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Xiangyi Cao
BGI College&Henan Institute og Medical and Pharmaceutical Sciences in Academy of Medical Science, Zhengzhou University

Qiong Kang
Dongcheng District Primary and Secondary School Health Care Centre

Deng Jiang
Institution of Health Service and Transfusion Medicine

Jun Xiao
Air Force Medical Center,PLA

Yanyu Zhang
Institute of Health Service and Transfusion Medicine

Ping Ma
Insitute of Health Servise and Transfusion Medicine

Xiaoang Yang
BGI College&Henan Institute of Medical and Pharmaceutical Sciences in Academy of Medical Science,Zhengzhou University

Liping Lv (lvlp2020@163.com)
Irish Blood Transfusion Medicine

Research

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Scavenger Receptor Class B Type I Is More Conducive to Hepatitis C Virus Invasion Compared with Low-Density Lipoprotein Receptor

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

1 BGI College & Henan Institute of Medical and Pharmaceutical Sciences in Academy of Medical Science，Zhengzhou University；

2 Dongcheng District Primary and Secondary School Health Care Centre；

3 Institute of Health Service and Transfusion Medicine, Beijing 100850, P. R. China

4 Air Force Medical Center，PLA

† Xiangyi Cao,Qiong Kang and Jiang Deng contributed equally to this work.

* Authors to whom correspondence should be addressed;

E-Mails:xiaoangyang@163.com (X.A.) ;lvlp2020@163.com(L.L.)

Abstract

Background: Hepatitis C virus is the major cause of chronic hepatitis which may deteriorate into liver cirrhosis or hepatocellular carcinoma. A number of studies have demonstrated that HCV cell entry is a complex multi-step process involving several cellular proteins, such as scavenger receptor class B type I (SR-BI), tetraspanin CD81, tight junction protein claudin-1 (CLDN-1) and occludin (OCLN). The low-density lipoprotein receptor (LDLR) is an important factor during the initial HCV particle-binding step, which interacts with the complex formed between the virus particle and the lipoprotein in the blood. However, the process of HCV early infection is not well-established, with many details remaining to be elucidated.This research aimed to study the early entry stage of HCV virus particles and the role of LDLR more effectively.

Methods:Recombinant murine cell models of HCV infection in vitro was constructed, that expressed human HCV receptors, such as LDLR, CD81, SR-BI, CLDN-1, and OCLN. These factors were also introduced to mice by hydrodynamic delivery to construct a humanized mouse model of HCV infection in vivo.Expression levels of the mRNA of HCV entry factors in

recombinant cells were measured by qRT-PCR.Western blotting was used to determine whether the recombinant cells successfully expressed cellular proteins. HCV RNA was assayed by q-PCR following the incublation of HCVsd and HCVcc with the transgenice .

Results：Transgenic murine cell lines and mice were developed successfully, and expressed four or five human HCV entry factors in tandem or individually, respectively.We found that all of these transgenic cells and mice were susceptible to HCV, and five entry factors (5EF) rendered higher infectivity. Additionally, we observed that four entry factors (4EF/hLDLR-) could facilitate abundant HCV entry, but four other factors (4EF/hSR-BI-) were less effective. Conclusions: Whether in vitro or in vivo, SR-BI is an essential factor in HCV invasion, and target cells and mice were more vulnerable to the virus in the presence of SR-BI than LDLR. These results suggested that SR-BI may be a potential drug target to inhibit HCV early infection, and the absence of LDLR could reduce the infectivity to the virus.

Keywords: Hepatitis C virus; Virus entry; model construction; LDLR; SR-BI

Introduction

Hepatitis C virus (HCV) infection is a global health problem. Approximately 130-170 million people are chronically infected with HCV in the world, the majority of which develop liver cirrhosis or hepatocellular carcinoma. The recently discovered direct-acting antivirals (DAA)[1] represent a promising treatment for the majority of patients. However, the lack of vaccines and entry inhibitors remains a pressing issue in the prevention of HCV infection. Suitable small animal models are needed for drug screening and vaccine assessment. Tremendous efforts have been made to establish small animal models of HCV infection. Among these models, the genetically humanized mouse model is the most promising. This model, first reported by Marcus Dorner[2],generated by expressing human HCV entry factors (EF).

HCV early infection is a complex process involving several factors. Many studies have demonstrated that HCV particles are associated with VLDL or LDL in the blood, which leads to the formation of lipoviral particles (LVPs)[3].This association permits the virus to bind to target cells through lipoprotein receptors as well as viral structural proteins. A number of cellular factors have been reported that mediate the HCV entry process, including glycosaminoglycans (GAGs) [4, 5],

low-density lipoprotein receptor (LDLR) [6-8], scavenger receptor type B class I (SR-BI) [9], tetraspanin CD81[10] , tight junction claudin1 (CLDN-1)[11], occludin (OCLN)[12, 13], epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2)[14] , transferrin receptor 1 (TfR1)[15] , and cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1)[16]. Among these factors, the evidence suggests that both LDLR and SR-BI could bind native LDL and play important roles in serum-derived HCV (HCV-sd) particles infection. However, there are several unresolved questions about the function of these two factors. One of these unaddressed issues is whether they play equally important roles or if one of them is predominant. To shed light on these questions, the study of interactions between HCV and target cells at the onset of the early viral infection is necessary. In addition to serum-derived HCV (HCV-sd), the recently discovered virus particle-cell culture grown HCV (HCVcc) is usually used in many experiments, including studying the potential involvement of LDLR [8, 17].

To recapitulate the details of HCV natural infection *in vitro*, the development of an available cell model is a prerequisite. In this study, we utilized lentivirus vectors expressing human LDLR, CD81, SR-BI, CLDN-1, and OCLN in tandem and obtained recombinant humanized murine cell lines by transfecting mouse hepatoma carcinoma cells Hepa1-6 with these human proteins. HCVcc and HCV-sd were used to test the HCV-susceptibility of the cell models. The recombinant murine hepatoma carcinoma cell lines stably expressing several human HCV entry factors (EF) were successfully established and were susceptible to HCV. These murine cell models offer an ideal platform for the screening and evaluation of antiviral drugs in the HCV early entry step. On the basis of these cell models, the function of LDLR has been discussed and the proportion with SR-BI in HCV entry was compared preferably. We detected the same components by establishing transgenic mice *in vivo*. Finally we found that SR-BI played an indispensable role in HCV infection and that LDLR could promote virus particle entry. Meanwhile it appeared as though the function of SR-BI was more important than LDLR in HCV early infection.

Materials and Methods

Cells, Plasmids, and Reagents

Murine hepatoma carcinoma (Hepa1-6) cells, human hepatoma (Huh7.5.1) cells, mouse embryonic fibroblast (NIH3T3) cells, hepatocellular carcinoma (HepG2) cells

and HEK293FT cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). Recombinant Hepa1-6 was cultured in DMEM with 10% FBS and a known amount of antibiotics.

Packaging plasmids (pMDLg/pRRE, pRSV-REV), and the lentiviruses pCDH-hLDLR-hSR-BI-hCD81-GFP, pCDH-hSR-BI-hCD81-GFP and pCDH-hCLDN-1-hOCLN-DsRed were obtained from Dr. LiPing Lv [18]. Linker TGGGG-GGGGSGGGGS was inserted between hLDLR and hSR-BI, and linker ASGGGG-GGGSGGGGS between hSR-BI and hCD81.hLDLR/hSR-BI/hCD81 ORF was amplified, respectively. The two or three fragments were digested and inserted into pCDH-EF1-MCS-GFPvector to yield pCDH-hSR-BI-hCD81-GFP and pCDH-hLDLR-hSR-BI-hCD81-GFP recombinant vectors. Linker GGGGSGGGGSGGGAS was inserted between hCLDN-1 and hOCLN. The hCLDN-1/hOCLNORF was also amplified, respectively. The two fragments were digested and inserted into pCDH-MCS-T2A-DsRed vector to yield pCDH-hCLDN-1-hOCLN-DsRed recombinant vector [18].

The polyclonal anti-SR-BI antibody was from BD Biosciences, and the monoclonal anti-HCV NS3 antibody and monoclonal anti-β-actin antibody were obtained from Abcam. The EndoFree^{R} Plasmid Maxi Kit (10) and the BCA Protein Assay Kit were purchased from the QIAGEN and CWbio Company. The Luciferase Assay System (10-Pack) was provided by Promega. Trizol and RIPA (C1053) were from Invitrogen and Millipore.

Virus Production

Three lentiviruses (pCDH-hLDLR-hSR-BI-hCD81-GFP, pCDH-hSR-BI-hCD81-GFP, pCDH-hCLDN-1-hOCLN-DsRed) were produced by co-transfection of HEK 293FT cells with the constructed recombinant pCDH- transfer vector, the packaging plasmids (pMDLg/pRRE, pRSV-REV) and the pCMV-VSV-G expressing vector of the vesicular stomatitis virus glycoprotein G, respectively. Genotype 2a HCVcc was collected from the supernatants of Huh7.5.1 cells that were transfected with genotype 2a strain JFH1-RLuc RNA from a ribozyme-modified HCV genome. In addition, 1b HCVcc was obtained from Dr. Fang Yan. Serum-derived HCV (HCV-sd) were acquired from the Beijing 302 hospital in compliance with approved protocols of institutional review boards (IRB).

For lentivirus production, supernatants of HEK293FT were harvested 48 hours

post-transfection, pooled, filtrated through a 0.45-μm pore-size membrane, and stored at -80℃. The lentivirus used for developing recombinant cells were concentrated through ultracentrifugation at 33500 rpm for 2 hours at 4℃ (Beckman SW41t) and stored at 4℃ after stockdilution in 400μl phosphate-buffered saline (PBS).

For 2a HCVcc production, vector FL-J6/JFH-5'C19Rluc2AUbi (provided by C. Rice, Rockefeller University, NY) encoding full-length JFH-1 was used to generate RNA by using the MEGAscript T7 kit (Ambion, USA) [18]. And RNA was transduced into Huh7.5.1 cells DMRIE-C (Invitrogen). 72 hours post- transduction, supernatants were collected and stored immediately at -80℃.

Generation of Humanized Murine Cell Lines

400μL of the virus pCDH-hCLDN-1-hOCLN-DsRed was used to infect 2×10^5 Hepa1-6 cells in a 6-well dish in the presence of polybrene (8μg mL^{-1}), at 37℃ for 1 hour. The cells were then incubated with DMEM containing 10% FBS for 72 hours. The cells were washed with PBS, and fresh DMEM, and suitable G418 was added daily. After 3 days, the remaining cells were incubated again with DMEM containing FBS and G418. Through flow cytometry and cell sorting, we obtained the first transgenic cell line hCLDN-1+/hOCLN+/Hepa1-6 (CO/Hepa1-6), which was observed red fluorescein DsRed under fluorescence microscopy. Two additional virus samples, pCDH-hLDLR-hSR-BI-hCD81-GFP and pCDH-hSR-BI-hCD81-GFP, were used to infect recombinant CO/Hepa1-6 cells by the same procedure. Finally, two transgenic cell lines, hLDLR/SR-BI/CD81/CLDN-1/OCLN+/Hepa1-6 (LSCCO/ Hepa1-6) and hSR-BI/CD81/CLDN-1/OCLN+/Hepa1-6 (SCCO/Hepa1-6), were developed, that co-expressed red and green fluorescein.

Focus Forming Unit Assay

A few humanized murine cells were incubated with DMEM overnight at 37℃. When those cells expressed GFP and DsRed as determined by fluorescence microscopy, the plates were washed with PBS three times and fixed for 15 minutes with 4% paraformaldehyde. The cells were stained for 10 minutes with DAPI in the dark. The recombinant cells were visualized and photographed by laser scanning confocal microscopy (LSCM).

Western Blot Analysis

Western blotting was employed to determine whether the recombinant cells successfully expressed hLDLR, hCD81, hSR-BI, hCLDN-1 and hOCLN. The cells were lysed with the lysis buffer RIPA containing proteinase inhibitors at 0℃ for 20 minutes and then centrifuged at 12 000 rpm for 20 minutes at 4℃ to remove nuclei. The samples were heated for 5 minutes at 100℃ before the run. 40mg protein was electrophoresed in 10% SDS-PAGE mini gels and transferred onto PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies overnight at 4 ℃. After washing with TBS-Tween 20 (0.15%), the membranes were incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse antibody (1:2000) for 1.5 hours at room temperature. Finally, antibody binding to proteins was detected using a chemiluminescence HRP Substrate (Millipore Corporation, Billerica, MA 01821 USA).

Quantitative Real Time PCR

Expression levels of the mRNA of HCV entry factors in recombinant cells were measured by qRT-PCR. Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA (1μg) was reverse transcribed with the relevant kit (ReverTra Ace^{R}qPCRRT Master Mix). The primers and the SYBR-PCR kit (SYBR^{R}qPCR Mix) were used for amplification. The reactions were incubated in a 96-well optical plate or an eight link tube at 98℃ for 2 minutes, followed by 40 cycles at 95℃ for 5 seconds, 56℃ for 20 seconds and 72℃ for 15 seconds. The PCR reactions were run on a Bio-Rad CFX Manager machine and the data were analyzed by the internal software package. Mouse GAPDH was used as the control.

The primer sequences were as follows: human LDLR, forward primer, CGACGTTGCTGGCAGAGGAAATGA, reverse primer, AAGACCCCCAGGCA-AAGGAAGACG; human SR-BI, forward primer, CGGATTTGGCAGATGACAGG, reverse primer, GGGGGAGACTCTTCACACATTCTAC; human CD81, forward primer, TGTTCTTGAGCACTGAGGTGGTC, reverse primer, TGGTGGATGATG-ACGCCAAC; human CLDN-1, forward primer, CACCTCATCGTCTTCCAAGCAC, reverse primer, CCTGGGAGTGATAGCAATCTTTG; human OCLN, forward primer, CGGCAATGAAACAAAAGGCAG, reverse primer, GGCTATGGTTATGGCTATG-GCTAC; and mouse GAPDH, forward primer, ACGGCCGCATCTTCTTGTGCA, reverse primer, ACGGCCAAATCCGTTCACACC.

Virus Infection

For synchronized infection, 3×10^4 cells were seeded in 48-well tissue culture plates overnight prior to infection. The cultures were washed with cold DMEM without FBS, and the medium was then replaced with 100μl HCVcc or diluted patient serum (10%); The cells were incubated for 2 hours at 4℃ for the binding assay and 6 hours at 37℃ for the entry assay, followed by washing away unbound viruses. After removal of the supernatant, the cells were washed twice with PBS, and lysed in 50μl passive lysis buffer for luciferase detection or RNA was collected for counting HCV copy number. For continuous transfection and infection, target plasmids were transfected with Lipofectamine 2000 (Invitrogen); after 60 hours, the medium was incubated at 37℃ with the antibody for 1 hour (anti-SR-BI was used at 10μg mL^{-1}) prior to infection. The culture was replaced with 100μl 1b HCVcc or HCV-Sd for 6 hours at 37℃ to calculate virus copy number.

Animal Studies

BALB/c mice (female, 5-6 weeks old, 16-20g body weight) were purchased from Vital River (Beijing, China). 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 via caudal vein with 1.25mg kg^{-1} lentivirus plasmids, which expressesd human HCV receptors. After 72 hours post-injection, mice were again injected via caudal vein with 10mL kg^{-1} HCVcc or HCV-sd. Total RNA was isolated from mouse liver using the Trizol Reagent according to the manufacturer's protocol. Human molecules and HCV genome copy number were quantified by qRT-PCR. The reactions were at 94℃ for 2 minutes, followed by 50 cycles at 94℃ for 30 seconds, 55℃ for 30 seconds and 72℃ for 40 seconds. The Primer sequences were as follows: HCV genome, forward primer, CTGTGAGGAACTACTGTCTTC, and reverse primer, CCCTATCAGGCAGTACCACAA.

Statistical Analysis

Data was presented as the means and standard deviations. Comparisons were made using the LSD-*t* test for experiments between groups. The differences among means were considered significant when $p < 0.05$.

Results

Humanized murine cell lines can stably express four or five exogenous human HCV entry factors.

To develop cell models of HCV infection and clarify the role of LDLR in its entry step, we infected Hepa1-6 with recombinant lentivirus particles expressing hCLDN-1-hOCLN. The transgenetic cell line (CO/Hepa1-6) was obtained by cell sorting according to the DsRed percentage (Fig 1.A). Approximately 96.6% of murine hepatocytes expressed these two human heterologous genes.Based on CO/Hepa1-6 cells lines, LSCCO/Hepa1-6 and SCCO/Hepa1-6 cell lines were established transfected with hLDLR-hSR-BI-hCD81 or hSR-BI-hCD81, which were co-localizationed by GFP and DsRed protein (Fig 1.B). Approximately 86% of murine hepatocytes expressed four or five human HCV entry factors.

Fluorescein GFP and DsRed that connected with target entry factors via ligase T2A on lentivirus vectors (Fig 2.E) co-localized in the cell cytoplasm under confocal microscopy (Fig 1.C). When they were translated in the host cells together with inserted human genes, fluorescent proteins were cut off at the ligase T2A site, and expressed human proteins were released. Thus, these human HCV entry factors may be expressed on the cell surface or in the cytoplasm (Fig 1.C) and optimization is needed afterward. Meanwhile, the amount of the two fluorescent proteins appeared to be dissimilar, because of size inequality of the vectors or because the murine cells lacked enough epitopes. Thus, the quantity of the target protein is not controlled. On the other hand, molecular mass of fusion proteins of hLDLR-hSR-BI-hCD81 and hSR-BI-hCD81 are 254KDa and 93KDa, respectively. Polyclonal anti–SR-BI was confirmed to bind to the human tandem protein expressed in the murine cell (Fig 1.E). To investigate the expression of four or five entry factors in recombinant murine cells (LSCCO/Hepa1-6, SCCO/Hepa1-6), we performed additional experiments at the level of mRNA (Fig 1.D). RNA was extracted from these two cell lines and was analyzed by quantitative qRT-PCR in contrast to GAPDH. Taken together, these data indicated that recombinant humanized murine cells could stably express human HCV entry factors via lentivirus transfection. This model is promising for the development of an animal model, and it can be used for investigating the function of those molecules during HCV infection.

Expression of human factors in murine cells results in obvious susceptibility to HCV, and human LDLR seems to increase HCV-sd uptake.

To evaluate the exploitability of the developed cell model in HCV infection research, we challenged the transgenic cell lines with genotype 2a HCVcc (JFH-1) and serum from Hepatitis C patient, with Huh7.5.1 cells as controls. In contrast to Hepa1-6, recombinant cells have obvious virus particle entry ($p < 0.05$) after co-culture with 2a HCVcc, and the RLU volumes are similar to the Huh7.5.1 cells (Fig 2.A). However, luciferase signals have also been detected in Hepa1-6 and we thought that background or virus residue may be the cause. To ascertain the non-specificity of Hepa1-6 to HCV, we gathered different murine cells, such as NIH3T3 and HepG2, and incubated them simultaneously with genotype 1b HCVcc. Detection of intra-cellular HCV RNA copies suggested that these mouse cell lines have no infectivity of HCV, in contrast to Huh7.5.1 cells ($p < 0.05$), despite the existence of the virus residue (Fig 2.D). Therefore, these two recombinant cells markedly produced susceptibility to HCV particles (Fig 2.A.B), and this demonstrated that construction of a humanized mouse model was feasible. Moreover, the nonstructural protein HCV-NS3 was detected in the target cells after HCVcc infection (Fig 2.C). Taken together, these data indicated that these recombinant humanized murine cells could be used to study the function of added human molecules in the HCV life cycle, and will facilitate the development of animal models in the future.

Although the role of LDLR in lipid transfer and the dependence of LVPs on lipid metabolism are well-established, it remains unclear how LDLR is involved in the HCV life cycle. LDLR is an important factor in HCV-sd entry, considering interaction with LVPs. We confirmed that there was more HCV-sd entry in LSCCO/Hepa1-6 cells than SCCO/Hepa1-6 cells ($p < 0.05$) (Fig 2.B). However, HCVcc is usually gained via transfection of HCV-RNA[19-21], and may be present in the formation of the virion. Therefore, it needs to be discussed whether LDLR plays an important role in HCVcc entry. In hLDLR+/- murine cell lines (LSCCO/Hepa1-6 and SCCO/Hepa1-6), we observed that HCVcc particle entry was similar, regardless of the presence of human LDLR (Fig 2.A). This result suggests that LDLR does not account for viral entry in the early HCVcc life cycle *in vitro*. In addition, constructed lentivirus vectors were reformed, and every entry factor was combined with ligase T2A, so that human molecules could express individually at translation after integration with host genes (Fig 2.E). When murine hepatoma carcinoma cells Hepa1-6 were transfected for 60h,

there was no difference in genotype 1b HCVcc entry between hLDLR+ and hLDLR- cells (Fig 3.A). Thus, the role of LDLR in the early viral entry stage was not affected by the expression of exogenous proteins. Similar data were obtained when Hepa1-6 cells were transfected with a single plasmid expressing human factor alone (Fig 3.B). Therefore, LDLR is not an essential factor in the early HCVcc life cycle *in vitro*, and other factors may have similar functions that compensate for its role during HCVcc entry.

SR-BI is an indispensable factor for HCVcc infection in vitro, compared to LDLR.

LDLR binds to lipoviral particles (LVPs) and regulates lipid transfer, but some studies have found that SR-BI also participates in the metabolism of lipids[22, 23] . We compared the function of LDLR and SR-BI, and explored whether alternatives may support HCV particle entry into target cells. Studies have reported that SR-BI can bind to both native high-density lipoprotein (HDL) and modified lipoproteins such as oxidized LDL[24]. This led us to hypothesize that LVPs may be mediated by SR-BI directly via lipid metabolism, obviating LDLR. First, we studied HCVcc infection of Hepa1-6 cells transfected with reformed lentivirus vectors and added SR-BI neutralized in the presence of anti-SR-BI antibodies (10μg mL^{-1}). The results confirmed that the addition of LDLR did not allow more HCVcc virus entry into target cells, and that SR-BI could promote virus to enter rather than LDLR ($p < 0.05$) (Fig 3.A). However, LDLR seemed to affect or even reduce viral entry. This may occur due to competition between LDLR and SR-BI in lipid metabolism. Furthermore, corresponding to tandem expression of human SR-BI in murine cells, we transfected Hepa1-6 cells with factor plasmid respectively. The results were in agreement with those previously described (Fig 3.B). On the other hand, we also found that polyclonal anti-SR-BI markedly inhibited HCVcc infection when the other four factors co-expressed, in contrast to the five factors (Fig 3.A). The same results were obtained when Hepa1-6 cells were transfected with every factor plasmid ($p < 0.05$) (Fig 3.B). Thus, SR-BI, rather than LDLR, could facilitate infectious HCVcc particle entry in the presence of CD81, CLDN-1 and OCLN *in vitro*. Although LDLR had an obvious important function in HCV natural infection (HCV-sd), unlike HCV-sd, SR-BI could directly influence HCVcc particle entry into host target cells, and the impact of LDLR is negligible *in vitro*. Taken together, these data indicated that SR-BI predominantly mediates HCVcc particle binding and entry into host cells.

The role of SR-BI is more important than LDLR in the HCV entry step in vivo.

Some studies have found that HCVcc may combine with lipoprotein. However, a defect in VLDL secretion has been observed in the Huh-7 cell line[25, 26]. HCVcc produced by the Huh7.5.1 cell line did not exactly resemble with infectious patient serum (HCV-sd)[27]. This virus usually contains a few LVPs and many viral particles *in vitro*. When HCVcc enters the blood circulation, it may bind with lipoprotein in the blood and produce generous LVPs. It is significant to contrast the contribution of LDLR and SR-BI in HCV infection *in vivo*. For this reason, we constructed some transgenic mice via injection of lentivirus plasmids expressing HCV receptors and detected human molecules by qRT-PCR at approximately 72 hours post-injection. Finally, transgenic mice expressing different HCV receptors have been built successfully (Fig 4.B-D).

On the basis of these transgenic mice, we determined that the absence of SR-BI attenuated HCV entry and affected the infectivity to the virus directly, irrespective of HCVcc or HCV-sd, in presence of the other four receptors (Fig 4.E-F). On the other hand, we observed that SR-BI could motivate HCV entry compared to LDLR, in the presence of CD81, CLDN-1 and OCLN (Fig4.E-F). Consistent with consequence above *in vitro*, these results indicated that SR-BI was primary to HCV early infection and was more advantageous than LDLR in virus cell entry.

Discussion

Recently, infection and transfection have been used to establish cell or animal models for research in infectious HCV particle entry. The team of Charles M. Rice constructed a cell model for HCVpp entry *in vitro* by transfecting the mouse embryonic fibroblast cell line NIH3T3 with adenovirus vectors expressing four human HCV receptors firstly[13]. Then, they constructed a humanized mouse model by the same method[2]. That model supplied many possibilities for studying the HCV life cycle *in vitro* and *vivo*. However, adenovirus does not stably express human proteins in host cells, and to co-localize four factors in a cell is very difficult. Thus, we transfected Hepa1-6 with lentivirus vectors expressing human HCV entry factors in tandem. To study the early entry stage of HCV virus particles and the role of LDLR more effectively, we developed humanized murine cell lines successfully by utilizing many methods. These cell lines constructed by us expressed stably up to four or five HCV entry factor proteins, which will benefit the manufacture and evaluation of

antiviral drugs, and further research in small animal models.

Although LDLR has been proposed as a potential factor in HCV early binding, it remains difficult to draw a clear conclusion on the role played by LDLR during the entry stage of infectious HCV particles. We introduced human LDLR into murine cells to investigate the function and compared it with that of SR-BI.

Hepatocytes secrete VLDL, which are made up of triglycerides, cholesterylesters, ApoB, and ApoE. When released into the plasma, HCV particles may interact with VLDL and become lipoviral particles (LVPs). VLDL is processed by lipoprotein lipase (LPL) and produces intermediate density lipoproteins (IDL). ApoE, located on IDL, interacts with LDLR on hepatocytes. The remaining IDL in the cycle is converted to LDL through a reaction catalyzed by hepatic lipase, and mediated by LDLR[8]. Nevertheless, as the ligand of VLDL, β-VLDL is a type of lipoprotein particle enriched in cholesteryl ester that is similar to IDL due to the dependence of apoE on the interaction between β-VLDL and LDLR[8]. SR-BI is a membrane glycoprotein that is highly expressed in liver and steroidogenic tissues[28-30]. Much work has shown that it participates in high-density lipoprotein (HDL) uptake[24, 31] and chylomicron metabolism[32, 33]. There are two different binding sites at the extracellular domain of SR-BI, a binding site for HDL and an LDL/lipid binding site, when it binds to a broad spectrum of ligands[34]. Adelman and St Clair confirmed that β-VLDL contains two binding sites: a high-affinity binding site, which exhibits many characteristics of LDLR, and another binding site, which displays many characteristics of a scavenger receptor class B[35]. Therefore both LDLR and SR-BI are involved in lipid metabolism. Although lipoviral particles (LVPs) may be mediated by LDLR or SR-BI, participation of the former molecule may intensify HCV-sd (Fig 2.B) and HCVcc entry (Fig 4.E) *in vivo*. In contrast to HCV-sd, there were more virus particles in HCVcc than LVPs, because of the defect of the Huh7 cell line in the secretion of VLDL[25, 26]. SR-BI can bind with the virus envelope E2 HVR1 directly[36] and is more likely to promote HCVcc to enter *in vitro*, compared with LDLR. Therefore, the role of LDLR has not been highlighted *in vitro*, when host cells were infected by HCVcc (Fig 2.A/ Fig 3.A.B).

Although LDLR is relatively crucial in HCV early infection, it seems that SR-BI is the key point to affect virus entry. We demonstrated that SR-BI was very important in HCV-sd and HCVcc infection (Fig 3, Fig 4.E.F) *in vivo* and *in vitro*. In other words, SR-BI may complete enough of the functions of binding and entry in the early life

cycle of HCV particles. Not only can it bind with LVPs[37] or HVR1 of HCV E2[36], but it also joins the HCV-CD81 interaction, considering that exposure of CD81 binding sites on HCV E2 depends on the lipid transfer function of SR-BI[23].

In brief, these conclusions demonstrate that SR-BI may be an important drug target in effectively preventing HCV virus infection.This provides other possibilities and a novel perspective for research in HCV infection.

Conclusions

In summary,we constructed recombinant murine cell models of HCV infection in vitro, that expressed multiple human HCV receptors.Then a humanized mouse model of HCV infection in vivo was constracted. These murine cell models offer an ideal platform for the screening and evaluation of antiviral drugs in the HCV early entry step.On the basis of cell models, the function of LDLR has been discussed and the proportion with SR-BI in HCV entry was compared preferably.Finally we found that SR-BI played an indispensable role in HCV infection and that LDLR could promote virus particle entry.In addition SR-BI was more important than LDLR in HCV early infection.

Abbreviations

HCV: Hepatitis C virus; SR-BI:scavenger receptor class B type I; CLDN-1:claudin-1; LDLR:low-density lipoprotein receptor;5EF:five entry factors;DAA:discovered direct-acting antivirals;EF:entry factors;LVPs:lipoviral particles; GAGs:glycosaminoglycans;EGFR:epidermal growth factor receptor;EphA2:ephrin receptor A2;TfR1:transferrin receptor 1;NPC1L1:Niemann-Pick C1-like 1; HCV-sd:serum-derived HCV; HCVcc:particle-cell culture grown HCV

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Authors’ contributions

LP, ZY, conceived the study. KQ, DJ, XJ,MP,performed the research. CY prepared the manuscript. LP, YA, edited the manuscript. All authors read and approved the fnal manuscript.

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Availability of data and materials

We can share our data if needed.

Ethics approval and consent to participate

BALB/c mice (female, 5-6 weeks) used in the current study came from Charles river (Beijing), which were housed under SPF conditions in National Beijing Center for Drug Safety Evaluation and Research (NBCDSER). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of NBCDSER and approved by the Animal Ethics Committee of NBCDSER(2020-680).

Consent to publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or fnancial relationships that could be construed as a potential confict of interest.

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Figures

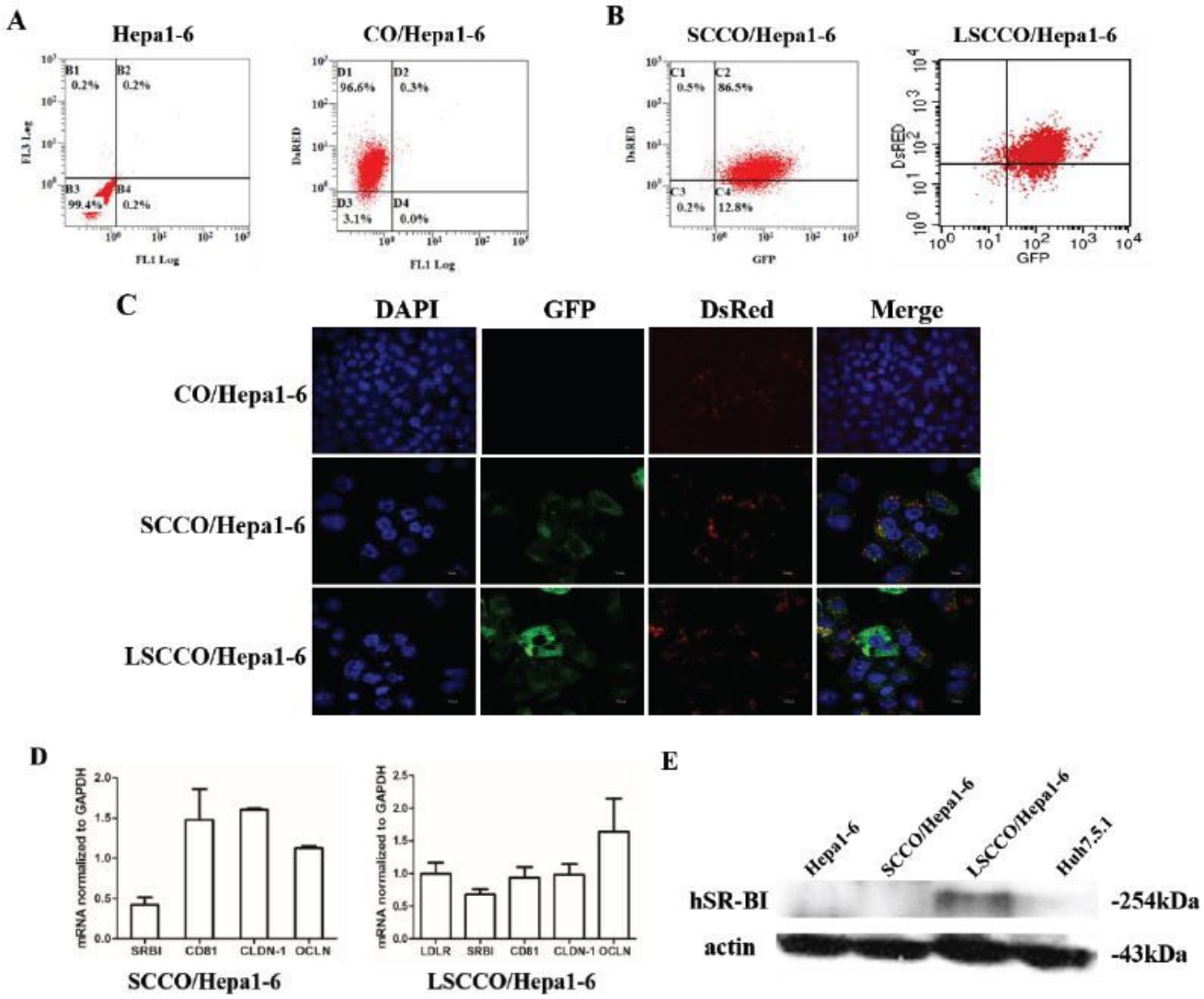

Figure 1

Production of recombinant humanized murine cell lines. DsRed-receptor lentivirus expressing human CLDN-1-OCLN infected Hepa1-6. The percentages of DsRed-positive cells were the first transgene cell (A). Percent of GFP and DsRed co-expression in other target cells after hCLDN-1-hOCLN/Hepa1-6 was transfected by GFP-receptor vector expressing human SR-BI, CD81 and/or LDLR (B). Proteins hLDLR-hSR-BI-hCD81, hSR-BI-hCD81 and hCLDN1-hOCLN in the recombinant cells were detected by immunofluorescence microscopy (C). Human LDLR, SR-BI, CD81, CLDN-1 and OCLN mRNA levels were determined by RT-quantitative PCR (D). The data were normalized to GAPDH expression. Means and SD of three experiments are shown. Modified SR-BI expression was assessed by immunoblotting (E).

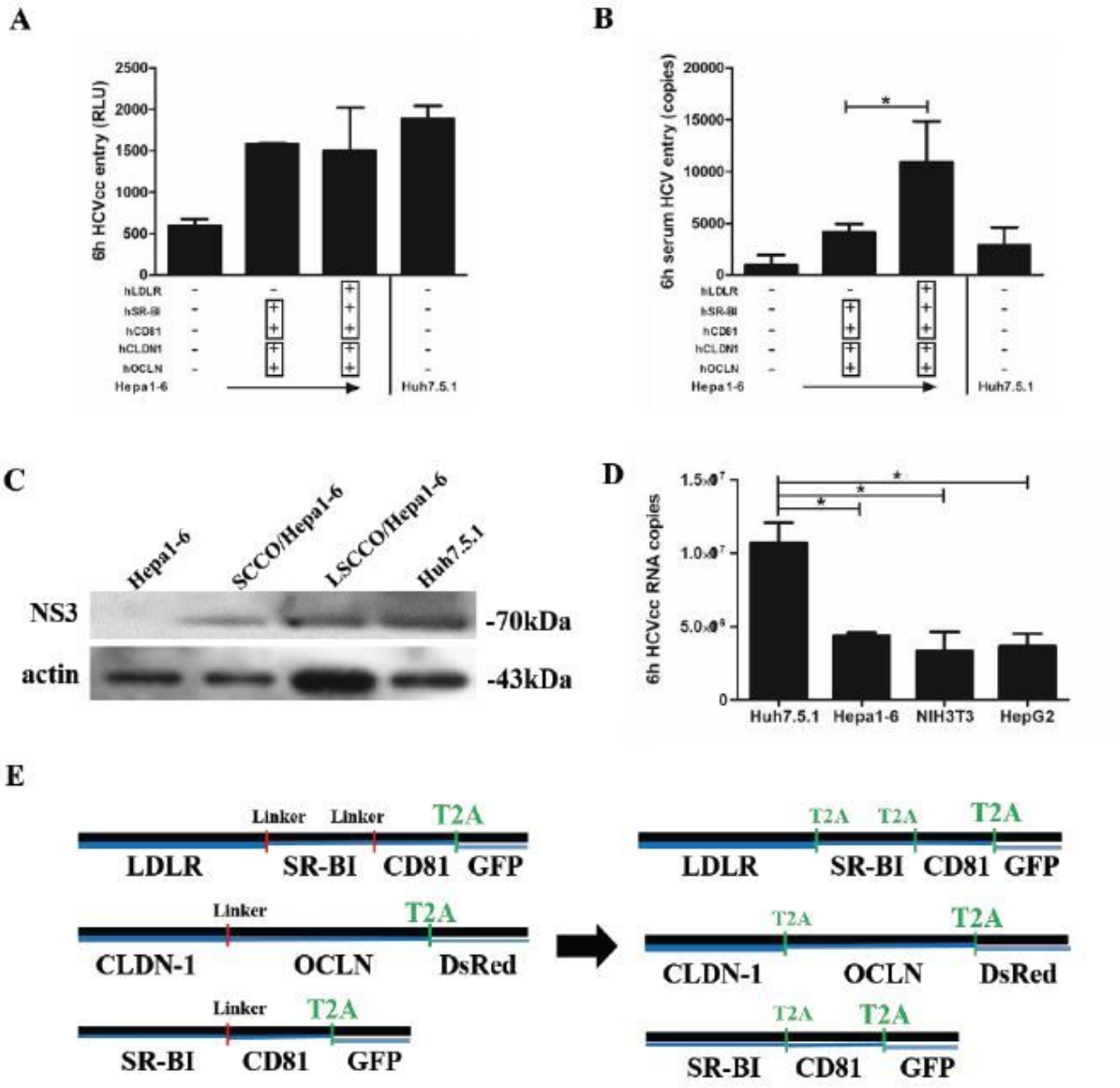

Figure 2

Expression of human HCV entry factors generated the susceptibility to infectious HCV particles in murine cells, and LDLR could not promote HCVcc uptake in vitro. Recombinant murine cells (solid lines represent tandem expressing cells) and Huh7.5.1 were incubated with genotype 2a HCVcc and patient serum for 6h. RNA copies and Luc-signals were detected as the infectivity (A, B). Recombinant cells infected with HCVcc were then lysed, and the expression of NS3 was determined by immunoblotting (C). Hepa1-6, NTH3T3 and HepG2 cells were incubated with genotype 1b HCVcc for 6h in contrast to Huh7.5.1. Detection of RNA copies reflects the infectivity of the HCV particles (D). Schematic diagram of constructed lentivirus vectors by connecting every human molecule via ligase T2A (E). Repeat the experiment three times.* $p<0.05$

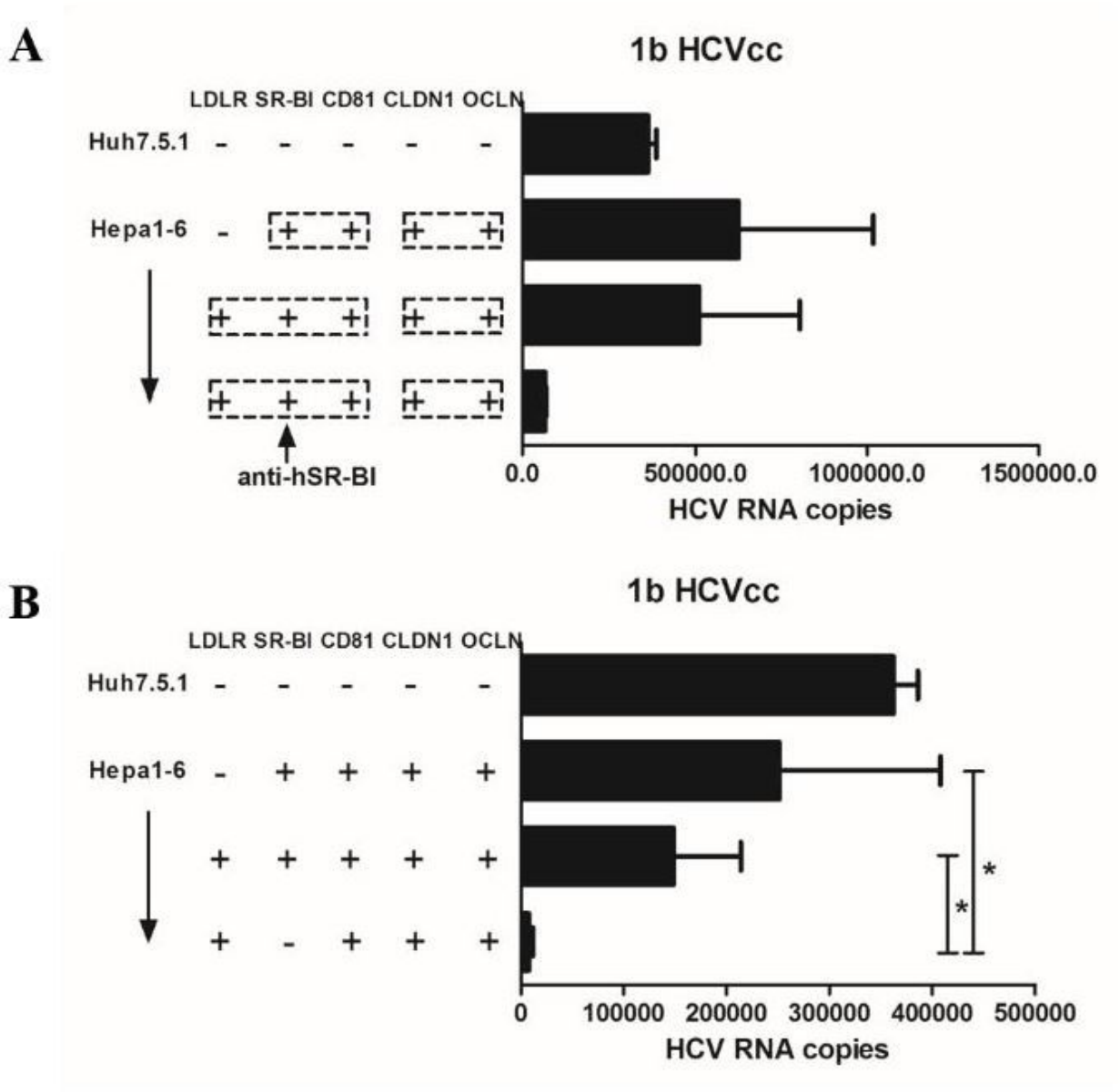

Figure 3

SR-BI is a capital factor in infectious HCV particles cell entry in vitro. Hepa1-6 cells transfected with remade vectors (A) or every plasmid individual expressing human molecule (B), the former group with or without neutralizing anti-SR-BI (10μg mL-1), were incubated with 1b HCVcc for 6h. RNA was collected to estimate entry.

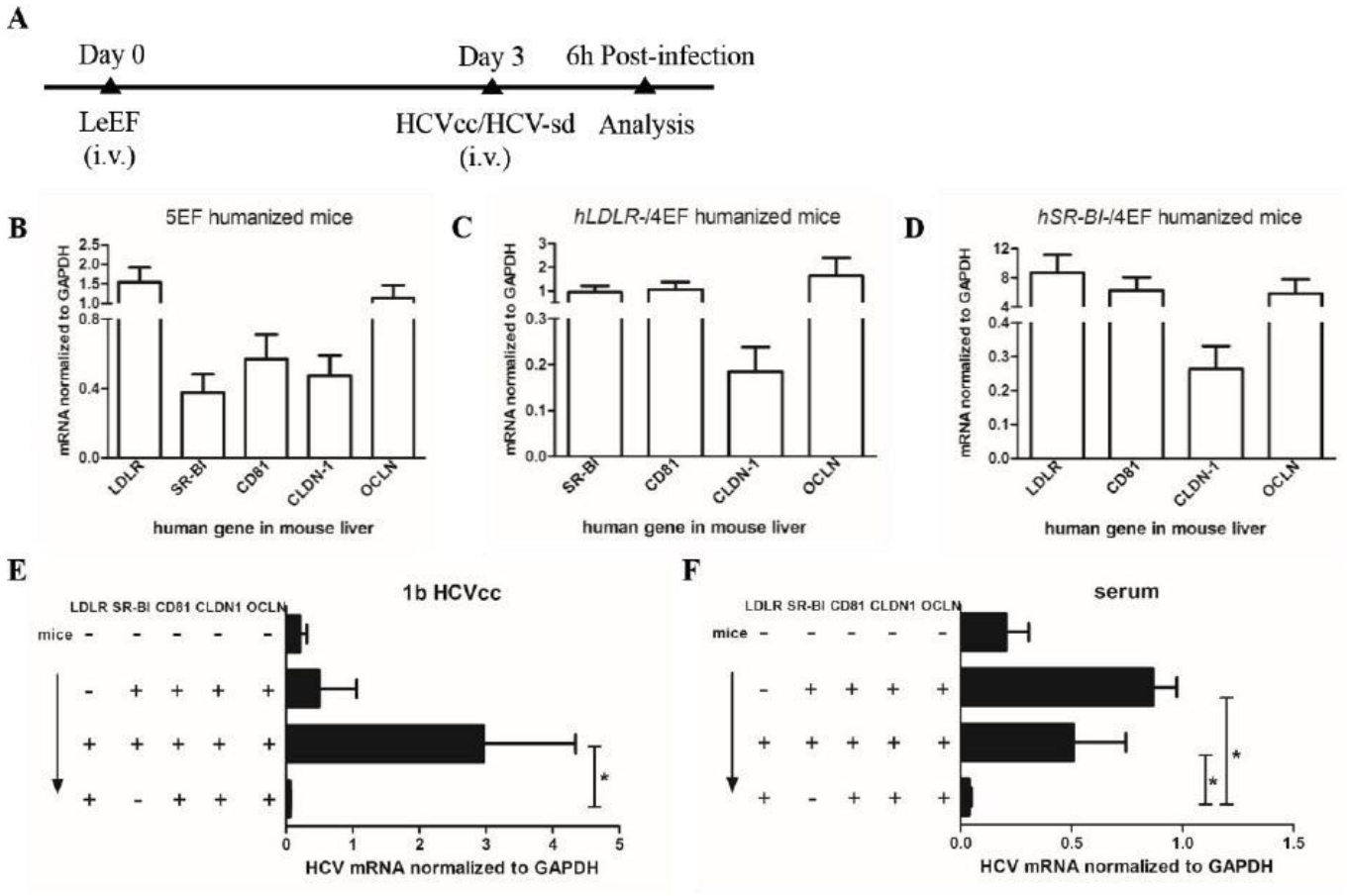

Figure 4

Humanized mouse models were developed and SR-BI is a primary factor in HCV early infection in vivo. The timeline for administration of lentivirus and HCV was shown as sketch (A). Many human receptors of HCV entry were transferred to BALB/c mice by hydrodynamic delivery according to different groups, such as 5EF humanized mice (LDLR, SR-BI, CD81, CLDN-1 and OCLN) (B), hLDLR-/4EF humanized mice (SR-BI, CD81, CLDN-1 and OCLN) (C) and hSR-BI-/4EF humanized mice (LDLR, CD81, CLDN-1 and OCLN) (D). The expression of human receptors was detected by RT-PCR. hLDLR+/- and hSR-BI- mice from one, two and three groups were injected with 1b HCVcc (E) or HCV-sd (F). Liver tissues were isolated and virus copies were detected by RT-PCR at 6h post-injection.* $p < 0.05$.