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Antiviral Effects of the Anti-Occludin Monoclonal Antibody on Persistent Hepatitis C Virus Infection in a Human Liver Chimeric Mouse Model

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Worldwide, ~71 million people are infected with hepatitis C virus (HCV). Claudin-1 (CLDN1) and occludin (OCLN), both tetraspanins of epithelial tight junctions, are entry receptors for HCV. Previously, we generated anti-CLDN1 and anti-OCLN monoclonal antibodies (mAbs), both of which strongly inhibit HCV entry into hepatocytes. However, the relevance of CLDN1 and OCLN in persistent HCV infection remains unclear. In the present study, we evaluated the involvement of CLDN1 and OCLN in persistent HCV infection using the mAbs against CLDN1 (clone 3A2) and OCLN (clone 1-3). Interestingly, both mAbs significantly reduced intracellular HCV RNA levels in a cell culture system. Additionally, the anti-OCLN mAb reduced serum HCV levels in chronic HCV-infected human liver chimeric (PXB) mice (often used as an *in vivo* HCV infection model), whereas the anti-CLDN1 mAb did not have this effect. These results suggest that the OCLN molecule contributes to maintaining persistent HCV infection *in vivo*. In further investigation, we determined whether combinations of NS5B inhibitor, nesbuvir, and the anti-OCLN mAb had anti-HCV effects on persistent HCV-infected PXB mice. Administration of nesbuvir and control IgG caused a breakthrough of serum HCV levels in all mice, whereas nesbuvir and anti-OCLN mAb combinations caused the breakthrough at a later phase in only one of three mice. Thus, anti-OCLN mAb seems to suppress the occurrence of resistant viruses against nesbuvir. Based on these results, we suggest that the anti-OCLN mAb, which could be combined with direct-acting antiviral agents, might be a potential candidate antiviral agent in HCV therapeutics.

Key words occludin, claudin-1, direct-acting antiviral agents, hepatitis C virus, persistent infection

INTRODUCTION

Hepatitis C virus (HCV) is a positive sense RNA virus belonging to the *Flaviviridae* family.[1)] Around 71 million people worldwide are estimated to be infected with HCV and around 80% of HCV-infected patients will develop chronic hepatitis, which itself is correlated with the development of cirrhosis and hepatocellular carcinoma.[2,3)] Recently, direct-acting antiviral agents (DAAs) targeting HCV proteins have been developed and used clinically against the virus; consequently, sustained virological responses and fewer side effects have been achieved relative to the effects of previously applied interferon alpha and ribavirin combination therapy.[4,5)] DAAs have three major molecular targets: HCV NS3/4 protease, NS5A, and NS5B polymerase. DAA-based therapy requires high-cost, long-term administration and, in rare cases, DAA treatment fails.[6)] Resistance-associated substitution can occur in chronic HCV patients that experience DAA treatment failure. HCV cure rates could therefore be improved by preventing the emergence and transmission of HCV mutants with DAA resistance-associated substitutions.

Tight junctions are intercellular adhesion complexes in epithelial cells that control paracellular permeability and regulate epithelial polarity.[7)] The main protein components of transmembrane strands are the claudin family (27 members in humans) and three junctional MARVEL domain-containing proteins: occludin (OCLN), tricellulin, and MARVELD3.[8,9)] Both claudin-1 (CLDN1) and OCLN are critical for HCV entry into hepatocytes.[10–13)] Previously, we succeeded in creating anti-CLDN1 monoclonal antibodies (mAbs) and anti-OCLN mAbs that recognize the intact conformation of extracellular domains; these mAbs can strongly block *in vitro* and *in vivo* HCV infection without cytotoxicity.[12,14)]

PXB mice are a chimeric mouse strain with humanized livers; they are generated from the albumin promoter/enhancer-driven urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mouse via an injection with human hepatocytes.[15)] Since PXB mice have a liver that is almost entirely repopulated with normal human hepatocytes, they can be persistently infected with HCV. This chronic HCV infection model can be used for anti-HCV drug studies under persistent HCV-infected conditions.[16)]

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In the current study, we examined the anti-HCV activity of our anti-tight junction mAbs *in vitro* and *in vivo* under persistent HCV-infected conditions; furthermore, we evaluated the effects of combining anti-OCLN mAb with DAA in the persistent HCV infection mouse model. Based on our analyses of anti-HCV effects, we propose that OCLN-targeting strategies could be applied in anti-HCV therapy to effectively treat chronic HCV patients.

MATERIALS AND METHODS

Ethics Statement All procedures using human liver chimeric mice (i.e., PXB mice) were approved by the Animal Ethics Committee of PhoenixBio Co., Ltd. (Hiroshima, Japan) (permission numbers 1477 and 1564); all animal experiments were performed following approval from the Committee on the Ethics of Animal Experiments of the National Institute of Infectious Diseases (permission numbers 815005 and 815006), under the guidelines of the National Institute of Infectious Diseases, and in accordance with the Guidelines for Proper Conduct of Animal Experiments established by the Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf).

Regents and Antibodies Nesbuvir (also known as HCV-796 and VB-19796) was obtained from Apex Bio (A3655, Boston, MA, USA). Monoclonal antibodies against OCLN (clone 1-3) and CLDN1 (clone 3A2) were established via the DNA immunization method and differential cell binding screening using each antigen-defective cell, as previously described.[12,14] Rat immunoglobulin G (IgG; ChromPure) was obtained from Jackson ImmunoResearch laboratories (West Grove, PA, USA). Mouse IgG was purchased from the R&D systems (Minneapolis, MN, USA).

Cell Lines Huh7.5.1-8 cells,[17] human sarcoma cell line HT1080 (JCRB, Japan), and HT1080 cells expressing human OCLN (HT1080/hOCLN cells)[13] were cultured in Dulbecco's modified Eagle's Medium supplemented with 4,500-mg/L glucose, 10% fetal bovine serum, 100-U/mL penicillin, and 100-μg/mL streptomycin.

Effects of Anti-CLDN1 and Anti-OCLN mAbs on Chronic HCV-Infected Mice PXB mice were prepared as previously described.[15] Briefly, male PXB mice (16 or 17 weeks old) were intravenously inoculated with HCV-PBC001 (10^4 copies; genotype 1b; lot 080811). After 42 d, these mice were chronically infected with HCV. Subsequently, the mice were intraperitoneally injected with anti-OCLN mAb (clone 1-3) or anti-CLDN1 mAb (clone 3A2) at 30 mg/kg (two anti-OCLN-treated and two anti-CLDN1-treated mice per group) on days 0, 3, 7, and 10, respectively. Sera were collected from mice on days 0, 7, 14, 21, and 28 for analyses.

Combination Therapy with Anti-OCLN mAb and Nesbuvir Applied to Chronic HCV-Infected Mice PXB mice chronically infected with HCV-PBC002 (genotype 1a; lot 07420) were intraperitoneally injected with anti-OCLN mAb (clone 1-3) or control rat IgG at 30 mg/kg (four anti-OCLN-treated and rat IgG-treated mice per group) on days 0 and 3, respectively, and then nesbuvir was administrated peroraly twice daily at 50 mg/kg. Sera were collected from mice on days 0, 1, 2, 3, 5, and 7 for analyses.

Measurements of Anti-OCLN mAbs in Mouse Serum Anti-OCLN mAb (clone 1-3) levels in mouse serum were determined by cell ELISA as previously described.[14] In brief, confluent fixed HT1080/hOCLN cells in 96-well plates were incubated for 2 h with 100 μL of each serum sample diluted with PBS containing 5% (wt/vol) skim milk. Subsequently, a 1:5,000 dilution of 100-μL horseradish peroxidase-conjugated goat anti-rat IgG (GE Healthcare) was added for 1 h at room temperature. To detect mAbs bound to cells, 1-Step Ultra TMB-ELISA substrate solution (Thermo Fischer Scientific) was used as the substrate. In each well, the absorbance at 450 nm was determined via an OpsysMR plate reader (Dynex Technologies GmbH). Purified mAb (clone 1-3) was used as a standard.

Additional Serum Measurements Measurements of HCV RNA, human albumin, alanine aminotransferase (ALT), and aspartate transaminase (AST) levels in sera were performed as previously described.[12,14]

RESULTS AND DISCUSSION

We previously demonstrated that rat anti-OCLN mAbs and mouse anti-CLDN1 mAbs prevented HCV entry both in an *in vitro* cell culture system and an *in vivo* human liver chimeric mouse model.[12,14] In the present study, we again used a cell culture system and a mouse infection model, but investigated the effect of anti-OCLN mAb and anti-CLDN1 mAb on chronic HCV infection.

First, we examined whether these mAbs have anti-HCV effects on persistent HCV-infected cultured cells; we used clone 1-3 for anti-OCLN mAb and clone 3A2 for anti-CLDN1 mAb because these mAbs strongly inhibit HCV entry.[12,14] Huh7.5.1-8 cells persistently infected with HCV-JFH1[18] (> 30 d after HCV infection) were treated with anti-OCLN mAb, anti-CLDN1 mAb, or control IgG at 50 μg/well (0.5 mL) for 12 d and at 5.0 μg/well for a further 8 d (Fig. 1A). We found that cellular HCV RNA levels gradually decreased over time up to almost 100-fold with both anti-OCLN and anti-CLDN1 mAb treatments (Fig. 1B). These results indicate that both

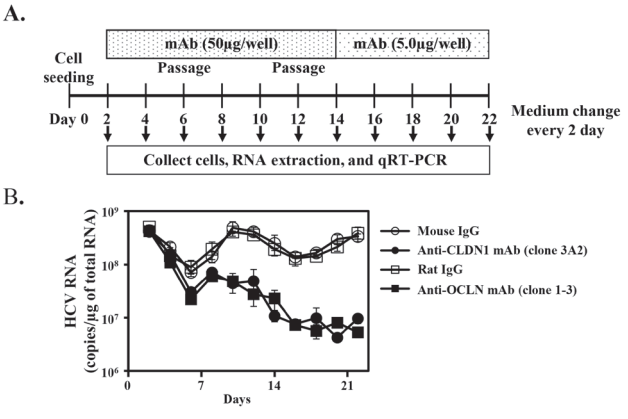

Fig. 1. Effects of Anti-OCLN mAb or Anti-CLDN1 mAb on the HCV RNA Levels in Cultured Cells Persistently Infected with HCV

(A) Experimental procedure. Huh7.5.1-8 cells were infected with HCV-JFH1-tau at MOI = 1 and maintained in culture for more than 3 weeks. These cells carrying replicable HCV RNA genomes were used as persistent HCV-infected cells. Subsequently, these cells were treated with anti-CLDN1 mAb (clone 3A2; n = 4), anti-OCLN mAb (clone 1-3; n = 4), or control antibodies (mouse IgG or rat IgG; n = 4) at the indicated doses and for the given periods (50 μg/mL for day 2–14 and 5 μg/mL for day 14–22). Culture media were changed and cell lysates were collected at the indicated time points (every 2 day). On day 6 and 12, cells were passaged at a split ratio of 1:8. Using total RNA fractions purified from cell lysates, intracellular HCV RNA levels were measured by qRT-PCR. (B) The cellular HCV RNA levels of each sample are expressed as number copies per μg of cellular total RNA. Open circles, mouse IgG; filled circles, clone 3A2; open squares, rat IgG; filled squares, clone 1-3. Data in each group are presented as the mean ± standard deviation (n = 4).

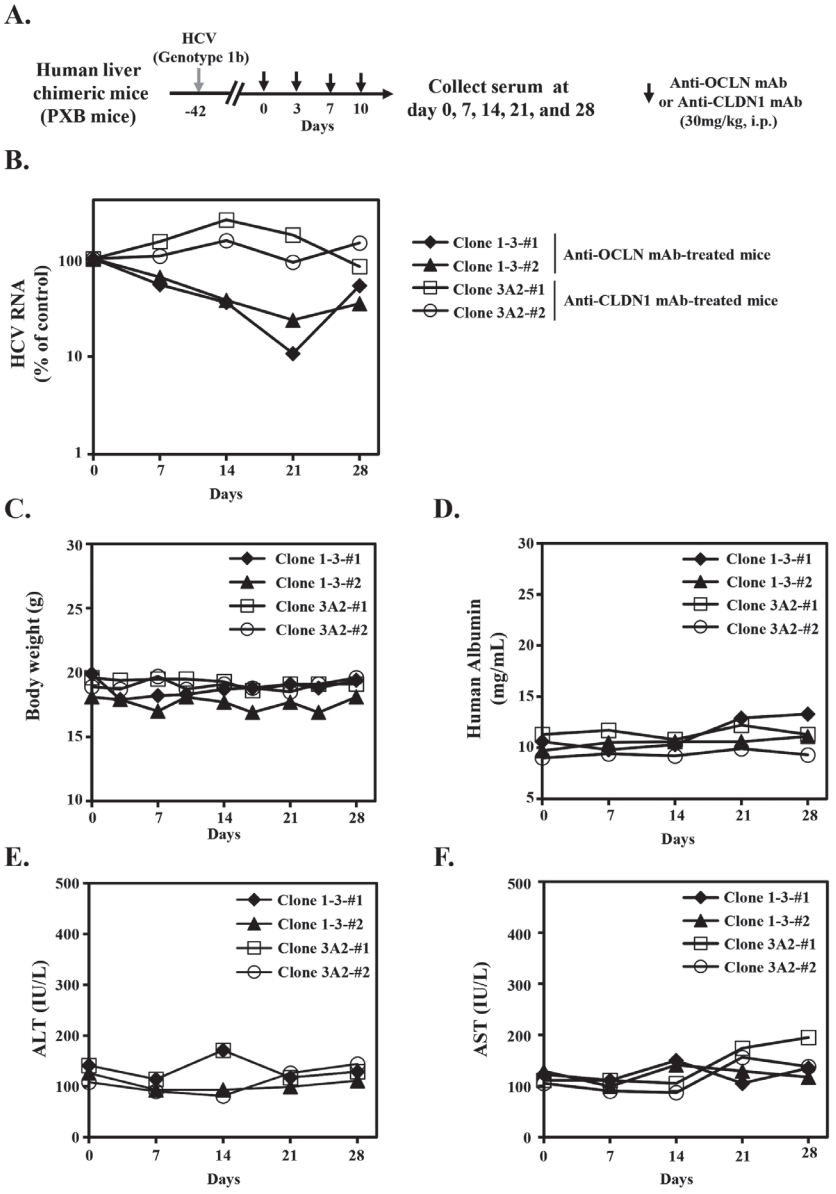

Fig. 2. Effects of Anti-OCLN mAb or Anti-CLDN1 mAb on Serum HCV RNA Levels in Chronic HCV-Infected PXB Mice

(A) Experimental procedure. Anti-CLDN1 mAb (clone 3A2; n = 2) or anti-OCLN mAb (clone 1-3; n = 2) were intraperitoneally administered into human liver chimeric PXB mice persistently infected with HCV at the indicated time points. Each serum sample was collected and analyzed as described in the Materials and Methods section. i.p., intraperitoneal injection. (B) Serum HCV RNA levels were determined by qRT-PCR. (C–F) The characteristics of the mAb-treated PXB mice: body weight (C) and serum levels of human albumin (D), ALT (E), and AST (F). Filled diamonds, clone 1-3-#1; filled triangles, clone 1-3-#2; open squares, clone 3A2-#1; open circles, clone 3A2-#2.

anti-OCLN mAb and anti-CLDN1 mAb can inhibit persistent HCV infection in a cell culture system.

Next, we assessed whether administration of anti-OCLN mAb and anti-CLDN1 mAb could reduce serum HCV RNA levels *in vivo* under chronic HCV infection conditions. Generally, small experimental animals, such as normal mice, are not susceptible to HCV infection. However, PXB mice are chimeric with > 70% human hepatocytes; thus, they can be used as a humanized model in HCV infection studies.[15] Indeed, in the present research, we used persistent HCV-infected human liver chimeric PXB mice, which are well-known to show steady serum HCV RNA contents,[16] as an *in vivo* chronic HCV infection model. We confirmed the model via serum HCV RNA values, which were > 6.0×10^6 copies/mL in the model mice (on day 0: clone 1-3-#1 had 1.4×10^7 copies/mL, clone 1-3-#2 had 1.7×10^7 copies/mL, clone 3A2-#1 had 1.2×10^7 copies/mL, and clone 3A2-#2 had 6.2×10^6 copies/mL). Mice were administered an intraperitoneal injection of anti-OCLN mAb or anti-CLDN1 mAb (30 mg/kg) on days 0, 3, 7, and 10 (Fig. 2A). We found that serum HCV RNA levels of anti-OCLN (clone 1-3)-treated mice gradually decreased and, at day 21, reached levels that were < 20% of those on day 0; however, the serum HCV RNA levels of anti-CLDN1 (clone 3A2)-treated mice did not show such a trend (Fig. 2B). Under these conditions, we did not detect any serious adverse effects on body weight, ALT, AST, and human albumin levels (Fig. 2C–F). Compared with the levels at day 21, serum HCV levels were apparently increased at day 28 (Fig. 2B). In tests of serum anti-OCLN concentrations, levels tended to increase immediately and then reach a plateau between day 3 and 14, after which they signif-

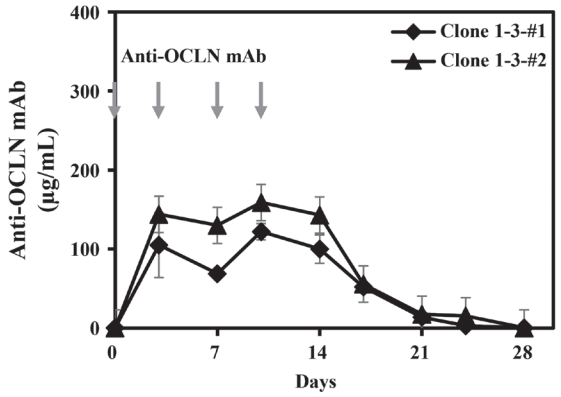

Fig. 3. Serum Anti-OCLN mAb Concentrations in PXB Mice After Intraperitoneal Administration of the Anti-OCLN mAb

Under the experimental conditions described in Fig. 2, anti-OCLN mAb (clone 1-3) contents in mice sera were determined using a cell ELISA-based assay (as described in the Materials and Methods section). Data are presented as means ± standard deviation (n = 3). The limit of detection was 0.5 μg/mL. Arrows indicate the time points at which anti-OCLN mAb was administered. Filled diamonds, clone 1-3-#1; filled triangles, clone 1-3-#2.

icantly decreased by 100-fold to the baseline level at day 28 (Fig. 3). Serum HCV levels seemed to be inversely correlated with serum anti-OCLN mAb concentrations. Unlike the results from the cell culture experiments (Fig. 1), treatment with anti-CLDN1 mAb did not affect serum HCV RNA levels in PXB mice. In our previous study, PXB mice that received the anti-CLDN1 mAb (clone 3A2) exhibited very low serum levels of 3A2 mAb, i.e., around 1–2 μg/mL,[12] which might not be sufficient for antiviral effects against persistent HCV infections.

In further analyses, we examined the combination effects of anti-OCLN mAb with a DAA in the persistent HCV-infected mouse model. Specifically, we combined HCV (genotype 1a) and nesbuvir, a selective inhibitor of HCV NS5B polymerase.[19] Breakthrough is known to occur due to nesbuvir administration alone and resistant viruses appear in PXB mice and patients infected with HCV.[20,21] The experimental design is shown in Fig. 4A. Results showed that when nesbuvir and control IgG were administered orally and intraperitoneally, respectively, into chronic HCV-infected PXB mice, serum HCV levels were transiently reduced up to day 3 and then returned almost to the original levels at day 7 (Fig. 4B). These breakthroughs suggest the emergence of nesbuvir-resistant viruses, because nesbuvir-resistant viruses are often detected early after its treatments.[21] In contrast, two anti-OCLN mAb-treated mice (#202 and #203) showed no breakthrough. One anti-OCLN mAb-treated mouse (#204), in which the reduction of serum HCV level was more gradual, showed breakthrough at a later time point after day 5. Another anti-OCLN mAb-treated mouse (#201) unexpectedly died; the cause is unknown, but sudden deaths often occur in chimeric mice. We also tested the toxic effects of coadministrations. As shown in Fig. 4C and Table 1, adverse changes in body weight and serum ALT, AST, and human albumin levels were not detected between days 1 and 7. These results strongly suggest that the treatment combination of nesbuvir and anti-OCLN mAb restrains the breakthrough caused by nesbuvir resistance and does so without apparent adverse effects.

In conclusion, by evaluating the effects of anti-CLDN1 and anti-OCLN mAbs on persistent HCV infection, we revealed the attractive possibility that anti-OCLN mAb (clone 1-3) inhibits persistent HCV infection. Therefore, we postulate that OCLN molecules contribute to the maintenance of chronic HCV infection (Fig. 5). Additionally, by assessing the effect anti-OCLN mAb combined with nesbuvir in a persistent HCV infection mouse model, we demonstrated that anti-OCLN mAb

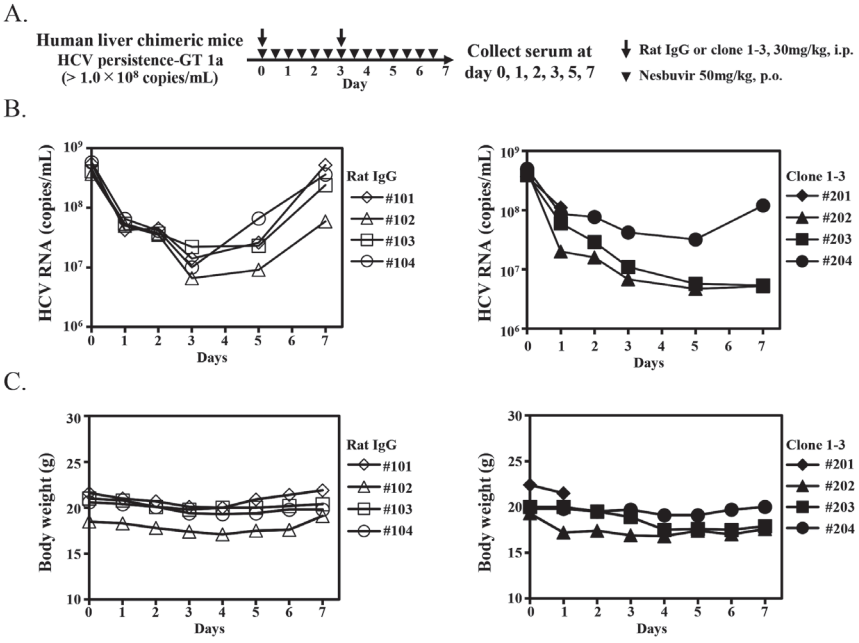

Fig. 4. Combination Effects of Nesbuvir and Anti-OCLN mAb on Chronic HCV Infection in a Human Chimeric Mouse Model

(A) Experimental procedure in which chronic HCV-infected human liver chimeric mice were used. These mice were treated with control rat IgG or anti-OCLN mAb (clone 1-3) (30 mg/kg) via intraperitoneal injection (i.p.) at the indicated times. All mice received nesbuvir (50 mg/kg) via peroral (p.o.) administration twice daily. (B) HCV RNA contents in mouse sera were measured by qRT-PCR at the indicated time points. (C) Body weights are shown for control rat IgG- or anti-OCLN mAb (clone 1-3)-treated mice (n = 4 animals per group). Open symbols, Rat IgG; closed symbols, clone 1-3.

Table 1. Body Weights and ALT, AST, and Human Albumin Levels of Human Liver Chimeric Mice at Various Time Points in the Combination Administration Experiments

	Body weight (g)		ALT (IU/L)		AST (IU/L)		Human Albumin (mg/dL)	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
#101	21.6	21.9	237	105	219	363	11.3	10.8
#102	18.5	19.1	252	138	318	357	12.6	11.8
#103	21.0	20.4	243	99	195	114	13.2	14.9
#104	20.6	19.8	120	99	150	129	11.2	11.8
#201	22.4	n/a	153	(582)	144	(1,467)	12.2	(10.2)
#202	19.3	17.6	234	84	216	102	13.5	13.5
#203	20.0	17.9	264	135	201	111	12.2	13.2
#204	19.8	20.0	234	114	207	126	11.8	12.8

(), levels at day 1; n/a, not applicable.

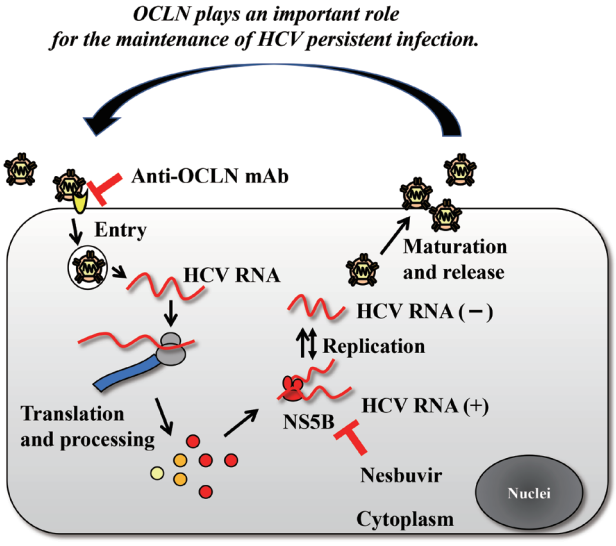

Fig. 5. Schematic Overview of the Antiviral Effects of Anti-OCLN mAb and the Emerging Role of OCLN in Chronic HCV Infection

(clone 1-3) prevents the breakthrough caused by DAA resistance. According to our results, although further *in vivo* analyses are needed, we propose that OCLN-targeting strategies could be promising anti-HCV therapies for effectively treating chronic HCV patients.

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Conflict of interest The authors declare no conflict of interest.

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