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Original Article

# Mouse homologues of hepatitis C virus human entry factors inhibit the entry of HCV pseudo-particles (HCVpp) into human hepatoma cells

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**ABSTRACT:** Hepatitis C virus (HCV) is a growing public health concern worldwide. No vaccine preventing HCV has yet been developed due to the hindrance of research on suitable small animal models. Moreover, it was known that human CD81, claudin-1 (CLDN1), scavenger receptor type B class I (SCARB1) and occludin (OCLN) (collectively called CCSO) are important factors for HCV entry into human hepatocytes. Therefore, we previously generated transgenic mice expressing CCSO in their hepatocytes, but these mice do not confer susceptibility to HCVpp or HCV entry. Earlier study also showed mouse hepatocytes had strong restriction factor(s) that inhibit HCV entry. Here, we tried to find out the inhibitory factor(s), and found mouse homologues (Cd81, Ocln) themselves are inhibitory for HCV entry in human hepatoma cells when they express the mouse homologues on their cell surfaces. So, deleting these inhibitory factors can make mouse hepatocytes more susceptible to HCV infectivity *in vivo*. This *in vitro* study would thus be helpful in making an efficient mouse model for HCV infection.

**KEYWORDS:** Cd81, Ocln, NPC1L1, Huh7.5.1, NIH3T3

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## INTRODUCTION

HCV causes a chronic liver disease and affects about 3% of the total population worldwide[1]. Narrow host tropism of HCV limits the advancement in basic and clinical studies of HCV infection[2]. Currently, chimpanzee is the widely recognized model for HCV infection[3] but its use is limited by ethical concerns. Although murine hepatocytes are resistant to HCV entry and show inefficient viral replication *in vivo*[4], mouse hepatic cells support the entry, replication, and production of infectious HCV particles *in vitro*[5, 6]. Recently, it was reported that only 0.4% of hepatocytes is infected by HCV in C57BL/6 transgenic mice expressing CCSO under defective innate immune system[7]. More recently, ICR background transgenic mice harboring human CD81 and OCLN genes can be infected by HCV[8], and the full-length HCV genome RNA under pol I promoter can be replicated in C57BL/6 transgenic mice[9] but the produced infectious particles in the sera of both the mice could be detected hardly. So, making an efficient mouse model for HCV infection is still unmet. HCV usually enters into human hepatocytes through the combined action of at least four host molecules: CD81[10], CLDN1[11], SCARB1[12], and OCLN[13]. This led us to hypothesize that the expression of CCSO in mouse hepatocytes could render them susceptible to HCV infection *in vivo.* We therefore previously generated C57BL/6 transgenic mice expressing CCSO in their hepatocytes under the control of albumin enhancer/promoter and investigated whether HCVpp or HCV from patients' sera could infect the livers of these mice. The report showed that CCSO expressing mice reject susceptibility to HCV entry despite having four human factors in their hepatocytes. Interestingly, the previous report also showed that the mouse hepatocytes contain dominant negative restriction factor(s) that inhibit HCV entry[14]. Hence, in this study, we tried to find out the inhibitory factor(s) present in mouse hepatocytes. We hypothesized that mouse homologues (Cd81, Ocln) themselves might be the inhibitory for HCV entry into the hepatocytes of CCSO-expressing mice. Because our previous study showed the HCV can bind CCSO receptors efficiently but could not internalize into mice hepatocytes[14]. This inability to internalization may be due to the interaction of mouse homologues (Cd81, Ocln) either with their respective human homologues (CD81, OCLN) or other factors involved in HCV entry. Two other mouse homologues (Cldn1, Scarb1) are not considered in inhibition because these two receptors both from mouse and human are equally capable of mediating HCV uptake in Chinese hamster ovary cells[13]. So, in the present study we made several permanent cell lines of Huh 7.5.1 (a highly permissive human hepatoma cell line for HCV entry) that could express mouse homologues (Cd81, Ocln) on their cell surfaces and examined whether these homologues inhibit the entry of HCVpp in these cells. We found that the mouse homologues inhibit the entry of HCVpp up to 31% in cells expressing these proteins compared to the native or empty vector transfected Huh7.5.1 cells. But we could not deny the necessity of

additional human factor(s) for making mouse hepatocytes more susceptible to HCV entry *in vivo*. So, in this study, we have also shown that the over-expression of an additional new human entry factor, NPC1L1 (Niemann-Pick C1-like 1) enhances the entry of HCVpp significantly in mouse embryonic fibroblast cells NIH3T3; conversely the knock down of NPC1L1 reduces HCVpp entry in Huh7.5.1 cells which is consistent with the previous report[15]. Therefore, these *in vitro* studies would be helpful for better understanding in making an efficient mouse model for HCV infection.

## MATERIALS AND METHODS

### *Cell culture*

Huh7.5.1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. D5796, Sigma-Aldrich Co., Missouri, USA). Mouse Cd81, mouse Ocln and empty vector (CXN2)-harboring Huh7.5.1 cells were grown in DMEM containing 0.4 mg/mL G418 disulfate salt (Cat. No. 04 727 878 001, Roche Diagnostics GmbH, Germany). Human NPC1L1 (antisense)-harboring Huh7.5.1 cells were grown in DMEM containing 6.0 µg/mL blasticidin S HCl (Cat. No. R210-01, Invitrogen, CA, USA). The four human factors (CCSO) and the five human factors (CCSO with NPC1L1)-expressing NIH3T3 cells were grown in DMEM containing 0.2 mg/mL G418 disulfate salt, and 2.0 µg/mL blasticidin S HCl with 0.2 mg/mL G418 disulfate salt respectively. In DMEM of all cell cultures, 10% fetal calf serum (Sigma-Aldrich Co., Missouri, USA) was supplemented.

### *Construction of plasmid DNAs*

### *Mouse Cd81-expressing plasmid*

In order to add the FLAG tag at the C-terminal end of mouse Cd81 protein, PCR was performed with the oligonucleotides, 5'ATGGGGGTGGAGGGCTGCAC3' (sense primer), 5'GGAACAGCTCCGTGTACGACTACAAGGACGACG ACGACAAGTGAGAATTC3' (antisense primer, C-terminal + FLAG tag) and with the mouse liver Cd81 cDNA as a template. The PCR product was sub-cloned into pBluescript II/KS (-) vector (Stratagene Co., CA, USA). From this recombinant plasmid, the EcoR1-cut insert was then ligated into a mammalian cell expression vector CXN2[16] that cut with the same restriction enzyme and named it CX-Cd81-FLAG (Figure 1A).

### *Mouse Ocln-expressing plasmid*

In order to add the HA tag at the C-terminal end of mouse Ocln protein, PCR was performed with the oligonucleotides, 5'GGTGACCAGTGACATCAGCC3' (sense primer), 5'CTCGAGTCAGGCGTAGTCGGCCACGTCATAGGG GTAAGGTTTCCGTCTGTCATAATCTCC3' (anti sense primer, C-terminal + HA tag) and with the mouse liver Ocln cDNA as a template. The Xho1 cut insert was then ligated into a CXN2 vector that was cut with the same restriction enzyme and named it CX-Ocln-HA (Figure 1B).

### *Human NPC1L1 (sense) Plasmid*

In order to add the FLAG tag at the N-terminal end of human NPC1L1 (sense), PCR was performed with the oligonucleotides, 5'GAATTCTCACTTGTCGTCGTCGTCCTTGTAGTCA TGGCGGAGGCCGGCCTG3' (sense primer, FLAG tag +N-terminal), 5'TTGTTACCCGCCGTCAAGAC3' (antisense primer) and with the human NPC1L1 cDNA as a template. The EcoRI cut insert was then ligated into a pCAGGS vector[16] that was cut with the same restriction enzyme and named it CX-FLAG-NPC1L1 (sense) (Figure 1C).

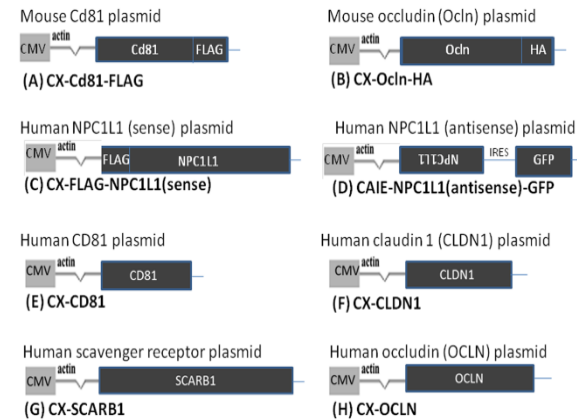


***Figure 1.*** *Schematic representation of eight constructs.* ***A****, CX-Cd81-FLAG, mouse Cd81-expressing construct in which the epitope, FLAG was tagged at the C-terminal end of mouse Cd81 protein.* ***B****, CX-Ocln-HA, mouse Ocln-expressing construct in which the epitope, HA was tagged at the C-terminal end of mouse Ocln protein.* ***C****, CX-FLAG-NPC1L1 (sense), human sense NPC1L1-expressing construct in which the FLAG tag was added at the N-terminal end of human NPC1L1.* ***D****, CAIE-NPC1L1 (antisense)-GFP, the human antisense NPC1L1-expressing construct in which the human antisense NPC1L1 gene was cloned into the GFP expressing vector, pCAIE.* ***E-H****, Human CD81, CLDN1, SCARB1 and OCLN-expressing plasmid constructs respectively.*

For adding the blasticidin resistance gene (BsR), we partially digested the CX-FLAG-NPC1L1 plasmid with the SalI enzyme and recovered the desired size insert from the agarose gel and then ligated with the BsR insert cut with the same enzyme from pJP1 plasmid (Cat. No. ATCC67080, ATCC, VA, USA).

### *Human NPC1L1 (antisense) Plasmid*

In order to make the human antisense NPC1L1 expressing plasmid (Figure 1D), we cut the NPC1L1-harboring pEGFP-N1 vector (Cat. No. 6085-1, Clontech, CA, USA) (a gift from Dr T. Takada, The University of Tokyo) with the EcoRI enzyme and recovered the NPC1L1 insert from the agarose gel. We then ligated the insert into a GFP expression mammalian plasmid pCAIE[17] that was cut with the same enzyme and transformed the recombinant plasmid into JM109 *E.coli* (Cat No. 9052, Takara Bio Inc., Tokyo, Japan) cells. The desired human antisense NPC1L1-harboring plasmid was isolated and confirmed by the nucleotide sequence analysis.

### *Human CD81, CLDN1, SCARB1, OCLN-expressing plasmids*

In order to clone the human CD81, CLDN1, SCARB1 and OCLN cDNAs into CXN2 vector individually, PCR was performed with the oligonucleotides: 5' GAATTCCAAGCCGGCAGAGGCCGGGC3' (sense primer), 5'TCAGCACAAGGCCCCGCGGA3' (antisense primer) and with the human CD81 cDNA as a template for making human CD81-expressing plasmid; 5'GAATTCATGGCCCTGCTGGGTGGGTG3' (sense

primer), 5'TCACACATACTCCTTGGAAG3' (antisense primer) and with the human CLDN1 cDNA as a template for human CLDN1-expressing plasmid; 5'TCTAGACGCCTGTGTCGTCTCTGTCG3' (sense primer), 5'TCAAAGCCCACCTGTGCCCC3' (antisense primer) and with human SCARB1 cDNA as a template for human SCARB1-expressing plasmid; 5'CTCGACATGTCATCCAGGCCTCTTGA3' (sense primer), 5'CTATGTTTTCTGTCTATCAT3' (antisense primer) and with the human OCLN cDNA as a template for human OCLN-expressing plasmid. The individual PCR products were sub-cloned into pBluescript II/KS (-) vector. The recombinant plasmids were cut with the EcoR1 for getting CD81, OCLN and cut with XbaI and XhoI for SCARB1and OCLN inserts respectively. The restriction enzymes-cut inserts were then ligated into CXN2 vector cut with the same enzymes and named them CX-CD81 (Figure 1E), CX-CLDN1 (Figure 1F), CX-SCARB1 (Figure 1G), CX-OCLN (Figure 1H). In order to make all the inserts-expressing plasmids, the nucleotide sequence of each insert was confirmed by sequencing each time.

***Transfection, detection of recombinant mouse proteins (Cd81, Ocln) in human hepatoma cells (Huh7.5.1) and human proteins (CCSO & NPC1L1) in mouse cells (NIH3T3) by Western blot***

The plasmid constructs, CX-Cd81-FLAG and CX- Ocln-HA, were transfected into Huh7.5.1 cells individually using the X-tremeGene HP DNA transfection reagent (Cat. No. 06366236001, Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. Transformed cells were selected in the growth medium containing 0.4 mg/mL G-418 disulfate salt. Single cells were cloned ~ 25 days post transfection of the plasmid constructs using the cylinder technique method[18] and expanded. The cell lysates obtained from the mouse Cd81, Ocln-expressing Huh7.5.1 cells with the Nonidet P-40 lysis buffer were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Cat. No. RPN2020F, GE Healthcare, Buckinghamshire, UK). The transferred membranes were first incubated with the mouse monoclonal anti-FLAG M2 antibody (Cat. No. F3165, Sigma-Aldrich Co., Missouri, USA) for the detection of mouse Cd81protein, and with the rabbit polyclonal anti-HA antibody (Cat. No. 561-8, MBL, Nagoya, Japan) for mouse Ocln protein. The membranes were then incubated with the HRP-conjugated anti-mouse IgG (Cat. No. P0447, Dako, Glostrup, Denmark) and anti-rabbit IgG (Cat. No. P0448, Dako, Glostrup, Denmark) as secondary antibodies, respectively. Finally, the signals of specific proteins were detected using the chemiluminescent ECL kit (Cat. No. NEL 104, NEL 105, Perkin-Elmer Life Sciences, MA, USA). In order to make the four human proteins (CCSO)-expressing NIH3T3 cells, the four plasmid constructs CX-CD81, CX-CLDN1, CX-SCARB1 and CX-OCLN were then transfected into NIH3T3 cells simultaneously using the X-tremeGene HP DNA transfection reagent. Transfected cells were selected in the growth medium containing 0.2 mg/mL G-418 disulfate salt. Single cells were cloned ~ 20 days post transfection of the plasmid constructs and expanded. The cell lysates from the CCSO-expressing NIH3T3 cells were subjected to Western blotting for the detection of CD81 (Anti-CD81 mAb, Cat. No. CBL579, Merck Millipore, Darmstadt, Germany), CLDN1 (Anti-CLDN1 mAb, Cat. No. H00009076-M01, Abnova, Taoyuan, Taiwan), SCARB1 (Anti-CLA-1 mAb, Cat. No. 610882, BD Biosciences Pharmigen, CA, USA), and OCLN (Anti-OCLN mAb, Cat. No. H00004950-M01, Abnova, Taoyuan, Taiwan). In this case, the HRP-conjugated anti-mouse antibody was used a secondary antibody and the signals were detected using the chemiluminescent ECL kit. Similarly, for making five human factors (CCSO with NPC1L1)-expressing NIH3T3 cells, we transfected the CX- FLAG-NPC1L1 plasmid construct into four human factors, CCSO-expressing NIH3T3 cells. Transfected cells were selected in the growth medium containing 0.2 mg/mL G-418 disulfate salt & 2.0 µg/mL blasticidin S HCl. The cell lysates from the five human factors-expressing NIH3T3 cells were subjected to Western blotting for the detection of CD81, CLDN1, SCARB1, OCLN and FLAG for NPC1L1.

***Transfection and detection of human antisense NPC1L1, recombinant mouse Cd81, Ocln proteins in Huh7.5.1 cells by confocal microscopy***

In order to make human NPC1L1-knocked down permanent cell line of Huh7.5.1, the plasmid construct CAIE-NPC1L1 (antisense)-GFP (Fig. 1D) was transfected into Huh7.5.1 cells using the X-tremeGene HP DNA reagent. Transformed cells were selected in the growth medium containing 6.0 µg/mL blasticidin S HCl. Single cells were cloned ~ 25 days post transfection of the plasmid, expanded and observed with a confocal microscope (FV-1000, Olympus, Tokyo, Japan) by GFP expression. The empty vector CXN2, CX-Cd81-FLAG, and CX-Ocln-HA-transfected Huh7.5.1 cells grown on cover slips were fixed with 3.5% formalin in phosphate-buffered saline (PBS) for 20 min at room temperature. The cells were first incubated with the primary antibodies mouse monoclonal anti-FLAG M2 antibody for the detection of mouse Cd81 and with the rabbit polyclonal anti-HA antibody for the detection of mouse Ocln proteins. The cells were then incubated with the secondary antibodies fluorescein isothiocyanate (FITC) conjugated polyclonal rabbit anti-mouse IgG (Cat. No. F0261, Dako, Glostrup, Denmark) and Alexa-647 conjugated polyclonal goat anti-rabbit IgG (Cat. No. A21244, Invitrogen, CA, USA), respectively. The cells were then rinsed with PBS and counter-stained with DAPI (EJ085, Dojindo Molecular Technologies, Kumamoto, Japan). The washed cells were observed with a confocal microscope.

***Pseudo-particles (HCVpp, VSVGpp, NENVpp) production***

Murine leukemia virus (MLV)-based HCVpp was generated as described previously[19]. Briefly, 8.1 µg of the Gag-Pol packaging construct, 8.1 µg of the transfer vector construct (luciferase), and 2.7 µg of the JFH-1 glycoprotein construct (pcDNAΔC-E1-E2, a kind gift from Dr. Thomas Pietschmann) were co-transfected into 293T cells using the calcium phosphate method. Supernatants containing the HCVpp were collected 40 h after transfection, and passed through a 0.45-µm filter (Cat. No. 431220, Corning Inc., NY, USA). The HCVpp were pelleted through 20% sucrose cushions by ultracentrifugation using a Beckman Coulter SW27 rotor (Cat. No. 331186, USA) at 25,000 rpm for 2 h at 4° C. Similarly, control pseudo-particles, VSVGpp (vesicular stomatitis virus G glycoprotein pseudo-particle used as positive control) and NENVpp (non-envelope glycoprotein pseudo-particle used as negative control), were generated as described previously[20].

***Infection with pseudo-particles and luciferase Assays***

For infection with the pseudo-particles, native Huh7.5.1 cells, empty vector CXN2-transformed Huh7.5.1 cells (used as controls), mouse Cd81, mouse Ocln-expressing Huh7.5.1 cells, human NPC1L1-knocked down-Huh7.5.1 cells, four human factors (CCSO)-expressing NIH3T3 cells (used as control), five human factors (CCSO with NPC1L1)-expressing NIH3T3 cells were seeded in 12-well-plates at a density of 8.0 x$10^4$ cells per well in 10% FCS supplemented DMEM and incubated overnight at 37° C in a 5% $C0_2$ incubator. On the next day, the cells were infected with the pseudo-particles for 3 h at 37° C. After 3 h, the supernatants were removed, and the cells were further incubated with the regular medium until 72 h. The luciferase activities of the cell extracts were measured according to the manufacturer's instructions (Cat. No. E1500, Promega Corporation, WI, USA). Briefly, 72 h post infection with pseudoparticles, carefully remove the growth media from cells and rinse with ice cold PBS and remove the PBS as much as possible. Add 200 µL 1X lysis buffer per well of 12-well-plates, shake at 145 rpm for 5 min at room temperature to ensure complete lysis of the cells. Transfer cells and all liquid to a microcentrifuge tube, centrifuge at 14000 rpm for 2 min at 4° C and collect the cell supernatant to a new tube. Dispense 100 µL of the Luciferase Assay Reagent into luminometer tube, one tube per sample. Add 20 µL of the cell supernatant to a luminometer tube containing the Luciferase Assay Reagent, mix by pipetting 2-3 times, place the tube in the luminometer (Lumicounter 700, Microtech Co., Chiba, Japan) and measure the luciferase activities of the cell extracts.

***Ethical Statement***

The experiments conducted in this manuscript were approved by the Recombinant DNA Experiment Safety Committee of Hamamatsu University School of Medicine, Japan.

**RESULTS**

***Generation of permanent cell lines of Huh7.5.1expressing recombinant mouse proteins (Cd81, Ocln) on their cell surfaces***

To investigate whether mouse homologues (Cd81, Ocln proteins) of HCV human entry factors inhibit the entry of HCVpp into the Huh 7.5.1 cells *in vitro*, we made two types of permanent cell lines of Huh 7.5.1 that could express these proteins on their cell surfaces. To detect the mouse proteins in these cells, we added the FLAG and HA tags at the C-terminal ends of Cd81 and Ocln proteins respectively (Figure 1A and B). We also made an empty vector (CXN2)-harboring permanent cell line of Huh7.5.1 used as control in the infectivity assay. Permanent cell lines of mouse Cd81, mouse Ocln-expressing Huh7.51 cells were made as described in the Materials and methods section. Next, using a Western blot analysis, we found the recombinant mouse proteins, Cd81 and Ocln to be synthesized from their respective cells (Figure 2A, lanes 2 and 3). Furthermore, in order to investigate whether the synthesized mouse proteins (Cd81, Ocln) were translocated properly in their respective cells, we did immunofluorescence staining and found these proteins with the expected cell surface localization (Figure 2 B, upper & middle panels for Cd81 & Ocln respectively).

***Generation of permanent cell lines of human NPC1L1-knocked down Huh7.5.1 and mouse NIH3T3 cells expressing five human factors (CCSO with NPC1L1)***

To determine whether the knock down of human NPC1L1 in Huh7.5.1 cells affects the entry of HCVpp, we made the human NPC1L1-knocked down permanent cell lines of Huh7.5.1 by transfecting the plasmid construct, CAIE-NPC1L1(antisense)-GFP (Figure 1D) into Huh7.5.1 cells. In order to confirm whether these cells produced the human antisense NPC1L1, we cloned the human antisense NPC1L1 cDNA into a GFP-expressing vector as described in the Materials and methods section. We found that these permanent cell lines could overexpress the human antisense NPC1L1 and translocate this protein properly (Figure 2B, lower panel). From the over-expression of human antisense NPC1L1, we could expect that the endogenous human sense NPC1L1 was totally knocked down in these cell lines.

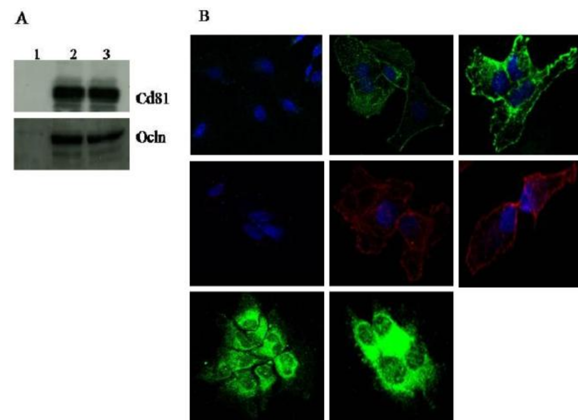


***Figure 2.*** *Detection of the recombinant mouse Cd81, Ocln proteins and the overexpression of human antisense NPC1L1 in Huh7.5.1 cells.* ***A****, The cell lysates obtained from the empty vector (CXN2)-harboring-Huh7.5.1 cells (lane 1) and the two clones of each Cd81-expressing-Huh7.5.1, Ocln-expressing-Huh7.5.1 cells (lanes 2 & 3) were subjected to the Western blotting against anti-FLAG (upper panel) and anti-HA antibodies (lower panel) for the detection of mouse Cd81 and Ocln proteins expression respectively.* ***B****, Localization of the mouse Cd81 (upper panel: middle and right, green for Cd81; blue, DAPI for nuclear staining) and mouse Ocln (middle panel: middle & right, red for Ocln; blue, DAPI for nuclear staining) proteins on the cell surfaces of each stably Cd81 and Ocln-expressing two clones of Huh7.5.1 cells. Empty vector (CXN2) transformed Huh7.5.1 cells served as a negative control (upper & middle panels: left). Detection of the over expression of human antisense NPC1L1 in the stably human antisense NPC1L1-expressing two clones of Huh7.5.1 cells observed by GFP expression (lower panel: left & middle). Photographs were obtained at 400X magnification.*

To investigate whether the introducing of another human entry factor in four human factors (CCSO)-expressing NIH3T3 cells facilitates the entry of HCVpp, we made the five human factors (CCSO with NPC1L1)-expressing NIH3T3 cells by transfecting the plasmid construct human sense NPC1L1 (Figure 1C) into CCSO-expressing NIH3T3 cells. To detect the expression of human NPC1L1 in these cell lines, we added the FLAG tag at the N-terminal end of human NPC1L1 (Figure 1C). We found that these cell lines could overexpress the human NPC1L1 (Figure 4A, lanes 2 and 3) and translocate this protein properly (Data not shown).

***Mouse homologues (Cd81, Ocln) and the knock down of human NPC1L1 inhibit the entry or infectivity of HCVpp in Huh7.5.1 cells***

The HCVpp we used here have HCV E1/E2 proteins in the envelope instead of MLV envelope protein, and a luciferase expression unit in the RNA genome[19]. To determine the specificity of HCVpp entry, we also made control pseudo-particles VSVGpp and NENVpp [20]. Next we infected two clones of each mouse Cd81-expressing Huh7.5.1 cells, mouse Ocln-expressing Huh7.5.1 cells, human NPC1L1-knocked down Huh7.5.1cells with the pseudo-particles. We also infected the native Huh7.5.1, empty vector CXN2-transfected Huh7.5.1 cells with the pseudo-particles as controls. 72 hours post infection; we obtained the cell extracts and measured the luciferase activities as mentioned in materials and methods section. From the luciferase activities, we obtained the results that the HCVpp entry or infectivity into the two clones of mouse Cd81-expressing Huh7.5.1 cells was reduced by average 31% and that into the two clones of mouse Ocln-expressing Huh7.5.1 cells by average 24.2% compared to the only empty vector-transfected Huh7.5.1 cells (Figure 3A).

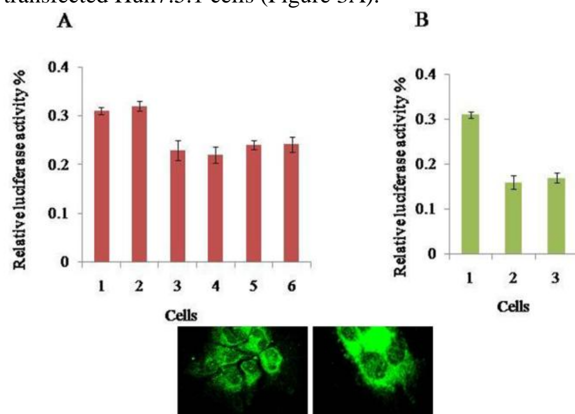


***Figure 3.*** *HCVpp entry or infectivity measured from the measurement of the relative luciferase activities of the cell extracts.* ***A****, The stably mouse proteins-expressing several cell lines (indicated below) of Huh7.5.1 were challenged in parallel with the HCVpp, VSVGpp and NENVpp. Relative luciferase activity was reported as the percentage of the titer of HCVpp divided by the titer of VSVGpp, after subtraction of the signals from the infection with NENVpp. HCVpp entry or infectivity measured from the relative luciferase activity by unitary method. The data shown were the means ± s.d of triplicate experiments. In the x-axis, no.1 & 2 indicated the native and the empty vector (CXN2)-harboring Huh7.5.1 cells respectively, 3 & 4 indicated the two clones of mouse Cd81-expressing Huh7.5.1cells, 5 & 6 indicated the two clones of mouse Ocln-expressing Huh7.5.1 cells.* ***B****, The stably human NPC1L1-knocked down-Huh7.5.1 cells were challenged in parallel with the HCVpp, VSVGpp, and NENVpp. Relative luciferase activity was reported as described previously and HCVpp entry or infectivity measured from the relative luciferase activity by unitary method. The data shown were the means ± s.d of triplicate experiments. In the x-axis, no.1 indicated the native Huh7.5.1 cells, 2 & 3 indicated the two clones of the human NPC1L1-knocked down-Huh7.5.1 cells.*

This result indicated that the mouse homologues (Cd81, Ocln) are directly inhibiting the entry or infectivity of HCVpp in Huh7.5.1 cells when these cells express the mouse proteins on their cell surfaces. Here the empty vector-transfected Huh7.5.1 cells were considered 100% infectivity with the HCVpp and unexpectedly, the native Huh7.5.1 cells showed 3% less infectivity compared to the empty vector-transfected Huh7.5.1 cells (Fig. 3A). It might be due to some technical errors. However, when we knocked down the human NPC1L1 in the two clones of Huh7.5.1 cells and infected these cells with the pseudo-particles, similarly we obtained the results from luciferase activities of the cell extracts and found that the HCVpp entry or infectivity was reduced by average 46.8% in these two clones of NPC1L1 knocked down Huh7.5.1 cells compared to the native Huh7.5.1 cells (Figure 3B). Here the native Huh7.5.1 cells were considered 100% infectivity with the HCVpp. This result indicated that human NPC1L1 is an important entry factor for HCVpp in Huh7.5.1 cells and this data also supports the previous study[15].

***The additional human factor NPC1L1 can make mouse NIH3T3 cells more susceptible to HCVpp entry or infectivity***

In order to investigate whether the introducing of another human entry factor in four human factors (CCSO) expressing NIH3T3 cells facilitates the entry of HCVpp, we made two permanent clones of the five human factors (CCSO with NPC1L1) expressing NIH3T3 cells. We then infected these two clones of five human factors expressing NIH3T3 cells with the pseudo-particles and simultaneously infected the four human factors expressing NIH3T3 cells as control. From the luciferase activities of the cell extracts obtained 72 h post infection, we obtained the result that the HCVpp entry or infectivity was increased by average 42.8% more in the two clones of five human factors expressing NIH3T3 cells compared to the four human factors expressing NIH3T3 cells (Figure 4B). This result

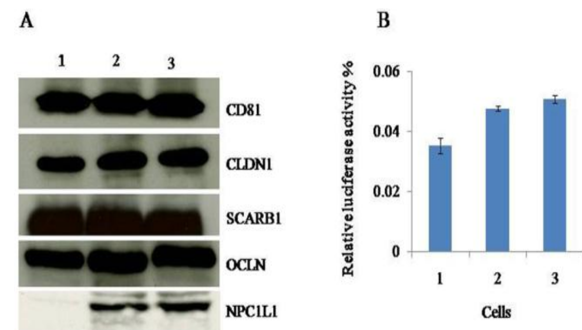


***Figure 4.*** *Detection of the human factors in mouse NIH3T3 cells, and HCVpp entry or infectivity measured.* ***A****, Detection of five human factors in NIH3T3 cells. Lanes 2&3, the cell lysates obtained from the two clones of stably five human factors expressing NIH3T3 cells were subjected to Western blotting against CD81, CLDN1, SCARB1, OCLN and anti-FLAG antibodies for the detection of human CD81, CLDN1, SCARB1, OCLN and NPC1L1 respectively. Lane 1 was the cell lysate obtained from the four human factors (CCSO)-expressing NIH3T3 cells.* ***B****, HCVpp entry or infectivity measured from the measurement of the relative luciferase activities of the cell extracts. The stably four and five human factors expressing several cell lines (indicated below) of NIH3T3 cells were challenged in parallel with the HCVpp, VSVGpp and NENVpp. Relative luciferase activity was reported as described previously and HCVpp entry or infectivity measured from the relative luciferase activity by unitary method. The data shown were the means ± s.d of triplicate experiments. In the x-axis, no.1 indicated the four human factors (CCSO)-expressing NIH3T3 cells, 2 & 3 indicated the two clones of five human factors (CCSO with NPC1L1)-expressing NIH3T3 cells.*

indicated that the additional human factor such as NPC1L1 can make mouse cells NIH3T3 more susceptible to HCVpp entry or infectivity when these cells express five human factors simultaneously.

## DISCUSSION

HCV follows several steps for successful infection in human hepatocytes. The first step is HCV entry into hepatocytes. The second step is the replication of HCV genomic RNA. The last step is viral assembly and budding from the host cells[21]. Many researchers are trying to make efficient mice models which will mimic humans in HCV infectivity, but so far very few were succeeded to some extent[7, 8]. The reason why not yet making an efficient mouse model for HCV infection might be due to the hindrance of HCV entry process into mouse hepatocytes not for the hindrance of post-entry processes. Because recently we have reported that mouse hepatocytes are suitable for HCV post-entry processes such as genomic RNA replication, assembly and release *in vivo*[9]. So, entry process might be the most important step for efficient viral infection in host cells. Our previous study also showed that mouse hepatocytes have entry inhibitory factor(s) for HCV[14], and in this study, we have found that mouse homologues (Cd81, Ocln) themselves are inhibitory. They prevent the entry of HCVpp significantly in the most permissive Huh7.5.1 cells when these cells express the mouse homologues on their cell surfaces. How these mouse homologues prevent the entry process of HCVpp, we need further study to clarify it. In this study, we have also shown that an additional human factor (NPC1L1) can make a non-permissive mouse cell (NIH3T3) to more permissive one for HCVpp entry when it expresses five human factors (CCSO with NPC1L1) simultaneously, whereas the knock down of NPC1L1 reduces HCVpp entry significantly in Huh7.51 cells which is consistent with the previous report[15]. So, we may expect that mouse hepatocytes may be more susceptible to HCV entry *in vivo* when they express human NPC1L1 along with four other known human factors (CCSO) simultaneously. Thus, these *in vitro* studies suggest that it is possible to make a more susceptible and immune-competent mouse model for HCV infection by deleting the mouse inhibitory factors (Cd81, Ocln) and introducing a new additional human entry factor (NPC1L1) when we make a mouse.

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