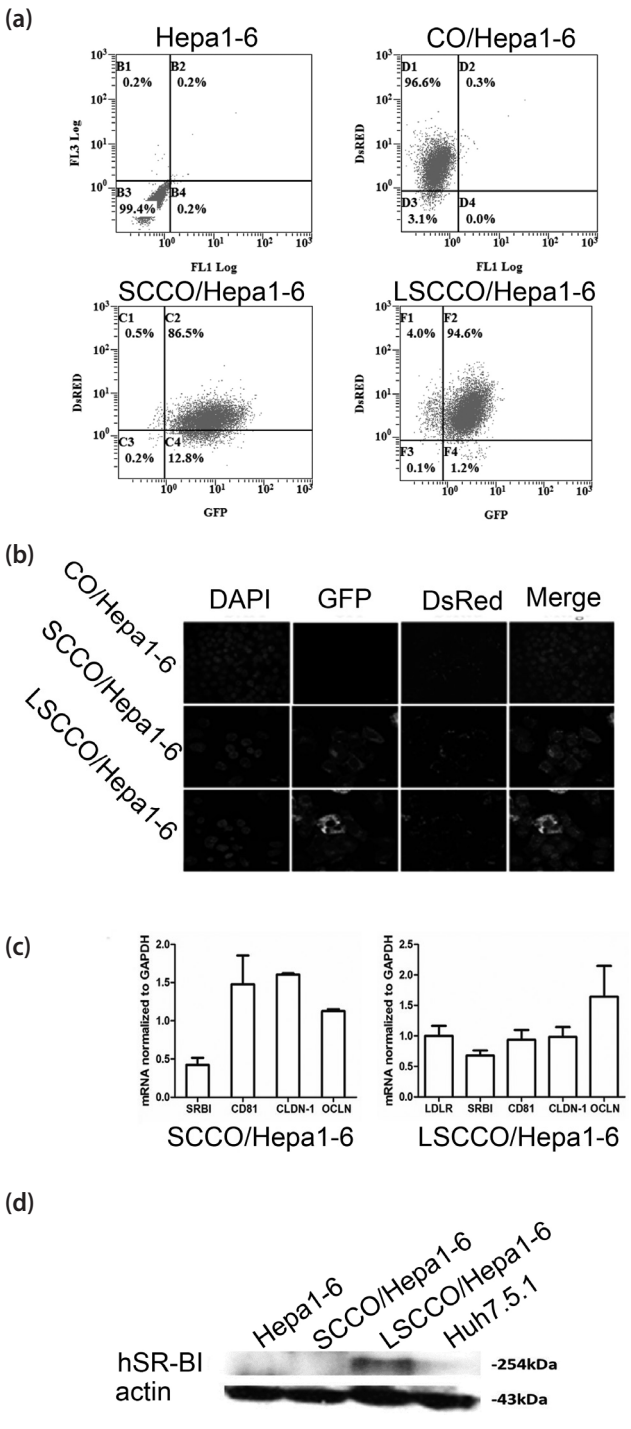


Fig. 1

Expression of human HCV entry factors in Hepa1-6 cells transduced with combinations of recombinant lentivirus expressing hLDLR-hSR-BI-hCD81-GFP, hSR-BI-hCD81-GFP, and hCLDN1-hOCLN-DsRed

(a) Flow cytometry analysis of the percent of GFP and DsRed positive cells. **(b)** Confocal microscopy of cells stably expressing hLDLR, hSCARB1, hCD81, hCLDN1, and hOCLN. **(c)** qRT-PCR analysis of hLDLR, hSR-BI, hCD81, hCLDN1, and hOCLN mRNA levels. **(d)** Western blot analysis of expression of hSCARB1.

10 ml/kg HCVcc or HCV-sd via the caudal vein. Total RNA was isolated from mouse livers using TRIzol Reagent according to the manufacturer's protocol at 6 h p.i. HCV entry factor mRNA and virus copy numbers were quantified by qRT-PCR.

Statistical analysis. Data were presented as means with standard deviations. Comparisons between groups were performed using the LSD-*t* test. Differences among means were considered significant at $p < 0.05$.

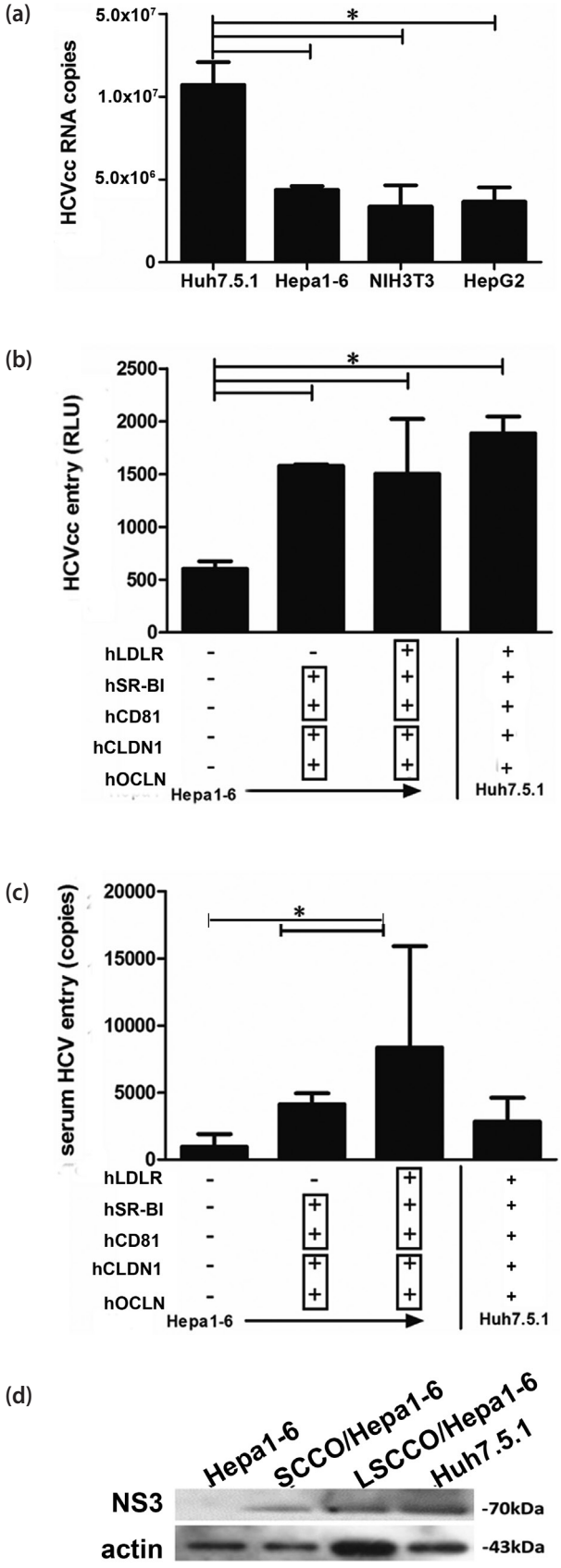
Results

Establishment of humanized murine hepatocytes

Entry factors play important roles in HCV binding and entry into target human hepatocytes. hSR-BI and hLDLR are also critically important components of HCV-sd infection that interact with LVPs. However, murine hepatocytes lack these receptors. To humanize mouse hepatocytes, we first established CO/Hepa1-6 by transfection with recombinant lentivirus particles expressing hCLDN1-hOCLN followed by a DsRed reporter. The percentage of $DsRed^+$ cells represented transgenic cells that expressed hCLDN1-hOCLN on their surfaces. Approximately 96.6% of murine hepatocytes expressed hCLDN1 and hOCLN (Fig. 1a). Based on CO/Hepa1-6 cell lines, LSCCO/Hepa1-6 and SCCO/Hepa1-6 cells were transfected with recombinant lentivirus particles expressing hLDLR-hSR-BI-hCD81 or hSR-BI-hCD81. More than 85% of murine hepatocytes expressed four entry factors or five entry factors (Fig. 1a). Confocal microscopy results indicated that GFP and DsRed proteins were co-localized in the cytoplasm (Fig. 1b). qRT-PCR analysis revealed that the desired genes were transcribed in humanized murine hepatocytes (Fig. 1c). We investigated whether the tandem expression of entry factors affected protein activity using western blotting. hSR-BI expression was observed in lysates from humanized murine hepatocytes (Fig. 1d). Taken together, these data indicate that humanized murine hepatocytes possessing entry factors have been successfully established.

Expression of human factors on murine hepatocytes results in observable susceptibility to HCV

To evaluate the utility of the developed cell models for HCV infection research, we challenged the transgenic cell lines with standardly used HCVcc (genotype 2a) and *HCV-sd*. An appropriate control is important for assessing virus infection. As Huh7.5.1 cells significantly increased HCVcc internalization (Fig. 2a), Huh7.5.1 cells were used as a positive control during this study. Humanized murine hepatocytes displayed higher RLU values, while



Hepa1-6 cells maintained a basal level (Fig. 2b) at 6 h p.i. This indicated that human factors in murine hepatocytes upregulated HCVcc (genotype 2a) internalization by assisting HCVcc (genotype 2a) entry. Furthermore, the same trend was observed after humanized murine hepatocytes were infected with HCV-sd. Compared to the other groups, HCV RNA levels were increased significantly ($p < 0.05$) in LSCCO/Hepa1-6 cells infected with HCV-sd (Fig. 2c). This result implied that hLDLR was a key factor in HCV-sd entry that functioned also for HCVcc (genotype 2a). Moreover, HCV-NS3 was detected in LSCCO/Hepa1-6 and SCCO/Hepa1-6 cells after HCVcc infection (Fig. 2d). These results indicated that humanized murine hepatocytes supported HCVcc binding and internalization to target cells in a manner similar to that of HCV-sd. Moreover, the magnitude of the increase was more robust in HCV-sd-infected cells than in HCVcc-infected cells.

hSR-BI is an indispensable factor for HCVcc (genotype 1b) infection in vitro

HCV 1b exhibits a high ratio in the general population of mainland China. To further determine whether humanized murine hepatocytes promoted HCVcc (genotype 1b) uptake, the cells were infected with HCVcc (genotype 1b). HCV entry factors induced an increase in HCVcc (genotype 1b) internalization. However, HCVcc (genotype 1b) entry was significantly decreased after humanized murine hepatocytes were exposed to anti-hSR-BI antibody (Fig. 3a). These results confirmed that hSR-BI significantly promoted virus entry compared to hLDLR ($p < 0.05$). The same results were obtained when Hepa1-6 cells were transfected with each factor plasmid ($p < 0.05$) (Fig 3b). Thus, hSR-BI could facilitate infectious HCVcc particle entry in the presence of hCD81, hCLDN1, and hOCLN *in vitro*. These observations suggest that hSR-BI predominantly mediates HCVcc (genotype 1b) particle binding and entry into host cells.

The role of hSR-BI is more important than that of LDLR in the HCV (genotype 1b) entry step in vivo

To estimate the hSR-BI determinants of HCV (genotype 1b) tropism *in vivo*, we generated transgenic humanized

Fig. 2

Entry of HCVcc and HCV-sd in humanized murine hepatocytes **(a)** Genotype 1b HCVcc infectivity of different cell lines. **(b)** Genotype 2a HCVcc entry assay in humanized murine hepatocytes. **(c)** HCV-sd entry assay in humanized murine hepatocytes. **(d)** Western blot analysis of the NS3 expression in HCV-infected humanized murine hepatocytes.

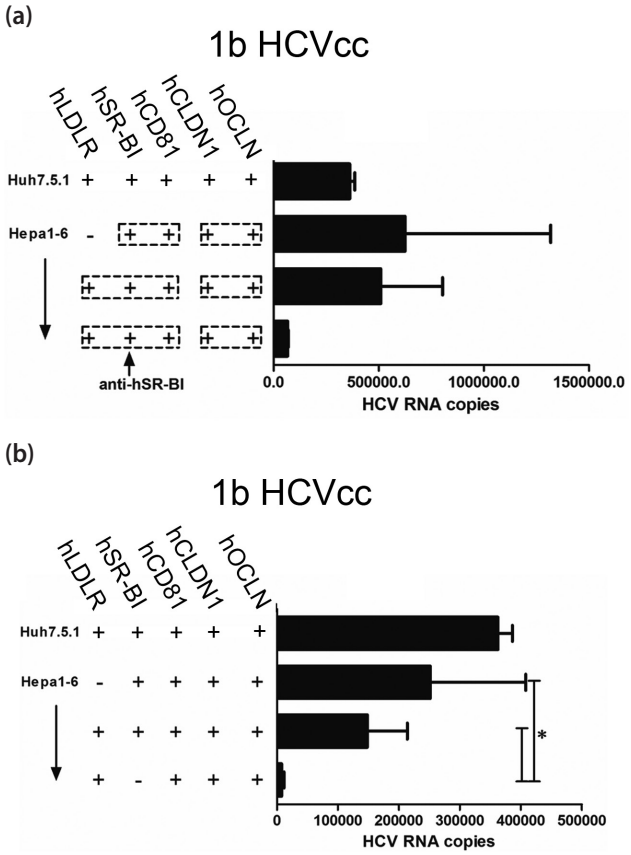


Fig. 3

Genotype 1b HCVcc infectivity and SR-BI mAb inhibition of Hepa1-6 cells transfected with recombinant expression vectors **(a)** Vectors expressing hLDLR-hSR-BI-hCD81-GFP, hSR-BI-hCD81-GFP, and hCLDN1-hOCLN-DsRed fusion proteins. **(b)** Vectors expressing hLDLR, hSR-BI, hCD81, hCLDN1, and hOCLN.

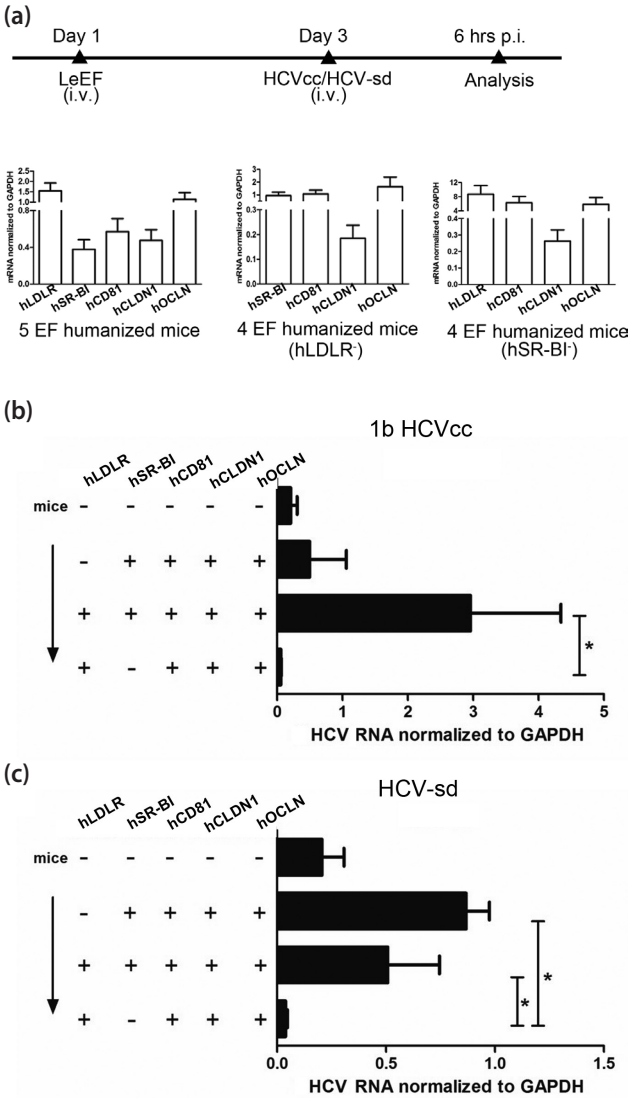


Fig. 4

HCV infectivity assay in transgenic humanized mouse model **(a)** Timeline for the administration of HCVcc and HCV-sd. **(b)** qRT-PCR analysis of hLDLR, hSR-BI, hCD81, hCLDN1, and hOCLN mRNA levels in the livers of transgenic mouse models. **(c)** qRT-PCR analysis of HCV RNA in mouse livers infected with HCVcc and HCV-sd.

mouse models expressing four or five human entry factors via hydrodynamic injection. After 3 days, entry factor mRNA was detected using qRT-PCR. Using this method, we determined that human entry factors were successfully transcribed in the livers of transgenic humanized mice (Fig. 4b). Furthermore, mice were inoculated with HCVcc (genotype 1b) via the caudal vein at 72 h post injection. All transgenic humanized mice expressing four or five human entry factors could be infected, and in these mice, the HCV RNA levels were significantly higher than background. However, HCV infection was largely inhibited by anti-SR-BI. Additionally, the same results were obtained when mice were inoculated with HCV-sd (Fig. 4c). In summary, these results indicate that hSR-BI promotes HCV entry more efficiently than does hLDLR in the presence of hCD81, hCLDN1, and hOCLN. Consistent with the *in vitro* results, these findings confirm that hSR-BI is primarily associated with HCV (genotype 1b) early infection and is more advantageous than hLDLR with regard to virus cell entry.

Discussion

The current limited understanding of HCV entry mechanisms has hampered the development of drug

screening techniques and vaccine assessment. The HCV life cycle consists of a complex process involving different virus subtypes and hepatocytes. Viral subtypes and host factors are closely associated with viral entry. The virus tropism depends on its subtype, the amount of entry factors, the sequence, location, and other factors. It remains difficult to delineate a clear interplay between HCV and hepatocytes, a mechanism that is important for the accurate treatment of patients.

Research has demonstrated that hCD81 and hOCLN are the minimal human factors required for HCV entry *in vitro* (Ploss *et al.*, 2009). hLDLR, hSR-BI, hCD81, hCLDN1, and hOCLN are all factors that assist HCV entry. However, only hLDLR and hSR-BI are acknowledged as factors involved in early HCV attachment. hLDLR is an important factor in HCV-sd entry owing to its interaction with LVPs. In this study, we observed a greater amount of HCV-sd entry in hLDLR transgenic mouse hepatocytes. However, when the hLDLR transgenic mouse hepatocytes were treated with HCVcc, this phenomenon disappeared. This was ascribed to the presence of HCV-sd in the lipoprotein-associated form in the serum. hSR-BI was identified as a co-receptor for HCV entry according to binding assays using a soluble form of E2 glycoprotein (Scarselli *et al.*, 2002). hSR-BI is a membrane glycoprotein that is highly expressed in the liver and in steroidogenic tissues (Acton *et al.*, 1996; Landschulz *et al.*, 1996; Krieger, 1999). Several studies have shown that it participates in high-density lipoprotein (HDL) uptake (Herijgers *et al.*, 2000; Van Eck *et al.*, 2008) and chylomicron metabolism (Out *et al.*, 2004, 2005). There are two different binding sites in the extracellular domain of SR-BI, a binding site for HDL and an LDL-binding site that allows it to bind to a broad spectrum of ligands (Gu *et al.*, 2000). Antibodies against SR-BI have been shown to inhibit HCV infection (Krzysztof *et al.*, 2012). In the present study, comparative binding assays were performed using HCVcc (genotype 2a), HCVcc (genotype 1b), and HCV-sd. Using humanized murine hepatocytes and transgenic humanized mouse models, we demonstrated that hSR-BI is an indispensable factor for the most prevalent 1b genotype HCV adsorption. Furthermore, this attachment could be completely prevented by treatment with mAb targeting hSR-BI.

In addition to entry factors, the HCV subtype is another key element that influences virus infectivity. The HCV 2a subtype is a traditional model virus used for studying HCV susceptibility (Spearman *et al.*, 2011). However, the HCV 1b subtype has been reported to rank first in terms of prevalence in the Chinese population (Chen *et al.*, 2017). HCV genotype 1b displays higher genetic variability in the hypervariable region 1 (HVR1) than do other genotypes (Janiak *et al.*, 2019). The tropism of the HCV 1b subtype requires further study. HCVcc (genotype 1b) and the corresponding HCV-sd were also used in our experiments. Surprisingly, we found that hSR-BI was more effective than was hLDLR in assisting HCV (genotype 1b) entry. Therefore, the role of hLDLR was not prominent when host cells were infected with HCV (genotype 1b).

In addition to the above aspects, HCV-existent forms should be taken into consideration. The association of HCV with lipoprotein is thought to increase infection and to allow for escape from immune detection, thus aiding the virus in maintaining persistent infection (Bartosch *et al.*, 2005). In serum, HCV particles interact with VLDL and become LVPs. VLDL contains two binding sites, including a high-affinity binding site that exhibits several characteristics of LDLR and another binding site that displays several characteristics of a scavenger receptor class B (Adelman and St, 1989). Therefore, both LDLR and SR-BI are involved in LVP binding. Although hLDLR is relatively crucial during HCV early infection, hSR-BI appears to be the key factor affecting virus entry. We demonstrated that hSR-BI is critically important for HCV (genotype 1b) infection *in vivo* and *in vitro*. Specifically, hSR-BI may function to complete the functions of binding and entry in the early life cycle of HCV (genotype 1b) particles. Not only can hSR-BI bind to LVPs (Bartosch, 2009) and/or HVR1 of HCV E2 (Bartosch *et al.*, 2005), but it also interacts with HCV-CD81 (Dao *et al.*, 2012).

In summary, we constructed recombinant murine cell models of HCV infection *in vitro* that express multiple human HCV receptors. Then, a humanized *in vivo* mouse model of HCV infection was constructed. The murine models offer an ideal platform for the screening and evaluation of antiviral drugs in the HCV early entry step. Based on cell models, the function of LDLR has been elucidated and exhibits a preferable participation for SR-BI in HCV entry. Finally, we confirmed that SR-BI plays an indispensable role in HCV infection and that LDLR can promote virus particle entry. Furthermore, SR-BI was found to be more important than was LDLR in early HCV infection, particularly the HCV 1b genotype that is predominant among the Chinese epidemic strains. Our conclusions demonstrate that SR-BI may be an important drug target for the effective prevention of HCV infection. This provides further possibilities and a novel perspective for HCV infection control.

Acknowledgments. The authors thank Prof. Charles M. Rice from Rockefeller University for his generous gift of the vector FL-J6/JFH-5'C19Rluc2AUbi. This work was supported by grant No. 81101296 from the National Natural Science Foundation of China, grant No. 7152113 from the Beijing Natural Science Foundation, and grant No. 2018ZX10302205 from the National Science and Technology Major Project of China.

References

Acton S, Rigotti A, Landschulz K, Xu S, Hobbs H, Krieger M (1996): Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 271, 518–520. https://doi.org/10.1126/science.271.5248.518

Adelman S, St Clair R (1989): Beta-VLDL metabolism by pigeon macrophages. Evidence for two binding sites with different potentials promoting cholesterol accumulation. Arteriosclerosis 9, 673–683. https://doi.org/10.1161/01.ATV.9.5.673

Agnello V, Abel G, Elfahal M, Knight G, Zhang Q (1999): Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA 96, 12766–12771. https://doi.org/10.1073/pnas.96.22.12766

Barth H, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, Von Weizsacker F, Blum HE, Baumert TF (2003): Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. 278, 41003–41012. https://doi.org/10.1074/jbc.M302267200

Bartosch B, Verney G, Dreux M, Donot P, Morice Y, Penin F, Pawlotsky JM, Lavillette D, Cosset FL (2005): An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. J. Virol. 79, 8217–8229. https://doi.org/10.1128/JVI.79.13.8217-8229.2005

Bartosch B (2009): Hepatitis C virus and its complex interplay with hepatic glucose and lipid metabolism. J. Hepatol. 50, 845–847. https://doi.org/10.1016/j.jhep.2009.02.009

Chen Y, Yu C, Yin X, Guo X, Wu S, Hou J (2017): Hepatitis C virus genotypes and subtypes circulating in Mainland China. Emerg. Microbes Infect. 6, e95. https://doi.org/10.1038/emi.2017.77

Dao Thi VL, Granier C, Zeisel MB, Guérin M, Mancip J, Granio O, Penin F, Lavillette D, Bartenschlager R, Baumert TF, Cosset FL, Dreux M (2012): Characterization of hepatitis C virus particle subpopulations reveals multiple usage of the scavenger receptor BI for entry steps. J. Biol. Ch. 287, 31242–31257. https://doi.org/10.1074/jbc.M112.365924

Dorner M, Horwitz JA, Robbins JB, Barry WT, Feng Q, Mu K, Jones CT, Schoggins JW, Catanese MT, Burton DR, Law M, Rice CM, Ploss A (2011): A genetically humanized mouse model for hepatitis C virus infection. Nature 474, 208–211. https://doi.org/10.1038/nature10168

Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wölk B, Hatziioannou T, McKeating JA, Bieniasz PD, Rice CM (2007): Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446, 801–805. https://doi.org/10.1038/nature05654

Gu X, Lawrence R, Krieger M (2000): Dissociation of the high density lipoprotein and low density lipoprotein binding activities of murine scavenger receptor class B type I (mSR-BI) using retrovirus library-based activity dissection. J. Biol. Chem. 275, 9120–9130. https://doi.org/10.1074/jbc.275.13.9120

Guo J, Yan R, Xu G, Li W, Zheng C (2009): Construction of the Vero cell culture system that can produce infectious HCV particles. Mol. Biol. Rep. 36, 111–120. https://doi.org/10.1007/s11033-007-9158-3

Herijgers N, Van Eck M, Korporaal SJ, Hoogerbrugge PM, Van Berkel TJ (2000): Relative importance of the LDL receptor and scavenger receptor class B in the beta-VLDL-induced uptake and accumulation of cholesteryl esters by peritoneal macrophages. J. Lipid Res. 41, 1163–1171. https://doi.org/10.1016/S0022-2275(20)32023-X

Janiak M, Perlejewski K, Grabarczyk P, Kubicka-Russel D, Zagordi O, Berak H, Osuch S, Pawełczyk A, Bukowska-Ośko I, Płoski R, Laskus T, Caraballo Cortés K (2019): Hepatitis C virus (HCV) genotype 1b displays higher genetic variability of hypervariable region 1 (HVR1) than genotype 3. Sci. Rep. 9, 12846. https://doi.org/10.1038/s41598-019-49258-y

Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T, Bartenschlager R (2006): Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J. Virol. 80, 5308–5320. https://doi.org/10.1128/JVI.02460-05

Krieger M (1999): Charting the fate of the „good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. Annu. Rev. Biochem. 68, 523–558. https://doi.org/10.1146/annurev.biochem.68.1.523

Landschulz KT, Pathak RK, Rigotti A, Krieger M, Hobbs HH (1996): Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. J. Clin. Invest. 98, 984–995. https://doi.org/10.1172/JCI118883

Lindenbach BD, Evans MJ, Syder AJ, WolkB, Tellinghuisen TL, Liu CC, MaruyamaT, Hynes RO, Burton DR, McKeating JA, Rice CM (2005): Complete replication of hepatitis C virus in cell culture. Science 309, 623–626. https://doi.org/10.1126/science.1114016

Liu S, Yang W, Shen L, Turner JR, Coyne CB, Wang T (2009): Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. J. Virol. 83, 2011–2014. https://doi.org/10.1128/JVI.01888-08

Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J, Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Doffoël M, Raffelsberger W, Poch O, McKeating JA, Brino L, Baumert TF (2011): EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nat. Med. 17, 589–595. https://doi.org/10.1038/nm.2341

Li DK, Chung RT (2019): Overview of Direct-Acting Antiviral Drugs and Drug Resistance of Hepatitis C Virus. Me-

thods Mol. Biol. 1911, 3–32. https://doi.org/10.1007/978-1-4939-8976-8_1

Lv LP, Jia SZ, Wang QL, Zhang YY, Zhou H, Xu JB (2009): Hepatitis C virus infection of mouse hepatoma cell expressing human CD81 or LDLR. Acta Virol. 53, 185–189. https://doi.org/10.4149/av_2009_03_185

Lv L, Kang Q, Yu X, Gao B, Hu T, Ma P, Zhang Y, Yan F, Xiao J, Deng J, Zhou X, Xu J (2015): Tandem overexpression of five human factors renders murine hepatocytes susceptible to hepatitis C virus. Acta Virol. 59, 20–26. https://doi.org/10.4149/av_2015_01_20

Martin DN, Uprichard SL (2013): Identification of transferrin receptor 1 as a hepatitis C virus entry factor. Proc. Natl. Acad. Sci. USA 110, 10777–10782. https://doi.org/10.1073/pnas.1301764110

Nielsen SU, Bassendine MF, Burt AD, Martin C, Pumeechockchai W, Toms GL (2006): Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. J. Virol. 80, 2418–2428. https://doi.org/10.1128/JVI.80.5.2418-2428.2006

Out R, Hoekstra M, de Jager SC, de Vos P, van der Westhuyzen DR, Webb NR, Van Eck M, Biessen EA, Van Berkel TJ (2005): Adenovirus-mediated hepatic overexpression of scavenger receptor class B type I accelerates chylomicron metabolism in C57BL/6J mice. J. Lipid Res. 46, 1172–1181. https://doi.org/10.1194/jlr.M400361-JLR200

Out R, Kruijt JK, Rensen PC, Hildebrand RB, de Vos P, Van Eck M, Van Berkel TJ (2004): Scavenger receptor BI plays a role in facilitating chylomicron metabolism. J. Biol. Chem. 279, 18401–18406. https://doi.org/10.1074/jbc.M401170200

Owen DM, Huang H, Ye J, Gale M, Jr (2009): Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor. Virology 394, 99–108. https://doi.org/10.1016/j.virol.2009.08.037

Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S (1998): Binding of hepatitis C virus to CD81. Science 282, 938–941. https://doi.org/10.1126/science.282.5390.938

Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM (2009): Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457, 882–886. https://doi.org/10.1038/nature07684

Sainz B Jr, Barretto N, Martin DN, Hiraga N, Imamura M, Hussain S, Marsh KA, Yu X, Chayama K, Alrefai WA, Uprichard SL (2012): Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. Nat. Med. 18, 281–285. https://doi.org/10.1038/nm.2581

Sainz B Jr, Barretto N, Martin DN, Hiraga N, Imamura M, Hussain S, Marsh KA, Yu X, Chayama K, Alrefai WA, Uprichard SL (2010): Infectivity of hepatitis C virus is influenced by association with apolipoprotein E isoforms. J. Virol. 84, 12048–12057. https://doi.org/10.1128/JVI.01063-10

Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A (2002): The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. 21, 5017–5025. https://doi.org/10.1093/emboj/cdf529

Spearman CW, Dusheiko GM, Hellard M, Sonderup M (2019): Hepatitis C. Lancet 394, 1451–1466. https://doi.org/10.1016/S0140-6736(19)32320-7

Van Eck M, Hoekstra M, Out R, Bos IS, Kruijt JK, Hildebrand RB, Van Berkel TJ (2008): Scavenger receptor BI facilitates the metabolism of VLDL lipoproteins in vivo. J. Lipid Res. 49, 136–146. https://doi.org/10.1194/jlr.M700355-JLR200