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Occludin-Knockout Human Hepatic Huh7.5.1-8-Derived Cells Are Completely Resistant to Hepatitis C Virus Infection

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It is well known that occludin (OCLN) is involved in hepatitis C virus (HCV) entry into hepatocytes, but there has been no conclusive evidence that OCLN is essential for HCV infection. In this study, we first established an OCLN-knockout cell line derived from human hepatic Huh7.5.1-8 cells using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 system, in which two independent targeting plasmids expressing single-guide RNAs were used. One established cell clone, named OKH-4, had the OCLN gene truncated in the N-terminal region, and a complete defect of the OCLN protein was shown using immunoblot analysis. Infection of OKH-4 cells with various genotypes of HCV was abolished, and exogenous expression of the OCLN protein in OKH-4 cells completely reversed permissiveness to HCV infection. In addition, using a co-culture system of HCV-infected Huh7.5.1-8 cells with OKH-4 cells, we showed that OCLN is also critical for cell-to-cell HCV transmission. Thus, we concluded that OCLN is essential for HCV infection of human hepatic cells. Further experiments using HCV genomic RNA-transfected OKH-4 cells or HCV subgenomic replicon-harboring OKH-4 cells suggested that OCLN is mainly involved in the entry step of the HCV life cycle. It was also demonstrated that the second extracellular loop of OCLN, especially the two cysteine residues, is critical for HCV infection of hepatic cells. OKH-4 cells may be a useful tool for understanding not only the entire mechanism of HCV entry, but also the biological functions of OCLN.

Key words hepatitis C virus; hepatocyte; occludin

Hepatitis C virus (HCV) infection is recognized as a major threat to global public health, with 185 million people being infected with the virus worldwide.1) When infected with HCV, 75–85% of patients develop persistent viremia and chronic hepatitis.2) Since chronic hepatitis is well correlated with the development of severe liver diseases, such as cirrhosis and hepatocellular carcinomas,3) anti-HCV therapy in patients with chronic HCV infection may be efficient in reducing the risk of these liver diseases.

HCV is an enveloped RNA virus in the Flaviviridae family, which possesses a single-stranded, positive-sense RNA genome of 9.6 kilobases (kb). The HCV RNA genome encodes a large polyprotein of approximately 3000 amino acids (aa), which is co- and post-translationally processed by host and viral proteases into 10 individual components including three structural (core, E1, E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B).4) The HCV particle consists of a nucleocapsid (core proteins and a viral genome) surrounded by an outer lipid envelope containing E1 and E2 glycoproteins. Nonstructural proteins play essential roles in viral RNA replication and assembly.

HCV entry into host cells is known to be mediated by various host factors, such as the scavenger receptor class B type I (SRBI),5) the cluster of differentiation 81 (CD81) molecule,6) claudin-1 (CLDN1),7) and occludin (OCLN).8) These host factors suggested to be involved in HCV entry co-operatively and serially, although the molecular mechanism remains poorly understood.9) We have isolated various hepatic cell mutants resistant to HCV infection, among which CD81-defective 751r cells and CLDN1-defective S7-A cells have been established as clones that are non-permissive to HCV infection.10,11) Using genetic approaches with these mutant cells, we have confirmed that CD81 and CLDN1 are essential for HCV entry into hepatic cells, which is consistent with other studies.6,7,12) There is as yet no conclusive evidence for the necessity of OCLN for HCV infection of hepatic cells, although OCLN has been shown by RNA interference (RNAi) experiments to be importantly involved in the infection.5,13–15)

In this study, we applied a genome-editing strategy using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system,16–18) which allowed us to establish OCLN-knockout human hepatic cells derived from Huh7.5.1-8 cells and to finally demonstrate that OCLN is essential for HCV entry into host hepatic cells. Furthermore, we analyzed HCV infection of hepatic cells using the OCLN-knockout cells.
MATERIALS AND METHODS

Cell Culture In this study, several human hepatic cell lines derived from Huh7.5.1 cells(9) were used: Huh7.5.1-8 cells, which are highly permissive to HCV(10); CD81-defective Huh7.5.1-5 cells, (10) renamed as 751r cells; and CLDN1-defective S7-A cells. (11) These cells and human embryonic kidney 293T (HEK 293T) cells were maintained at 37°C in an atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 0.1 mM nonessential amino acids, 100 units/mL of penicillin G, and 100 μg/mL of streptomycin sulfate. Cells were routinely passaged every 3–5 d at a density of >5×10^5 cells/10-cm dish.

Antibodies Rabbit monoclonal antibody (mAb) against SRBI was purchased from Abcam (Cambridge, U.K.). Rabbit anti-SRBI polyclonal antibodies (pAbs) were purchased from Novus Biologicals (Littleton, CO, U.S.A.). Mouse mAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rabbit anti-CLDN1 pAbs and a mouse anti-SRBI polyclonal antibodies (pAbs) were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL, U.S.A.). Mouse control immunoglobulin G (IgG) was purchased from Southern Fisher Scientific, Inc. (Waltham, MA, U.S.A.). Mouse mAb against the low-density lipoprotein receptor (LDLR) was purchased from EMD Millipore (San Jose, CA, U.S.A.). Mouse mAb against the liver-specific S7-A cells. (11) These cells and human embryonic kidney 293T (HEK 293T) cells were maintained at 37°C in an atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 0.1 mM nonessential amino acids, 100 units/mL of penicillin G, and 100 μg/mL of streptomycin sulfate. Cells were routinely passaged every 3–5 d at a density of >5×10^5 cells/10-cm dish.

Flow Cytometric Analysis Cells were detached with 0.05% (w/v) Trypsin/0.5 mM ethylenediaminetetraacetic acid or cell dissociation buffer (Thermo Fisher Scientific, Inc.) and then treated with anti-SRBI pAbs (Novus) at a dilution of 1:100, anti-CD81 mAb (clone JS-81) at 1 μg/mL, or anti-CLDN1 mAb (clone 3A2) at 2 μg/mL in phosphate-buffered saline (PBS) containing 2% FCS for 60 min at 4°C. After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit IgG (Thermo Fisher Scientific, Inc.) at 2 μg/mL in PBS containing 2% FCS for 30 min at 4°C. After washing with PBS, cells were analyzed using a FACS caliber flow cytometer (BD Biosciences).

Construction of Retroviral Expression Vectors The pC7-Flag and pCX4-bsr vectors were obtained from the Osaka Bioscience Institute (Osaka, Japan). Human OCLN (hOCLN) cDNA (GenBank accession number NM_001205254) was amplified by PCR using a human liver cDNA library (Clontech Laboratories, Inc., Mountain View, CA, U.S.A.), and the resultant OCLN cDNA was cloned into the pCX4-bsr vector. The constructed vector was named pCX4-bsr-hOCLN. The pCX4-bsr-hOCLN vector was constructed by inverse PCR (iPCR) from pCX4-bsr-hOCLN using the following primers: 5'-GAGCATGACAGAATGTCACCCAGCTTCTTTGGATTAGCTACACCATCC-3' and 5'-AAAAAGGACTTGGATTCGTCCAGCGGTAGAGGTA-3'. Each pCX4-bsr-FLAG-hOCLN deletion mutant was constructed by iPcr from pCX4-bsr-FLAG-hOCLN using the following oligonucleotides: 5'-GGAGGCTATACAGACCGAGACGACGGGCAACTCAG-3' and 5'-GCCATTCTGTGCACGAGGATGAGGAC-3'. The resultant plasmid was named pCX4-bsr-FLAG-hOCLN deleted mutant was constructed by iPcr from pCX4-bsr-FLAG-hOCLN using the following oligonucleotides: 5'-GGAGGCTATACAGACCGAGACGACGGGCAACTCAG-3' and 5'-GCCATTCTGTGCACGAGGATGAGGAC-3'. The resultant plasmid was named pCX4-bsr-FLAG-hOCLN deleted mutant was constructed by iPcr from pCX4-bsr-FLAG-hOCLN using the following oligonucleotides: 5'-GGAGGCTATACAGACCGAGACGACGGGCAACTCAG-3' and 5'-GCCATTCTGTGCACGAGGATGAGGAC-3'. The resultant plasmid was named pCX4-bsr-FLAG-hOCLN deleted mutant was constructed by iPcr from pCX4-bsr-FLAG-hOCLN using the following oligonucleotides: 5'-GGAGGCTATACAGACCGAGACGACGGGCAACTCAG-3' and 5'-GCCATTCTGTGCACGAGGATGAGGAC-3'.
medium was replaced with 2 mL of fresh medium, and cells were cultured overnight. The retrovirus-containing medium was collected, filtrated through a 0.45-µm filter, and immediately used for infection. Infected cell populations were selected on blasticidin S (5 µg/mL).

**In Vitro HCV Infection**

Infectious HCV-JFH1\(^{19,20}\) was...
prepared from culture supernatants of Huh7.5.1-8 cells that had been transfected with in vitro-transcribed HCV-JFH1 RNA and passed a few times on Huh7.5.1-8 cells.11) Other infectious HCV-JFH1 chimera strains, Jc1 (genotype 2a/2a) and TNS2J1 (genotype 1b/2a), were prepared as previously described.21–23) Detection of HCV infection was performed using quantitative reverse transcription-PCR (qRT-PCR) and immunohistochemistry as previously described.10,11)

HCV Pseudoparticle Infection HCV pseudoparticles (HCVpp) were generated as previously described.24) Briefly, a Gag–Pol packaging construct (Gag–Pol 5349), a transfer vector construct (Luc 126), and an envelope glycoprotein (E1 and E2)-expressing vector [H77, genotype 1a (GenBank accession number JX472009.1); TH, genotype 1b (GenBank accession number AB985268.1); J6, genotype 2a (GenBank accession number AB047639); JFH1, genotype 2a (GenBank accession number AB047639); or VSV-G (GenBank accession number M27165)] were transfected into HEK 293T cells. The medium from transfected cells was collected and used as the HCVpp source. HCVpp infection and a luciferase reporter assay were previously described.25)

HCV Replication Activity Assay with Subgenomic Replicons The subgenomic replicon plasmids pSGR-JFH1-wt and pSGR-JFH1-GND26) carrying the luc gene were based on the HCV-JFH1 sequence. The latter contained a GDD-to-GND synthetic HCV-JFH1 sequence. The latter contained a GDD-to-GND mutation in NS5B, which abolishes RNA polymerase activity. These plasmids were linearized and transfected into HEK 293T cells and cultured for 1–4 d. Cells were lysed, and the luciferase activity of the lysates was measured.10)

Cell-to-Cell HCV Transfer Assay Huh7.5.1-8/Aequorea coerulescens green fluorescent protein (AcGFp)-Nuc cells11) with green nuclear staining were infected with HCV-JFH1 and cultured for 1 d. HCV-infected Huh7.5.1-8/AcGFp-Nuc cells were mixed with S7-A, 751r or OKH-4 cells, at a cell number ratio of 1 : 10 in each case, and plated onto a 24-well plate. After 5 d, cells were immunostained with anti-HCV core antibody at a 1:100 dilution, and 16 cell clones were isolated. No OCLN protein was detected in OKH-4 cells, and there were no differences in cell growth and morphology between parental Huh7.5.1-8 and OCLN-knockout OKH-4 cells (data not shown).

HCV entry into host cells is mediated by multiple host factors, including LDLR, SRBI, OCLN, CLDN1, and CD81. We compared expression levels of these HCV entry factors between Huh7.5.1-8 and OKH-4 cells by immunoblot analysis. No OCLN protein was detected in OKH-4 cells, and there were no differences in cellular expression levels of the LDLR, SRBI, CLDN1, and CD81 proteins between Huh7.5.1-8 and OKH-4 cells (Fig. 1E). When cell surface expression of CD81, CLDN1, and SRBI proteins was examined using flow cytometry, Huh7.5.1-8 and OKH-4 cells exhibited similar expression levels of these HCV entry factors (Fig. 1F).

OCLN Is Essential for the HCV Entry Step into Human Hepatic Cells We then evaluated the permissiveness of OCLN-knockout OKH-4 cells to HCV infection. Parental Huh7.5.1-8 cells and each HCV entry factor-defective cell type (CD81-defective 751r, CLDN1-defective S7-A, and OKH-4 cells) were infected with HCV-JFH1 and then HCV RNA contents in these cells and the culture supernatants were measured by qRT-PCR (Figs. 2A, B). HCV RNA was at undetectable levels not only in 751r and S7-A cells but also in OKH-4 cells. We further tested entry activities of different genotypes of HCVpp (H77, 1a; TH, 1b; and J6 and JFH1, 2a) into each HCV entry factor-defective cell line. Entry of all HCVpp genotypes was impaired in OKH-4 cells as well as in 751r and S7-A cells (Fig. 2C). These results indicated that OCLN-knockout OKH-4 cells were non-permissive to HCV infection.

For OCLN complementation experiments, we established OKH-4 cells stably expressing FLAG-tagged hOCLN (OKH-4/FLAG-hOCLN cells) (Fig. 2D). Huh7.5.1-8, OKH-4/ mock, and OKH-4/FLAG-hOCLN cells were infected with HCV-JFH1, and cellular HCV RNA contents were measured. The Huh7.5.1-8 and OKH-4/FLAG-hOCLN cells exhibited very similar time courses of HCV RNA production; however, OKH-4/mock cells showed no HCV RNA production (Fig. 2E). These results indicated that OCLN is essential for HCV-
Fig. 2. Hepatic OCLN-Knockout OKH-4 Cells Do Not Support HCV Infection

(A, B) HCV-JFH1 infection. Huh7.5.1-8, OKH-4, 751r, and S7-A cells were infected with HCV-JFH1 at an MOI of 0.1. At 4d post-infection (dpi), HCV RNA contents in cells (A) and culture supernatants (B) were quantified by qRT-PCR. Data in each graph are presented as the mean±standard deviation (S.D.) (n=3). (C) HCVpp infection. Huh7.5.1-8, OKH-4, 751r, and S7-A cells were infected with HCVpp for 6h. At 2dpi, luciferase activities of cell lysates were measured using a luminometer. H77 (genotype 1a, white), TH (genotype 1b, light gray), J6 (genotype 2a, dark gray), and JFH1 (genotype 2a, black). Relative luminescence units (RLUs) were corrected for vesicular stomatitis virus pseudoparticle (VSVpp) infectivity. Data are presented as the mean±S.D. (n=3). (D–F) OCLN complementation experiments. OKH-4 cells were stably transduced with retroviruses without (mock) or with the FLAG-hOCLN gene. Huh7.5.1-8, OKH-4/mock, and OKH-4 cells stably expressing FLAG-hOCLN (OKH-4/FLAG-hOCLN) were lysed, and equal protein amounts of each cell lysate (6.5 µg) were subjected to immunoblotting for OCLN and GAPDH proteins (D). These cells were infected with HCV-JFH1 at an MOI of 0.1 and cultured for the indicated times. Cellular HCV RNA contents were quantified by qRT-PCR (E). Circle, Huh7.5.1-8 cells; square, OKH-4/mock cells; triangle, OKH-4/FLAG-hOCLN cells. The dotted line shows the blank level (7.46±2.29×10^4 copies/µg of total RNA). Data in each graph are presented as the mean±S.D. (n=3). (F) Huh7.5.1-8 and OKH-4 cells were infected with HCV (JFH1, Jc1, or TNS2J1) at an MOI of 1. At 4 dpi, the cells were fixed and stained with the anti-HCV core protein mAb (green) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue).
JFH1 infection of human hepatic cells. We also investigated whether OCLN-dependent infection is observed with different infectious HCV strains, Jc1 (genotype 2a/2a chimera) and TNS2J1 (genotype 1b/2a chimera), in addition to JFH1 (genotype 2a), by immunohistochemistry analysis. At 4 d post-infection (dpi) with these infectious strains, both Huh7.5.1-8 and OKH-4/FLAG-hOCLN cells, but not OKH-4/mock cells, were able to be stained with the anti-HCV core protein mAb (Fig. 2F). Based on these complementation experiments, we confirmed that OCLN is essential for HCV infection of human hepatic cells.

As shown in Fig. 2C, OCLN is critical at least for the entry step of HCV infection. Next, we checked whether OCLN is involved in other steps of the HCV life cycle. When HCV RNA replication activities in Huh7.5.1-8 and OKH-4 cells were analyzed using subgenomic replicons, SGR-JFH1 wild-type (SGR-JFH1-wt) and replication-defective SGR-JFH1-GND mutant, similar time courses of replication were observed in these cells (Fig. 3A). We further examined HCV production activities in Huh7.5.1-8 and OKH-4 cells when the HCV entry process was skipped using transfection of these cells with HCV genomic RNA. There was no difference between these cell lines in HCV RNA contents in cells and supernatants (Figs. 3B, C). These data demonstrated that OCLN was mainly involved in the entry step of the HCV life cycle in hepatic cells.

The Second Extracellular Loop of OCLN Is Critical for HCV Infection in Hepatic Cells

Previous reports have suggested that the second extracellular loop (ECL2) of OCLN is important for HCV infection in the experimental systems using artificial HCVpp and non-hepatic cells. However, there is no clear evidence for the significance of ECL2 in intact HCV infection of hepatic cells. We then evaluated that using our OCLN-knockout hepatic OKH-4 cells and intact HCV. We established OKH-4 cells stably expressing FLAG-hOCLN mutants (ΔECL1 and ΔECL2) with deletions in the respective ECLs (Fig. 4A). At 4 dpi with HCV-JFH1, both OKH-4/FLAG-hOCLN-ΔECL1 and OKH-4/FLAG-hOCLN-ΔECL2 cells were comparable with those in Huh7.5.1-8 cells, but HCV RNA was undetectable in OKH-4/FLAG-hOCLN-ΔECL2 cells, indicating that the latter were non-permissive to HCV-JFH1 infection (Fig. 4B). Similar results were obtained using different genotypes of HCVpp (Fig. 4C). Michta et al. have reported that two cysteines in OCLN ELC2 are critical for HCV infection. We then established OKH-4 cells stably expressing FLAG-hOCLN mutants (C216S, C237S) with each cysteine substituted by serine (Fig. 4D). At 4 dpi with HCV-JFH1, both OKH-4/FLAG-hOCLN-C216S and OKH-4/FLAG-hOCLN-C237S cells showed undetectable levels of cellular HCV RNA (Fig. 4E), indicating a defect in HCV-JFH1 infection. These cells were also resistant to infection with different genotypes of HCVpp (Fig. 4F).
Based on these results, we confirmed that OCLN ECL2, especially the two cysteine residues, is essential for HCV infection in hepatic cells.

OCLN Is Essential for Cell-to-Cell Transmission It has been known that there are two modes of HCV infection: one is infection through culture medium (cell-free infection) and direct infection between contacted cells (cell-to-cell infection). Although OCLN has been suggested to be important for cell-to-cell HCV infection, there has been no definitive data showing whether OCLN is essential for cell-to-cell transmission. Therefore, we evaluated this issue using OCLN-knockout OKH-4 cells. HCV-preinfected Huh7.5.1-8/ΔC16S or Huh7.5.1-8/ΔC237S cells were mixed with OKH-4 cells. Under these conditions, OKH-4 cells did not show cell-to-cell transmission (Fig. 5 and Supplementary Fig. 1). Based on these results, OCLN as well as CLDN1 play essential roles not only in cell-free HCV infection but also in cell-to-cell HCV transfer.

DISCUSSION A number of previous studies have suggested that OCLN is very important for HCV infection, but there has been no conclusive evidence whether OCLN is essential for HCV infection of hepatic cells. In several studies, the involvement of OCLN in HCV infection has been investigated using RNAi. Because RNAi knockdown of OCLN is partial, it has not...
been determined whether HCV infection critically depends on OCLN expression in hepatic cells. On the other hand, non-hepatic cells that do not express OCLN have also been used to show the OCLN-dependent HCV entry \cite{8,13,29,30,33}; however, artificial infection systems, such as HCVpp, have to be used in these experiments because genuine HCV cannot efficiently replicate in non-hepatic cells. Indeed, it has been reported that the structure of HCVpp is different from that of HCV particles.\cite{34} In this study, we established OCLN-knockout cells derived from human hepatic Huh7.5.1-8 cells using the CRISPR/Cas9 system (Fig. 1) and concluded that OCLN is essential for both HCV cell-free infection and cell-to-cell transmission (Figs. 2, 5), and it plays a role in the entry step of HCV infection (Fig. 3). Since there were no effects on expression levels of other entry factors LDLR, SRBI, CLDN1, and CD81, by OCLN knockout (Figs. 1E, F), it is also strongly suggested that OCLN and these factors are involved in HCV entry co-operatively.

To generate OCLN-knockout cells, we used two sgRNA expression vectors (pX330-site A, pX330-site B) to target two different sites in the \textit{OCLN} gene. (There are four sgRNA target sites in the \textit{OCLN} gene.) We first tried to target each site using individual sgRNA expression vectors but failed to obtain OCLN-knockout cells (data not shown). Then, simultaneous targeting of the two sites in the \textit{OCLN} gene (site A: antisense, 184–206, and site B: sense, 328–350) was performed using two corresponding sgRNA expression vectors, which resulted in the successful knockout of the \textit{OCLN} gene (Fig. 1). This approach to simultaneously target two sites may be advantageous not only for more effective gene knockout but also for checking gene deletions by genomic PCR (Fig. 1C). In OKH-4 cells, nucleotides at 190–344 would have been deleted by non-homologous end joining after double-strand breaks had occurred at both sites, 3 bp upstream of the protospacer adjacent motifs in site A and site B target sequences (Fig. 1D). It is possible for OKH-4 cells to express an N-terminal short fragment of OCLN and some C-terminal OCLN fragments that can be translated from downstream in-frame alternative start codons. However, no OCLN fragments were detected in OKH-4 cells by immunoblot analysis using antibodies recognizing the N- or C-terminus of OCLN (Fig. 1E, data not shown). Because Huh7.5.1-8 and OKH-4/FLAG-hOCLN cells exhibited very similar HCV infection levels (Figs. 2E, F), the presence of OCLN fragments in OKH-4 cells, if any, had little effect on permissiveness to HCV infection.

This study strongly suggests a promising anti-HCV strategy \textit{via} targeting the host entry factor OCLN; \textit{e.g.}, OCLN-binding probes, such as monoclonal antibodies, may prevent HCV infection. Indeed, Sourisseau \textit{et al.}\cite{35} reported that various FLAG-tag-inserted OCLN constructs were expressed in human renal carcinoma 786-O cells, which are normally not able to support HCV entry owing to insufficient OCLN expression,\cite{8} and that HCVpp entry into cells, mediated by some of the constructs, was inhibited by anti-FLAG antibodies. Previously, we have successfully developed, using a differential screening strategy for parental Huh7.5.1 cells and Huh7.5.1-derived CLDN1-knockout S7-A cells, anti-CLDN1 monoclonal antibodies that markedly inhibited HCV infection.\cite{11} In this system, we could efficiently select specific monoclonal antibodies recognizing intact extracellular loops of CLDN1 because the only major difference between these cells was their ability or inability to express intact CLDN1. Based on a similar strategy using OKH-4 cells, we may be able to obtain in the future anti-OCLN monoclonal antibodies to prevent HCV infection.

In addition, OCLN-knockout hepatic OKH-4 cells and their derivatives that we established are useful for investigating the OCLN-dependent mechanism of HCV entry and also as cell biological tools for analyzing cellular functions of OCLN.

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