

# Hepatitis C virus utilizes VLDLR as a novel entry pathway

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**Various host factors are involved in the cellular entry of hepatitis C virus (HCV). In addition to the factors previously reported, we discovered that the very-low-density lipoprotein receptor (VLDLR) mediates HCV entry independent of CD81. Culturing Huh7.5 cells under hypoxic conditions significantly increased HCV entry as a result of the expression of VLDLR, which was not expressed under normoxic conditions in this cell line. Ectopic VLDLR expression conferred susceptibility to HCV entry of CD81-deficient Huh7.5 cells. Additionally, VLDLR-mediated HCV entry was not affected by the knockdown of cellular factors known to act as HCV receptors or HCV entry factors. Because VLDLR is expressed in primary human hepatocytes, our results suggest that VLDLR functions in vivo as an HCV receptor independent of canonical CD81-mediated HCV entry.**

virus entry | VLDLR | CD81 | hypoxia | hepatitis C virus

**H**epatitis C virus (HCV) infects more than 170 million people worldwide and is a major cause of chronic liver disease. The virus persists in 80% of infected individuals and can lead to chronic liver diseases including fibrosis, cirrhosis, steatosis, and hepatocellular carcinoma. HCV, an enveloped positive-stranded virus, enters host cells by using various host factors that function as receptors and mediate endocytosis. Several host factors, including CD81 (1), claudin-1 (CLDN1) (2), occludin (OCLN) (3), and scavenger receptor class B member 1 (SR-BI) (4), have been identified as receptors. Heparan sulfate glycosaminoglycan represents the first attachment site (5) before the interaction of the virus with these factors. Because all the entry factors are required for productive HCV infection, HCV entry seems to be the result of an orchestrated process involving these factors. Additionally, low-density lipoprotein receptor (LDLR) (6), Niemann-Pic C1-like 1 (NPC1L1) (7), transferrin receptor 1 (TfR1) (8), and epidermal growth factor receptor (EGFR) (9) have been shown to play a role in HCV entry. CD81 was the first factor to be identified as an HCV receptor, and it plays an important role in this process by binding with the HCV envelope glycoprotein E2 (10, 11). Indeed, CD81-deficient cell lines such as HepG2 do not permit the entry of HCV (2, 3).

Recent studies have demonstrated that HCV RNA replication in Huh7.5 cells is enhanced under hypoxic conditions (12). Because the oxygen content in liver tissue in vivo is estimated to be lower (with a gradient of 9–3%) than the oxygen content under in vitro culture conditions (13), the HCV life cycle may differ significantly from that observed using in vitro culture systems. The very-low-density lipoprotein receptor (VLDLR) is induced under hypoxic conditions. In turn, this receptor enhances the uptake of low-density lipoproteins (LDLs) and very-low-density lipoproteins (VLDLs) (14), possibly through the recognition of ligands (such as apolipoprotein) that associate with the lipoproteins (15). VLDLR is a type I transmembrane lipoprotein receptor belonging to the LDLR family (16). The expression of VLDLR increased 4.2-fold and 3.5-fold in HCV cirrhotic and HCV-HCC patients, respectively, as compared with normal

controls without liver disease (17). In vitro analysis has shown that during the early stage of infection HCV recognizes lipoprotein receptors such as SR-BI and LDLR on target cells via the association of the virus with apolipoprotein E (ApoE) or other related ligands (18). However, the cell lines that have been widely used for the analysis of HCV infection/replication (i.e., Huh7 and its derivatives) do not express VLDLR under conventional culture conditions (12), thereby preventing analysis of the role of VLDLR in HCV infection.

The HCV particle is a lipo-viro-particle (LPV) that contains lipoprotein components such as triglycerides, apolipoprotein B-100 (ApoB), and ApoE (19, 20). Because hypoxia affects the uptake of lipoproteins and therefore might influence HCV entry and replication, we hypothesized that the HCV life cycle might be influenced by oxygen levels.

Here, we elucidate the presence of a novel HCV entry pathway that uses VLDLR. Under hypoxic conditions, HCV entry into an in vitro cell-culture system was increased by up-regulating VLDLR expression. Moreover, VLDLR-mediated HCV entry was independent of the CD81-mediated HCV entry pathway.

## Results

**Increase in HCV Infection Under Hypoxic Conditions.** It has been shown that hypoxic conditions enhance HCV replication (12). We analyzed HCV infection in Huh7.5 cells under hypoxic conditions and observed increased infectivity of JFH1 (HCVcc<sup>JFH1</sup>), an infectious HCV clone (Fig. 1A). The HCVcc<sup>JFH1</sup> titer was approximately

## Significance

**Hepatitis C virus (HCV) utilizes various host factors to enter host cells. During the process of HCV entry, cell surface-residing lipoprotein receptors such as scavenger receptor class B member 1 (SR-BI) play important roles through interactions with virus envelope protein E2 and virus-associated apolipoproteins such as apolipoprotein E (ApoE). CD81 plays a crucial role during this process in association with HCV. Here, we identified another pathway for HCV entry that does not use CD81. This pathway involves an association with the very-low-density lipoprotein receptor (VLDLR) and does not require previously reported host factors such as claudin, occludin, or CD81. This finding may shed new light on the process of HCV entry.**

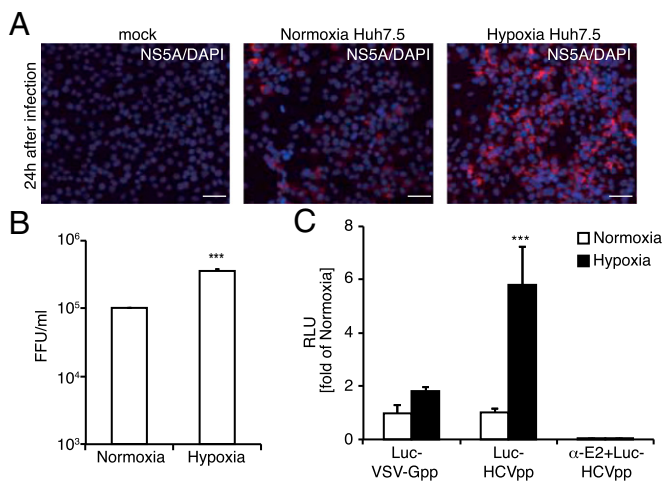
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**Fig. 1.** Increase in HCV entry under hypoxic conditions. (A) Huh7.5 cells cultured under normoxic or hypoxic conditions (1% O<sub>2</sub>) were infected with HCVcc<sup>JFH1</sup> with a multiplicity of infection (MOI) of 1. At 24 h postinfection, the cells were stained with NS5A (red). (Scale bars, 50  $\mu$ m.) (B) Analysis of HCV infectivity. Huh7.5 cells cultured under normoxic or hypoxic conditions were infected with serially diluted HCVcc<sup>JFH1</sup> for 24 h. Then HCV-infected cells stained with anti-HCV NS5A antibody were counted to obtain focusing-forming units (FFU) (average  $\pm$  SD;  $n = 3$ ). (C) The effect of Huh7.5 cells cultured under normoxic or hypoxic conditions on HCV entry. Huh7.5 cells cultured under normoxic (white bar) or hypoxic (black bar) conditions were infected with Luc-VSV-Gpp and Luc-HCVpp (genotype 2a). At 24 h postinfection, luciferase activity was quantified (average  $\pm$  SD;  $n = 3$ ). Treatment with the E2 antibody (15  $\mu$ g/mL) was included as a control. RLU, relative light units. \*\*\* $P < 0.005$  (Student's  $t$ -test).

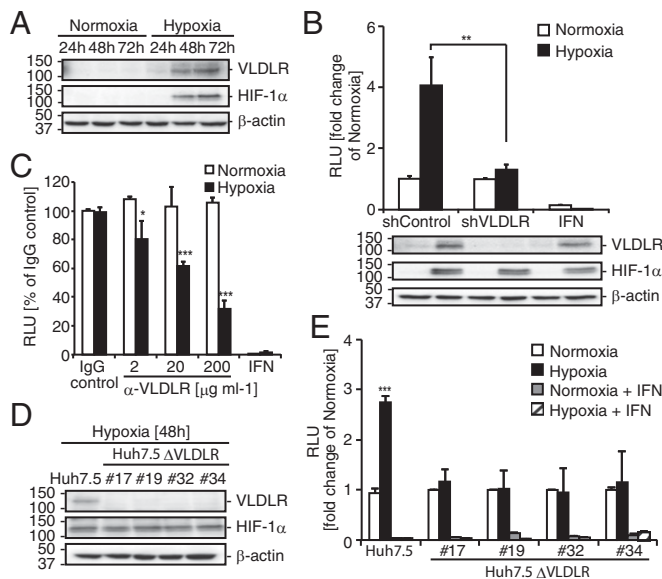
threefold higher under hypoxic conditions (Fig. 1B). To analyze whether the increased infection by HCVcc<sup>JFH1</sup> under hypoxic conditions is dependent not only on postinfection events but also on virus entry events, an HCV entry analysis was performed with luciferase-encoded HCV genotype 2a enveloped pseudoparticles (Luc-HCVpp) constructed with a lentivirus vector system (Fig. 1C). Luc-HCVpp specifically monitor the effects of HCV entry. Compared with vesicular stomatitis virus G protein pseudoparticles (Luc-VSV-Gpp) infection levels, which were unaffected by O<sub>2</sub> conditions, the luciferase activity in cells infected with Luc-HCVpp was approximately sixfold higher under hypoxic conditions. Then we analyzed the expression of various factors known to be involved in HCV entry to see if the enhanced virus entry was the consequence of an enhancement of the conventional entry mechanism. Protein and mRNA expression levels of CD81, SR-BI, LDLR, and NPC1L1 were unchanged under hypoxic or normoxic conditions, but expression of CLDN1 and OCLN were slightly increased under hypoxic conditions (Fig. S1A and B), and EGFR expression was reduced under hypoxic conditions. Because ectopic expression of CLDN1, OCLN, and EGFR did not alter the level of HCV infection (Fig. S1C), it is unlikely that these factors are involved in increased HCV infection under hypoxic conditions. This evidence led us to hypothesize that a yet-to-be-identified receptor or entry factor is involved in HCV entry under hypoxic conditions.

**HCV Entry Is Enhanced by the Induced Expression of VLDLR Under Hypoxic Conditions.** Infectious HCV constitutes a complex with lipid components such as triglycerides, ApoB, and ApoE, resulting in the formation of an LVP (19). The association of virus-associated ApoE with lipoprotein receptors on the cell surface is thought to be required for infectivity (21, 22). The uptake of LDL and VLDL is increased in hepatocytes under hypoxic conditions because of the induction of VLDLR expression and the association

with ApoE (14, 15). These reports led us to analyze the role of VLDLR in HCV entry.

The expression of VLDLR in Huh7.5 cells was induced under hypoxic conditions at the protein (Fig. 2A) and mRNA (Fig. S2A) levels. To test whether VLDLR affects HCV entry, VLDLR was knocked down transiently in Huh7.5 cells using shRNA (Fig. S2B), and the infection of Luc-HCVcc<sup>JFH1</sup> was verified using siRNA#1 because this cell line had the best knockdown efficiency (Fig. 2B and Fig. S2B). Under hypoxic conditions an approximately threefold reduction in Luc-HCVcc<sup>JFH1</sup> infection was observed in shVLDLR#1-treated Huh7.5 cells as compared with shControl cells, even though the infection in shVLDLR#1-treated and shControl cells was unchanged under normoxic conditions (Fig. 2B). Moreover, we examined the effect of a VLDLR antibody on HCV infection. The inhibition of Luc-HCVcc<sup>JFH1</sup> entry was observed in a dose-dependent manner in Huh7.5 cells grown under hypoxic conditions, but no effect was observed in the cells grown under normoxic conditions (Fig. 2C).

To investigate further the role of VLDLR in HCV entry, we established a VLDLR-knockout Huh7.5 cell line (Huh7.5  $\Delta$ VLDLR) using the clustered, regularly interspaced short-palindromic-repeat (CRISPR)/Cas9 system targeting a consensus sequence of mRNAs for all VLDLR isoforms (Fig. 2D). All cell clones lacking expression of the VLDLR gene failed to induce VLDLR expression under hypoxic conditions. The ability of HCV to infect each clone was nearly unchanged under normoxic conditions (Fig. S2C). Although none of these cells exhibited increased Luc-HCVcc<sup>JFH1</sup> infection under hypoxia (Fig. 2E), Luc-HCVcc<sup>JFH1</sup>



**Fig. 2.** HCV entry is enhanced by VLDLR under hypoxic conditions. (A) VLDLR, hypoxia-induced factor 1-alpha (HIF-1 $\alpha$ ), and  $\beta$ -actin levels were analyzed 24, 48, and 72 h after culturing under normoxic or hypoxic conditions. (B) shControl- or shVLDLR#1-transfected Huh7.5 cells were infected with Luc-HCVcc<sup>JFH1</sup> (MOI = 0.1). Luciferase activity was analyzed 24 h postinfection (average  $\pm$  SD;  $n = 3$ ). Treatment with IFN- $\beta$  (100 IU/mL) was included as a control. The VLDLR knockdown effect was verified by immunoblotting. (C) Hypoxic or normoxic cultured Huh7.5 cells were preincubated with IgG as a control or with anti-VLDLR for 1 h at 37  $^{\circ}$ C. After treatment, the cells were infected with Luc-HCVcc<sup>JFH1</sup> (MOI = 0.1) for 24 h (average  $\pm$  SD;  $n = 3$ ). (D) Immunoblot analysis of VLDLR, HIF-1 $\alpha$ , and  $\beta$ -actin levels 48 h after culture of Huh7.5 or Huh7.5  $\Delta$ VLDLR cells (#17, #19, #32, and #34) under hypoxic conditions. (E) Infection of Luc-HCVcc<sup>JFH1</sup> (MOI = 0.1) in Huh7.5  $\Delta$ VLDLR clones cultured under normoxic or hypoxic conditions. Cell lysates were analyzed 24 h after infection (average  $\pm$  SD;  $n = 3$ ). The data represent three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$  (Student's  $t$ -test).

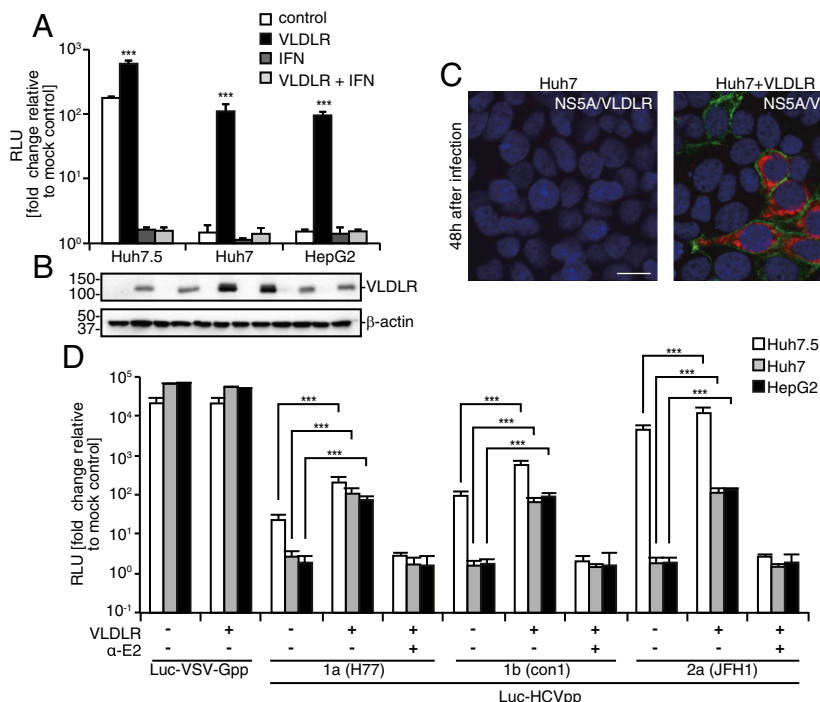
infection was rescued by the ectopic expression of VLDLR in all Huh7.5  $\Delta$ VLDLR clones, with the level of rescue varying from three- to 13-fold, depending on the clone (Fig. S2D). These results suggest that Luc-HCVcc<sup>JFH1</sup> infection was increased by the induced expression of VLDLR under hypoxic conditions.

**Ectopic Expression of VLDLR Variant 2 Showed the Greatest Entry of Luc-HCVcc<sup>JFH1</sup> Under Normoxic Conditions.** The VLDLR mRNA encodes four splicing variants (Fig. S3A) (23, 24). Variants 1 and 2 were the major variants induced in Huh7.5 cells under hypoxic conditions (Fig. S3B). To test whether ectopic expression of a variant of VLDLR influences HCV infection under normoxia, Huh7 cells were transfected with plasmids expressing VLDLR variants 1–4 followed by challenge with Luc-HCVcc<sup>JFH1</sup>. Cells transfected with VLDLR variant 2 showed the highest Luc-HCVcc<sup>JFH1</sup> infection; cells transfected with the other variants showed only a marginal increase in Luc-HCVcc<sup>JFH1</sup> infection (Fig. S3C). To analyze the effect of VLDLR variant 2 on other cells under normoxic conditions, Huh7.5, HepG2, and Huh7 cells were transfected with a plasmid expressing VLDLR variant 2 followed by Luc-HCVcc<sup>JFH1</sup> infection (Fig. 3A). The VLDLR expression levels are shown in Fig. 3B. Luciferase activity was increased fivefold in Huh7.5 cells expressing VLDLR (Fig. 3A), and Huh7 and HepG2 cells expressing VLDLR showed a 100-fold and 95-fold increase, respectively. HCVcc<sup>JFH1</sup> infection was detected by immunostaining in Huh7 cells expressing ectopic VLDLR (Fig. 3C). To analyze the effect of VLDLR on HCV replication, we assessed the levels of HCV RNA and HCV proteins in HCV full-length RNA replicon cells, NNC#2, with or without the expression of VLDLR. We found no differences in the level of HCV RNA and proteins (Fig. S4A and B). Moreover, the activity of the HCV internal ribosome entry site (IRES) examined by the HCV IRES-luc plasmid was not affected by VLDLR (Fig. S4C and D). Subsequently, using the Luc-HCVpp lentivirus vector system, we analyzed whether VLDLR-dependent infection was affected by the HCV genotype. VLDLR did not affect infection by Luc-VSV-Gpp. However, Luc-HCVpp infection was increased irrespective of HCV genotype in VLDLR-expressing cells (Fig. 3D). Therefore, we think that the increase in HCV infection was caused by the enhanced entry of HCV resulting from VLDLR expression.

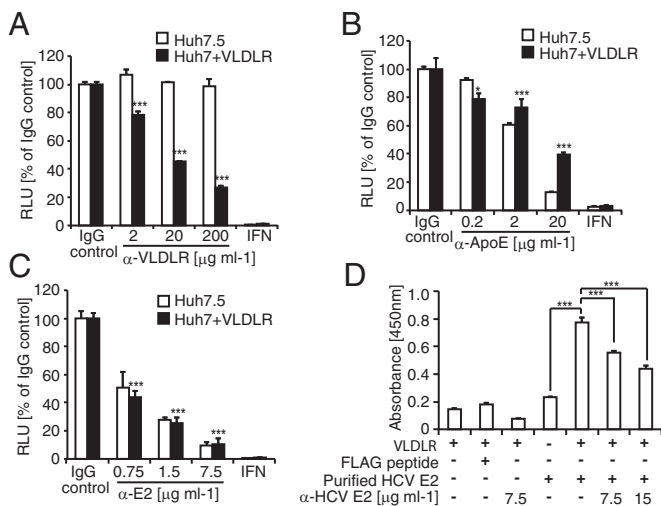
**VLDLR-Mediated HCV Entry Requires HCV E2 and ApoE.** To clarify the role of VLDLR in HCV entry, we examined the effect of ApoE, a ligand for VLDLR, and HCV E2. The HCVcc<sup>JFH1</sup> infection of VLDLR-expressing Huh7 cells was inhibited by the dose dependence of the anti-VLDLR antibody, whereas no effect was observed in Huh7.5 cells (Fig. 4A). This finding confirms that VLDLR is an HCV entry factor. Next, we tested the inhibition of HCV infection by antibodies directed against ApoE and HCV E2. Both antibodies suppressed Luc-HCVcc<sup>JFH1</sup> luciferase activity in a dose-dependent manner in Huh7.5 cells and in Huh7 cells expressing VLDLR (Fig. 4B and C). The effect of ApoE and HCV E2 on VLDLR-dependent HCV infection also was examined using CD81-knockout VLDLR-bearing Huh7.5 cells. HCV infection in the cells also was inhibited by the antibodies against ApoE and HCV E2 (Fig. S5A). The binding of VLDLR to HCV E2 was observed by ELISA using recombinant VLDLR and purified HCV E2 (Fig. 4D). This binding was specific, because it was competitively inhibited by the addition of an HCV E2 antibody (Fig. 4D). Additionally, VLDLR and HCV E2 interaction was confirmed by an immunoprecipitation experiment (Fig. S5B). These results suggest that ApoE and HCV E2 play roles in VLDLR-mediated HCV entry.

The NPVY domain of VLDLR is important for clathrin-dependent endocytosis (16). To ascertain the role of VLDLR-mediated endocytosis in HCV entry, we analyzed HCV entry in cells expressing a VLDLR with the NPVY motif in the VLDLR cytoplasmic domain mutated to AAVA (Fig. S6A). The mutated VLDLR did not allow the entry of Luc-HCVcc<sup>JFH1</sup> (Fig. S6B). Furthermore, treatment with chlorpromazine, an inhibitor of clathrin-dependent endocytosis, reduced Luc-HCVcc<sup>JFH1</sup> infection in VLDLR-expressing Huh7.5 $\Delta$ CD81#19 cells (Fig. S6C). Thus, HCV uses clathrin-dependent endocytosis via VLDLR to enter the target cells.

**VLDLR-Mediated HCV Entry Does Not Require Known HCV Receptors and Entry Factors.** To determine whether VLDLR-mediated HCV entry is CD81 dependent, we examined HCV infection in CD81-deficient Huh7.5 (Huh7.5#26) cells ectopically expressing VLDLR. The CD81-deficient Huh7.5#26 cell line was established from an analysis of Huh7.5 cells that showed a resistant



**Fig. 3.** Ectopic expression of VLDLR increased HCV infection even under normoxic conditions. (A) Cells transfected with a VLDLR-expressing or empty plasmid were infected with Luc-HCVcc<sup>JFH1</sup> (MOI = 0.1). Luciferase activity was analyzed 48 h postinfection (average  $\pm$  SD;  $n = 3$ ). (B) Immunoblot of VLDLR and  $\beta$ -actin. (C) Huh7 cells transfected with VLDLR-expressing or empty plasmid were infected with HCVcc<sup>JFH1</sup> (MOI = 1). The cells were stained for NS5A (red) and VLDLR (green) 48 h postinfection. Images were analyzed by confocal microscopy. (Scale bars, 20  $\mu$ m.) (D) Cells transfected with an empty or VLDLR-expressing plasmid were infected with luciferase-encoding pseudoparticles bearing the indicated envelopes. Luciferase activity was analyzed 72 h postinfection (average  $\pm$  SD;  $n = 3$ ). The data shown represent three independent experiments. \*\*\* $P < 0.005$  (Student's  $t$ -test).



**Fig. 4.** Effect of HCV E2 and ApoE on VLDLR-mediated HCV entry. (A) Huh7.5 or Huh7 cells transfected with a VLDLR-expressing plasmid were preincubated with IgG as a control or with anti-VLDLR for 1 h at 37 °C. (B and C) After treatment, the cells were infected with Luc-HCVcc<sup>JFH1</sup> (MOI = 0.1) for 48 h (average  $\pm$  SD;  $n = 3$ ). Luc-HCVcc<sup>JFH1</sup> was preincubated with an IgG control or anti-ApoE (B) and anti-HCV E2 (C). Huh7.5 or Huh7 cells transfected with a VLDLR-expressing plasmid were infected with antibody-treated Luc-HCVcc<sup>JFH1</sup> (MOI = 0.1) for 48 h (average  $\pm$  SD;  $n = 3$ ). (D) Recombinant VLDLR-coated plates were reacted with purified HCV E2 or with purified HCV E2 treated with anti-E2 antibody. The signal was detected using an anti-FLAG antibody and HRP-conjugated mouse IgG. Light absorbance was measured at 450 nm (average  $\pm$  SD;  $n = 3$ ). The data shown represent three independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.005$  (Student's *t*-test).

phenotype to HCV infection. The low level of CD81 expression in Huh7.5#26 cells was confirmed by flow cytometry (Fig. 5A). As expected, HCV infection of Huh7.5#26 cells was not observed using immunofluorescence (Fig. S7A). However, a 70-fold increase in Luc-HCVcc<sup>JFH1</sup> infection was evident in Huh7.5#26 cells expressing VLDLR (Fig. 5B). HCV infection was confirmed by immunostaining (Fig. S7A and B). More importantly, similar results were observed in VLDLR-expressing HepG2 cells that were CD81 deficient (Fig. S7B). Moreover, we established Huh7.5ΔCD81 clones #14 and #19 using the CRISPR method (Fig. 5C). These clones do not express VLDLR when grown under normoxic conditions and are resistant to HCV infection. However, they became susceptible to HCV infection when transduced with CD81 or VLDLR (Fig. 5D). Importantly, HCV infection in VLDLR-expressing Huh7.5ΔCD81#14 and #19 cells was not affected by CD81 antibody treatment (Fig. S7C). These results clearly indicate that VLDLR-mediated HCV infection is CD81 independent. Next, the requirement for the previously identified HCV receptors and entry factors in VLDLR-mediated HCV entry was analyzed by knockdown of each factor by siRNA. The target siRNA sequences are shown in Table S1. The siRNA knockdown efficiency was confirmed by Western blotting (Fig. S7D). We observed that knockdown of the factors CLDN1, OCLN, SR-BI, LDLR, and NPC1L1 did not suppress Luc-HCVcc<sup>JFH1</sup> entry into VLDLR-expressing Huh7.5 and Huh7.5#26 cells (Fig. S7E). However, in the absence of exogenous VLDLR expression, as expected, the inhibition of Luc-HCVcc<sup>JFH1</sup> infection ranging from 20–40% was observed in Huh7.5 cells after treatment with antibodies against SR-BI, LDLR, and NPC1L1 (Fig. S7F). Luciferase activity in VLDLR-expressing Huh7.5#26 cells that lacked CD81 expression after infection by Luc-HCVcc<sup>JFH1</sup> was not suppressed by treatment with these antibodies. Moreover, VLDLR-mediated Luc-HCVpp entry was observed in CLDN1-deficient 293FT cells (Fig. S7G). These results suggest that SR-BI, LDLR, NPC1L1,

and CLDN1 are not directly involved in VLDLR-mediated HCV infection.

Finally, we investigated whether VLDLR-mediated HCV entry resulted in abortive or productive infection. Supernatants were recovered from VLDLR-expressing Huh7.5#26 cells infected with HCVcc<sup>JFH1</sup> and were applied to Huh7.5 cells to analyze infection (Fig. S8A and B). Infected cells were observed by confocal microscopy, indicating that VLDLR-mediated HCV entry into the cells culminates in productive release.

**Mouse VLDLR Is Capable of Mediating HCV Entry.** Mouse hepatocytes become permissive for HCV entry when human CD81 and OCLN are expressed (3). However, it is likely that other factors expressed in mouse hepatocytes can be substituted and can function cooperatively in HCV entry. Transgenic mice expressing human CD81 and OCLN also support HCV entry (25). The discovery of the involvement of mouse VLDLR in HCV entry in Huh7.5#26 cells raised the question of whether HCV entry into mouse hepatocytes occurs exclusively via the CD81-dependent pathway. VLDLR expression was not observed in the mouse liver (26), and we confirmed this result (Fig. S9A). Thus, it is possible that the mouse liver does not take in HCV via the VLDLR pathway. However, a potential role for mouse VLDLR as a HCV receptor cannot be ruled out completely. To analyze this issue further, we molecularly cloned the mouse ortholog of the VLDLR gene and analyzed its function in HCV infection by ectopic expression in Huh7.5#26 cells that lack expression of endogenous VLDLR (Fig. S9B). HCVcc infection was observed in mouse VLDLR-transfected Huh7.5#26 cells (Fig. S9B). VLDLR expression was not observed in the mouse Hepa1-6 cell line (Fig. S9D). However, expression of mouse VLDLR in Hepa1-6 cells enabled the entry of Luc-HCVpp without affecting the entry of the Luc-VSV-Gpp control (Fig. S9C and D). Therefore, we propose that the lack of HCV infection in mouse cells in the absence of the human CD81 and OCLN genes results from a lack of sufficient expression of VLDLR and that HCV infection may occur through the VLDLR pathway if VLDLR expression is induced by environmental stimuli.

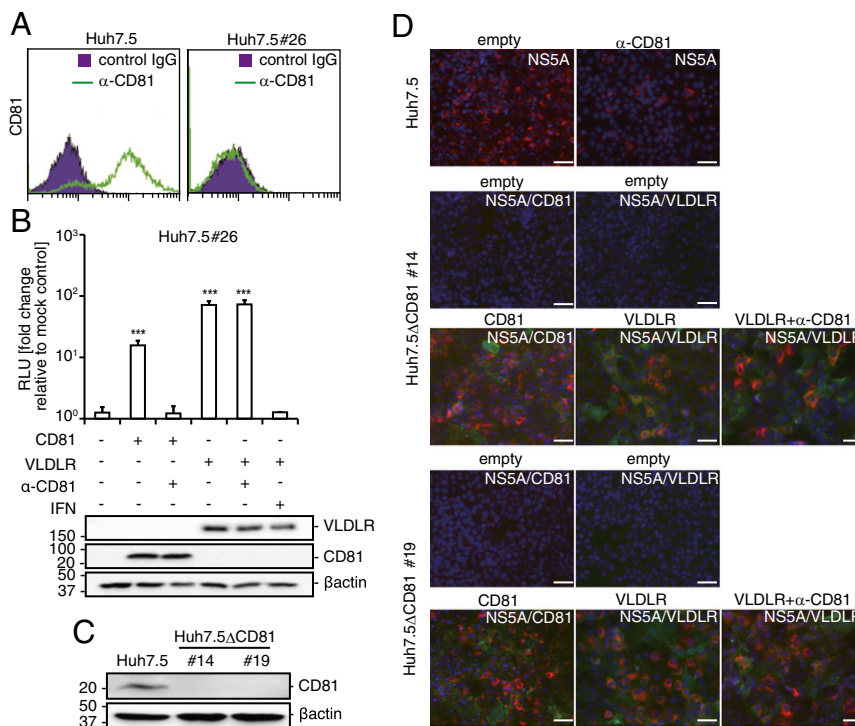
#### VLDLR-Mediated HCV Entry Occurs in Primary Human Hepatocytes.

The molecular mechanism of HCV entry was revealed using an *in vitro* HCV infection/replication system that is dependent primarily on the use of Huh7.5 and related cell lines. As described here, VLDLR is not expressed in Huh7.5 cells under normal culture conditions. Therefore, the role of VLDLR under physiological conditions has not been fully demonstrated.

To investigate the significance of VLDLR-mediated HCV entry *in vivo*, we analyzed the expression of VLDLR in cDNA derived from human liver specimens (Fig. 6A). Additionally, VLDLR protein expression levels were analyzed in primary human hepatocytes (PHH) derived from urokinase-type plasminogen activator severe-combined immunodeficiency (uPA/SCID) mice bearing human hepatocytes (Fig. 6B). VLDLR mRNA and protein expression were not observed in Huh7.5 cells but were observed in human liver tissue and PHHs. Thus, VLDLR is expressed in the liver under physiological conditions. Next, we investigated whether VLDLR is used for HCV entry in PHHs by adding a VLDLR antibody during infection (Fig. 6C). The VLDLR antibody inhibited the entry of HCVcc<sup>JFH1</sup> by 45% (Fig. 6C). Moreover, cotreatment with CD81 and VLDLR antibodies blocked the entry of HCVcc<sup>JFH1</sup> by 75% (Fig. 6C). These results suggest the involvement of VLDLR-mediated HCV entry under physiological conditions.

#### Discussion

The process of HCV entry into a target cell uses various host factors that seem to function via an orchestrated mechanism because infectivity is severely suppressed by the knockdown of any



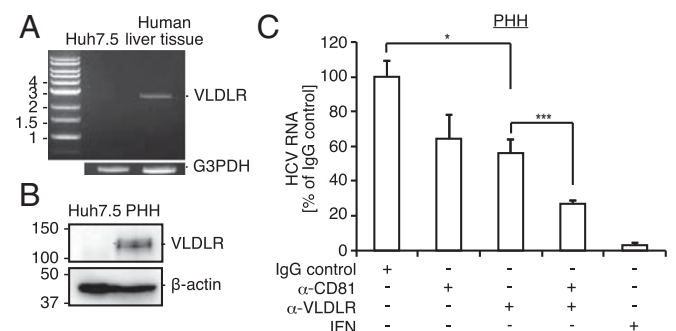
**Fig. 5.** VLDLR-mediated HCV entry using a CD81-independent pathway. (A) Huh7.5 or Huh7.5#26 cells were stained for CD81 (solid green line). The purple area indicates isotype-control staining. (B) Huh7.5#26 cells transfected with an empty, CD81-, or VLDLR-expressing plasmid were infected with Luc-HCVcc<sup>JFH1</sup> (MOI = 0.1). CD81 antibody (10 μg/mL) or IFN-β (100 IU/mL) was used to pretreat or treat cells, respectively. Luciferase activity was measured after 48 h (average ± SD; n = 3). VLDLR and CD81 expression levels were analyzed by immunoblotting. (C) Expression of CD81 in Huh7.5, Huh7.5ΔCD81#14, and Huh7.5ΔCD81#19 cells were verified by immunoblotting. (D) Huh7.5, Huh7.5ΔCD81#14, and Huh7.5ΔCD81#19 cells transfected with empty, CD81-, or VLDLR-lentiviral vector were infected with HCVcc<sup>JFH1</sup> (MOI = 1). The CD81 antibody (10 μg/mL) was pretreated for 1 h at 37 °C. The cells were stained for NS5A (red) and VLDLR (green) or CD81 (green) 48 h postinfection. Images were analyzed by fluorescent microscopy. (Scale bars, 50 μm.) The data shown represent three independent experiments. \*\*\*P < 0.005 (Student's *t*-test).

of these factors. The role of CD81 (to our knowledge the first identified HCV entry factor) in this process has been well characterized. CD81 interacts with the HCV E2 protein and SR-BI during an early stage of infection. Knockdown of CD81 or the use of CD81-deficient cells abolishes HCV infection, thereby demonstrating the importance of this molecule in HCV infection. During analysis of HCV entry using the Huh7.5 cell line (the cells most susceptible to HCV infection), we noticed that this cell line lacks expression of VLDLR. However, VLDLR expression was increased in Huh7.5 cells cultured under hypoxic conditions, leading us to analyze the role of this molecule in HCV infectivity.

The induced expression of VLDLR under hypoxic conditions increased HCV infectivity. Importantly, we found that the VLDLR-mediated HCV entry pathway was independent of CD81. In fact, HCV could enter Huh7.5 cells that lacked CD81 expression when VLDLR was ectopically expressed. HCV infection using VLDLR does not require CD81 and also does not require other factors that previously were demonstrated to function as host factors for HCV infection, because there was no reduction in infection following the knock down of CLDN1, OCLN, SR-BI, LDLR, and NPC1L1 in Huh7.5 or Huh7.5#26 cells transduced with VLDLR (Fig. S7E). This result suggests that the mechanism of VLDLR-mediated HCV infection is different from previously reported mechanisms (18).

There are several isoforms of VLDLR, variants 1–4. Variants 1, 2, and 3 were expressed under hypoxic but not normoxic conditions in Huh7.5 cells at different levels of expression, with the highest expression detected for variant 2 (Fig. S3B). Ectopic expression of VLDLR variant 2 induced the highest susceptibility to HCV infection (Fig. S3C). To determine whether VLDLR-dependent signaling plays a role in HCV infection, we

generated mutants of VLDLR variant 2 (Fig. S6A). The conversion of the NPVY motif in the cytoplasmic domain of VLDLR to AAVA resulted in a striking reduction in HCV infection (Fig. S6B). Furthermore, chlorpromazine, an inhibitor for clathrin-mediated endocytosis, inhibited VLDLR-mediated



**Fig. 6.** VLDLR-mediated HCV entry occurs in PHHs. (A) Expression of VLDLR in human liver tissue cDNA and Huh7.5 mRNA was analyzed by PCR or RT-PCR, respectively. G3PDH was used as the internal control. (B) VLDLR expression in Huh7.5 cells and PHHs was assessed by immunoblotting. (C) PHHs was preincubated with anti-VLDLR (20 μg/mL), anti-CD81 (1 μg/mL), anti-VLDLR (20 μg/mL) + anti-CD81 (1 μg/mL), or an IgG control for 1 h at 37 °C before infection with HCVcc<sup>JFH1</sup> (MOI = 1). HCV RNA was quantified by quantitative RT-PCR (qRT-PCR) 72 h postinfection (average ± SD; n = 3). The data shown represent three independent experiments. \*P < 0.05, \*\*\*P < 0.005 (Student's *t*-test).

HCV infection (Fig. S6C). These data strongly suggest that HCV entry uses VLDLR signaling and endocytosis.

Similar to SR-BI, VLDLR variant 2 interacted with the HCV E2 protein (Fig. S5B).

VLDLR-mediated HCV entry requires HCV E2 (Figs. 3D and 4C). Anti-ApoE suppressed VLDLR-mediated HCVcc entry (Fig. 4B). Because VLDLR binds to all types of ApoE isoforms, the interaction of ApoE with VLDLR may facilitate the entry of HCV. However, the precise role of ApoE in VLDLR-dependent HCV entry should be clarified further.

It is not known whether VLDLR-mediated HCV infection occurs along with CD81-dependent infection under physiological conditions in humans.

In addition to the mouse primary hepatocytes, we observed the expression of VLDLR in cDNA derived from normal human liver tissues and human hepatocytes derived from uPA/SCID mice expressing human hepatocytes (27). Furthermore, 55% of HCV entry into human hepatocytes derived from uPA/SCID mice was blocked following treatment with an anti-VLDLR antibody (Fig. 6C).

The expression of VLDLR mRNA in normal human liver specimens and the increased expression of VLDLR in HCV-infected individuals raise the possibility that VLDLR-mediated entry of HCV occurs under physiological conditions. Furthermore, because VLDLR expression is induced to variable degrees by environmental stimuli such as endoplasmic reticulum (ER) stress (28), the degree of VLDLR-dependent HCV entry compared with CD81-mediated entry may be affected by various factors in different individuals. A detailed analysis of this possibility warrants further investigation to obtain a conclusive result.

In summary, VLDLR is a novel HCV receptor and constitutes an HCV entry pathway that is distinct from the

CD81-dependent pathway. VLDLR expression in hepatocytes is induced under hypoxic conditions. ER stress also induces VLDLR expression in hepatocytes in vivo (28). Thus, we can speculate that HCV infection of individuals is affected by environmental conditions that alter the hepatocyte physiology. In this regard, clarification of the mechanism of the VLDLR-dependent entry of HCV may be relevant to therapeutic approaches.

## Materials and Methods

For details of antibodies and reagents, plasmids, RNAi, infection with HCVpp and HCVcc, assays of infectivity and HCV IRES activity, RT-PCR, ELISA, immunostaining, and statistical analyses, please see *SI Materials and Methods*.

Huh7.5, Huh7, HepG2, 293FT, NNC#2 (HCV full-length replicon genotype 1b) (29), Huh7.5#26, Huh7.5  $\Delta$ VLDLR (clones #17, #19, #32, and #34), and Hepa1-6 cells were used in this study. Huh7.5#26 cells (CD81-deficient Huh7.5 cell as to Huh7.5#26) were obtained by screening for an HCVcc<sup>RFH1</sup>-resistant phenotype. VLDLR-knockout Huh7.5 cells and CD81-knockout Huh7.5 cells were isolated using the CRISPR/Cas9 knockout system. Hepa1-6, a mouse liver cell line, was provided by the RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. Primary human hepatocytes were purchased from PhoenixBio.

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