Monoclonal Antibodies against Occludin Completely Prevented Hepatitis C Virus Infection in a Mouse Model

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ABSTRACT Hepatitis C virus (HCV) entry into host cells is a multistep process requiring various host factors, including the tight junction protein occludin (OCLN), which has been shown to be essential for HCV infection in *in vitro* cell culture systems. However, it remains unclear whether OCLN is an effective and safe target for HCV therapy, owing to the lack of binders that can recognize the intact extracellular loop domains of OCLN and prevent HCV infection. In this study, we successfully generated four rat anti-OCLN monoclonal antibodies (MAbs) by the genetic immunization method and unique cell differential screening. These four MAbs bound to human OCLN with a very high affinity (antibody dissociation constant of <1 nM). One MAb recognized the second loop of human and mouse OCLN, whereas the three other MAbs recognized the first loop of human OCLN. All MAbs inhibited HCV infection in Huh7.5.1-8 cells in a dose-dependent manner without apparent cytotoxicity. Additionally, the anti-OCLN MAbs prevented both cell-free HCV infection and cell-to-cell HCV transmission. Kinetic studies with anti-OCLN and anti-claudin-1 (CLDN1) MAbs demonstrated that OCLN interacts with HCV after CLDN1 in the internalization step. Two selected MAbs completely inhibited HCV infection in human liver chimeric mice without apparent adverse effects. Therefore, OCLN would be an appropriate host target for anti-HCV entry inhibitors, and anti-OCLN MAbs may be promising candidates for novel anti-HCV agents, particularly in combination with direct-acting HCV antiviral agents.

IMPORTANCE HCV entry into host cells is thought to be a very complex process involving various host entry factors, such as the tight junction proteins claudin-1 and OCLN. In this study, we developed novel functional MAbs that recognize intact extracellular domains of OCLN, which is essential for HCV entry into host cells. The established MAbs against OCLN, which had very high affinity and selectivity for intact OCLN, strongly inhibited HCV infection both *in vitro* and *in vivo*. Using these anti-OCLN MAbs, we found that OCLN is necessary for the later stages of HCV entry. These anti-OCLN MAbs are likely to be very useful for understanding the OCLN-mediated HCV entry mechanism and might be promising candidates for novel HCV entry inhibitors.

KEYWORDS antiviral agents, hepatitis C virus, monoclonal antibodies, occludin

Hepatitis C virus (HCV) is an enveloped RNA virus in the *Flaviviridae* family that possesses a single-stranded, positive-sense RNA genome. An estimated 185 million people are infected with HCV worldwide (1). Persistent HCV infection can result in liver

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cirrhosis and hepatocellular carcinomas (2). The recent development of direct-acting antiviral agents (DAAs) against HCV has markedly improved the outcome of antiviral treatments without serious side effects. The latest generation of DAA therapies is not prone to drug resistance; however, extensive and long-term use of DAAs might cause the emergence of drug-resistant viruses, which could be a major obstacle in successful pharmacological treatment of HCV in the future. Conversely, host-targeting agents exhibit a high genetic barrier to drug resistance and thus may be candidates for next-generation HCV therapies, even though there is some concern regarding adverse effects.

Although the detailed mechanism remains unclear, HCV entry into hepatocytes is a multistep process involving various host entry factors such as the low-density lipoprotein receptor (LDL-R) (3), glycosaminoglycans (GAGs) (4), the high-density lipoprotein receptor scavenger receptor class B type I (SR-BI) (5), the tetraspanin cluster of differentiation 81 (CD81) (6), the cholesterol transporter Niemann-Pick disease type C1 like 1 (7), epidermal growth factor receptor (8), and the tight junction (TJ) proteins claudin-1 (CLDN1) (9) and occludin (OCLN) (10). We previously showed that both CLDN1 and OCLN are essential for HCV infection of human hepatic cells using *CLDN1*- and *OCLN*-defective hepatic cells (11, 12). Using a pooled CRISPR/Cas9 genetic screening strategy, Marceau et al. recently demonstrated that among these entry factors, *CD81*, *CLDN1*, and *OCLN* would be essential for HCV infection (13). HCV entry inhibitors targeting host CD81, SR-BI, CLDN1, Niemann-Pick disease type C1 like 1, and epidermal growth factor receptor exhibit broad pangenomic activities (12, 14–19). Further, Colpitts et al. reported that anti-CLDN1 monoclonal antibodies (MAbs) inhibited infection of hepatic cells with DAA-resistant strains of HCV and showed synergistic inhibition with current DAAs (20). From the genetic studies, *cd81* knockout mice were found to have defects in development and fertility (21, 22), and *cldn1* knockout mice died within 1 day of birth with wrinkled skin (23), whereas *OCLN* knockout mice showed no apparent abnormal phenotypes (24). Hence, among the host entry factors, OCLN may be a promising target for novel host-targeting anti-HCV agents. However, the lack of OCLN-specific binders has hindered the development of OCLN-targeting drugs against HCV infection.

In this study, we created anti-human OCLN (hOCLN) MAbs that recognize the intact extracellular loop domains of OCLN using DNA immunization methods and screening of differential cell binding. The anti-hOCLN MAbs prevented both *in vitro* and *in vivo* HCV infections without apparent adverse effects. Based on these results, we propose the use of OCLN-targeting agents as potential anti-HCV drugs and the usefulness of our anti-hOCLN MAbs for understanding HCV entry mechanisms mediated by OCLN.

RESULTS

Development and characterization of MAbs against extracellular domains of hOCLN. To create MAbs that recognize the extracellular domains of intact hOCLN, rats were subcutaneously immunized with an expression vector encoding hOCLN, and plasma cells isolated from the immunized rats were fused with mouse myeloma cells (Fig. 1A). The resultant hybridomas were used to screen differential cell binding using two sets of cells: Chinese hamster ovary (CHO)-K1 cells, which either express undetectable levels of endogenous OCLN (10) or transiently express hOCLN (CHO-K1 and CHO-K1/hOCLN cells, respectively), and human hepatoma Huh7.5.1-8 cells, which express intact hOCLN (25) or have defective hOCLN expression (Huh7.5.1-8 and OKH-4 cells, respectively) (11), as described in Materials and Methods. Finally, four hybridoma clones (1-3, 32-1, 37-5, and 44-10) were established. Flow cytometry analysis showed that all MAbs from these hybridomas bound to native OCLN-expressing Huh7.5.1-8 cells but not to *OCLN* knockout OKH-4 cells (Fig. 1B). These established MAbs were also found to interact with human fibrosarcoma HT1080 cells that stably expressed hOCLN (HT1080/hOCLN cells) but not with those carrying empty vectors (HT1080/mock cells), which did not express OCLN (Fig. 1C). OCLN is a member of the MARVEL family of proteins; however, four clones did not react with the cell surface domains of MARVEL

FIG 1 Establishment of MAb against extracellular domains of hOCLN. (A) Strategy for development of MAb against hOCLN. s.c., subcutaneous. (B and C) Huh7.5.1-8 cells or Huh7.5.1-8-derived OKH-4 (OCLN knockout [KO]) cells. HT1080 cells carrying empty vectors (HT1080/mock) (B) and those expressing (Continued on next page)

family proteins (i.e., tricellulin and MARVELD3) (Fig. 2). These results confirmed that clones 1-3, 32-1, 37-5, and 44-10 specifically recognized intact OCLN on the cell surface.

Isotype analysis of these MABs classified clones 1-3, 37-5, and 44-10 as immunoglobulin IgG2b and clone 32-1 as IgG2a, whereas sequencing data analysis showed that the homology of each V_H and V_L region among these MABs was less than 50%, except for the homology of the V_H region between clones 32-1 and 37-5, which was 73%.

Flow cytometry analysis using HT1080 cells stably expressing deletion mutants of the first or second extracellular loop of hOCLN (referred to as HT1080/ Δ EL1 and HT1080/ Δ EL2, respectively) showed that clone 1-3 bound to HT1080/ Δ EL1 cells but not to HT1080/ Δ EL2 cells, whereas clones 32-1, 37-5, and 44-10 bound to HT1080/ Δ EL2 cells but not to HT1080/ Δ EL1 cells (Fig. 3). These results indicated that clones 32-1, 37-5, and 44-10 recognized the first extracellular loop of hOCLN, whereas clone 1-3 recognized the second extracellular loop of hOCLN. Meanwhile, only clone 1-3 bound to HT1080 cells stably expressing mouse OCLN (HT1080/mOCLN).

The binding properties of the MABs were also examined using a cell-based enzyme-linked immunosorbent assay (ELISA) with HT1080/hOCLN cells, which showed that all anti-OCLN MABs bound to HT1080/hOCLN cells in a dose-dependent manner but did not bind to HT1080/mock cells. All apparent antibody dissociation constant (K_d) values were in the picomolar range (580 ± 31 [clone 1-3], 590 ± 52 [clone 32-1], 720 ± 84 [clone 37-5], and 630 ± 48 [clone 44-10] pM), indicating that these MABs bind with a very high affinity to cell surface hOCLN (Fig. 4).

Immunocytochemical analysis under nonpermeabilized conditions revealed that cell-cell contact regions in polarized human hepatic HepG2/hCD81 cells were mainly stained with these anti-OCLN MABs but not those in OCLN-knockout HepG2/hCD81 (HG2-OK/hCD81) cells (Fig. 5), suggesting that each of these MABs can recognize intact extracellular domains of OCLN.

Anti-OCLN MABs did not affect cell viability or TJ barrier function. OCLN is one of the major proteins localized to TJs and plays an important role in the regulation of polarity and barrier function in epithelial cells (26). Therefore, we next investigated whether anti-OCLN MABs affected the TJ barrier function using human colorectal adenocarcinoma Caco-2 cells, which form well-constructed TJ barriers in a cell monolayer culture system. Indeed, we observed that cell-cell contact regions were stained in Caco-2 cell monolayers by anti-OCLN MABs (Fig. 6A), similar to staining of HepG2 cells (Fig. 5), and these anti-OCLN MABs had no cytotoxic effects on Caco-2 cells (Fig. 6B). Commercially available anti-OCLN MAB (clone OC-3F10) that recognizes the cytosolic domain of OCLN, which is usually used as a TJ marker, showed sharper stains of cell-cell contact regions than our anti-OCLN MABs (Fig. 6A), although this may be due to the use of permeabilized cells. Future studies are needed to assess the possibility that intact OCLN has broader distributions beyond TJ regions. When polarized Caco-2 cell monolayers were treated with control rat IgG, anti-OCLN MABs, or the TJ breaker C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), transepithelial electrical resistance (TEER) values were reduced only in C-CPE-treated cells (Fig. 6C). These results demonstrated that the established anti-OCLN MABs did not disrupt the TJ barrier of cultured epithelial cells.

Anti-OCLN MABs prevented HCV infection *in vitro*. Studies using the cell culture infection system have shown that OCLN is involved in HCV infection (10, 27), and our group confirmed that OCLN is essential for HCV infection of human hepatic cells (11). We then investigated whether our anti-OCLN MABs could prevent HCV infection in a cell culture infection system. We first tested the effect of these MABs on the viability of hepatic cells using the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt] cell viability assay, which showed that none of the MABs affected

FIG 1 Legend (Continued)

full-length hOCLN (HT1080/hOCLN) (C) were incubated with control rat IgG or each anti-OCLN MAB (clone 1-3, 32-1, 37-5, or 44-10) and treated with Alexa Fluor 488-conjugated anti-rat IgG. Stained cells were analyzed by flow cytometry. The vertical dashed lines indicate the peak value of each cell stained with control rat IgG (background levels).

FIG 2 Established anti-OCLN MAbs were specifically bound to OCLN. HT1080 cells exogenously expressing human MARVEL family proteins OCLN (hOCLN), tricellulin (hTricellulin), MARVELD3 (hMARVELD3), or not expressing any protein (mock) were incubated with control rat IgG or each anti-OCLN MAb (clone 1-3, 32-1, 37-5, or 44-10) and treated with Alexa Fluor 488-conjugated anti-rat IgG. Stained cells were analyzed by flow cytometry. The vertical dashed lines indicate the peak value of each cell stained with control rat IgG.

the viability of HCV-permissive Huh7.5.1-8 cells (Fig. 6D) as well as Caco-2 cells (Fig. 6B), even at high concentrations (50 μ g/ml). Huh7.5.1-8 cells next were infected with cell culture-derived HCV-JFH1 in the presence of control rat IgG or each anti-OCLN MAb, and cellular HCV RNA levels were measured by quantitative reverse transcription-PCR

FIG 3 Clone 1-3 recognized the second loop of hOCLN and mOCLN, whereas the three other clones recognized the first loop of hOCLN. HT1080 cells carrying an empty vector (mock) and those expressing full-length hOCLN (hOCLN), OCLN mutants deleted of either extracellular loop (ΔEL1 and ΔEL2), or mouse OCLN (mOCLN) was incubated with control rat IgG or each anti-OCLN MAb and then treated with Alexa Fluor 488-conjugated anti-rat IgG. Stained cells were analyzed by flow cytometry. The vertical dashed lines indicate the peak value of each cell stained with control rat IgG.

(qRT-PCR) at 4 days postinfection (dpi). As shown in Fig. 7A, all anti-OCLN MABs inhibited HCV RNA production in a dose-dependent manner. For clone 1-3, which recognizes the second extracellular loop of OCLN, the HCV RNA level was strongly decreased to the background level, and clone 32-1 showed relatively weak inhibition of

FIG 4 Binding of anti-OCN MABs to cell surface hOCLN with high affinity. After fixation, HT1080 cells carrying an empty vector (HT1080/mock cells) (A) or those expressing hOCLN (HT1080/hOCLN cells) (B) in 96-well plates were incubated with serial dilutions of each anti-OCN MAB (clone 1-3, 32-1, 37-5, or 44-10) or control rat IgG, followed by horseradish peroxidase-conjugated anti-rat IgG. The binding of each antibody to cell surface hOCLN was detected as described in the cell ELISA section of Materials and Methods. Data are presented as means $\pm$ SD ($n = 3$).

HCV RNA production. The concentration of each MAB that resulted in a 90% inhibition of cellular HCV RNA production was 0.280 ± 0.180 (clone 1-3), 33.4 ± 25.1 (clone 32-1), 5.32 ± 4.62 (clone 37-5), and 6.54 ± 5.64 (clone 44-10) nM. Further immunocytochemical analysis was conducted to examine the effect of these MABs on infection with two other infectious HCV strains, TNS2J1 (genotype 1b/2a chimera) and Jc1 (genotype 2a/2a chimera). Similar to the case of the HCV-JFH1 strain, our results showed that anti-OCN MABs decreased the amount of cellular HCV core protein in Huh7.5.1-8 cells infected with both the TNS2J1 and Jc1 strains (Fig. 7B).

HCV pseudoparticle (HCVpp) infection is a valid model of HCV entry into host cells. Therefore, we tested whether anti-OCN MABs block the entry of various genotypes of HCVpp into Huh7.5.1-8 cells. As shown in Fig. 8, treatment of cells with control rat IgG did not reduce HCVpp infection, whereas treatment with each anti-OCN MAB decreased the entry of all tested genotypes of HCVpp (1a, H77c; 1b, Con1 and TH; 2a, JFH1 and J6) in a dose-dependent manner. These anti-OCN MABs did not affect the entry of vesicular stomatitis virus pseudoparticle (VSVpp). These results showed that anti-

FIG 5 Cell surface OCLN staining with anti-OCN MABs. HepG2/hCD81 cells (upper) or OCLN knockout HG2-OK/hCD81 cells (lower) derived from HepG2/hCD81 cells were fixed and incubated with anti-OCN MAB (clone 1-3, 32-1, 37-5, or 44-10) or control rat IgG, followed by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; blue) and Alexa Fluor 488-conjugated anti-rat IgG (green). Stained cells were observed by confocal microscopy. Scale bars, 20 μ m.

FIG 6 Anti-OCN MABs had no effect on cell viability and TJ barrier function. (A) Fixed Caco-2 cells were incubated with control rat IgG, the established anti-OCN MABs, or commercially available MAB that recognizes the cytosolic domain of OCLN (clone OC-3F10) and then were treated with DAPI (blue) and Alexa Fluor 488-conjugated anti-rat IgG or anti-mouse IgG (green). Cells stained with clone 3F10 were pretreated with 0.2% Triton X-100–PBS for permeabilization. Stained cells were observed by confocal microscopy. Scale bars, 20 μ m. (B and D) Caco-2 (B) and Huh7.5.1-8 (D) cells were treated with the indicated concentrations of control rat IgG, anti-OCN MABs, or the TJ barrier breaker C-CPE for 4 days. Cell viability was then determined using the XTT assay. Values are expressed as percentages of control values. (C) Polarized Caco-2 cells in 24-well Transwell filters were cultured until the formation of a confluent cell monolayer and then either left untreated or treated with 5 μ g/ml of control rat IgG or each anti-OCN MAB (clone 1-3, 32-1, 37-5, or 44-10) or with 50 μ g/ml of C-CPE. TEERs of each cell monolayer were measured at the indicated time points. Data are presented as means $\pm$ SD ($n = 3$).

OCN MABs can specifically prevent HCV infection through the interruption of viral entry into hepatic cells. Anti-OCN MABs, particularly clone 1-3, may be promising candidates for the development of HCV entry inhibitors.

OCN is involved in the later step of HCV entry. HCV entry into hepatocytes is known to be mediated by various host factors, including SR-BI, CD81, CLDN1, and OCLN (28), suggesting that these host entry factors are engaged in HCV entry both cooperatively and serially. A study of HCV infection with artificial FLAG-tagged OCLN and

HCV strains

FIG 7 Anti-OCN MABs inhibited *in vitro* HCVcc infection. (A) Huh7.5.1-8 cells were preincubated with increasing concentrations of each control rat IgG or anti-OCN MAB for 30 min at room temperature and then infected with HCV-JFH1 for 2 h at 37°C. Cells were further cultured in the presence of each Ab for 4 days. Cellular HCV RNA was measured by qRT-PCR. Values are expressed as percentages of control values (treatment without Ab). Data are presented as means $\pm$ SD ($n = 4$). The limit of detection was 0.021 ± 0.027 . (B) Huh7.5.1-8 cells were preincubated with 5 μ g/ml of each rat IgG for 30 min at room temperature and then infected with various infectious HCVcc strains (JFH1, TNS2J1, or Jc1) at an MOI of 1.0. At 4 dpi, the cells were fixed and stained with anti-HCV core MAB (green). Scale bars, 100 μ m.

anti-FLAG antibody revealed that OCLN seems to act late in the HCV entry process, during the postbinding stage and after the use of CD81 and CLDN1 (29). To confirm this finding, the timing of OCLN usage during HCVpp infection was analyzed using our anti-OCN and anti-CLDN1 functional MABs. In brief, HCVpp was incubated with HCV-permissive HepG2/hCD81 cells at 4°C, and unbound particles were washed with phosphate-buffered saline (PBS). The culture temperature was then shifted to 37°C to allow postbinding infection. After 2 days of culture, HCVpp entry was gauged using the luciferase reporter assay. Anti-OCN and anti-CLDN1 MABs were added at different times before or after the binding phase. Treatment of cells with each MAB at 2 h after HCVpp binding still inhibited HCVpp entry. Meanwhile, the addition of anti-CLDN1 MAB (clone 3A2) 3 h after virus binding had no effect on HCVpp entry, but similar addition of the anti-OCN MABs (clones 1-3 and 37-5) significantly inhibited HCVpp entry (Fig. 9). These results showed that OCLN is used after CLDN1 in HCV infection, as previously suggested (29).

Anti-OCN MABs inhibited cell-to-cell HCV infection. It is well known that there are two types of HCV invasion into hepatocytes: infection through viruses floating free

FIG 8 Anti-OCN MAb inhibited *in vitro* HCVpp infection. Huh7.5.1-8 cells were preincubated with the indicated amounts of control rat IgG or each anti-OCN MAb for 30 min at room temperature and then infected with various genotypes of HCVpp (1a, H77c; 1b, Con1 and TH; 2a, JFH1 and J6), VSVpp, or pseudoparticles with no envelope proteins (–Env) for 6 h at 37°C. At 2 dpi, cellular luciferase activity was determined as described in Materials and Methods. Values are expressed as percentages of control values (treatment without Ab). Data are presented as means $\pm$ SD ($n = 3$).

in the medium (cell-free infection) and direct infection between neighboring cells (cell-to-cell infection [30, 31]). In this context, OCLN is suggested to be important for cell-to-cell (31, 32) as well as cell-free HCV infection. We also demonstrated that OCLN is essential for cell-to-cell infection using *OCLN* knockout hepatic cells (11). Here, we evaluated whether anti-OCN MAb can inhibit cell-to-cell HCV transmission. We first developed a visualization system to monitor cell-to-cell HCV infection using a mitochondrial antiviral signaling protein (MAVS)-based reporter. Mitochondrially tethered MAVS (33), which is also known as IPS-1/VISA/Cardiff, is a known cellular substrate of

FIG 9 Analysis of the timing of entry factor usage during HCV infection. HepG2/hCD81 cells were infected with HCVpp (JFH1) at 0 h. To monitor the relative timing of each step in the entry process, anti-CLDN1 MAb (clone 3A2) or anti-OCN MAb (clone 1-3 or 37-5) was added to the culture medium at the indicated time points. At 2 dpi, cellular luciferase activity was determined. Values are expressed as percentages of control values (at 5 h without Ab treatment). Data are presented as means $\pm$ SD ($n = 3$). *, $P < 0.01$.

the HCV NS3-4A protease, and the nonmembranous cleaved MAVS is formed shortly after HCV infection (34). We used MAVS with an N-terminal fluorescent mCherry tag and a simian virus 40 (SV40) nuclear localization sequence (mCherry-NLS-MAVS) as an indicator, which is predicted to be transferred from the mitochondria (cytoplasm) to the nucleus upon HCV infection. Indeed, in Huh7.5.1-8 cells stably expressing mCherry-NLS-MAVS, which are highly permissive to HCV infection (25), we observed the movement of mCherry-NLS-MAVS (red fluorescence) from the cytoplasm to the nucleus during HCV-JFH1 infection, and this nuclear localization of red fluorescence was completely blocked by the anti-OCLN MAb clone 1-3 (Fig. 10A). Previously, we isolated CD81-defective 751r cells from Huh7.5.1 cells, which are nonpermissive to cell-free HCV infection but weakly permissive to cell-to-cell infection (11, 12, 25). Additionally, in this MAVS indicator system, 751r cells that stably expressed mCherry-NLS-MAVS (751r/mCherry-NLS-MAVS) showed nuclear localization of red fluorescence after cellular transfection of HCV RNA (Fig. 10B), but this did not occur after infection with HCV-JFH1 (Fig. 10C). When HCV-preinfected Huh7.5.1-8 cells were mixed with naive 751r/mCherry-NLS-MAVS cells and cocultured for 4 days, a significant portion of the 751r/mCherry-NLS-MAVS cells adjacent to the HCV-infected Huh7.5.1-8 cells had nuclear localization of red fluorescence, indicating cell-to-cell HCV transmission from Huh7.5.1-8 cells to 751r/mCherry-NLS-MAVS cells (Fig. 10D and E). In this coculture system, the addition of anti-OCLN MAbs (clones 1-3 and 37-5) completely blocked nuclear localization of red fluorescence, but the addition of control rat IgG did not (Fig. 10F). Taken together with the above-described data, these results demonstrated that anti-OCLN MAbs can inhibit not only cell-free infection but also cell-to-cell infection.

Anti-OCLN MAbs completely prevented HCV infection *in vivo*. We next tested whether administration of anti-OCLN MAbs could inhibit HCV infection *in vivo* using human liver chimeric mice, a well-known animal model of HCV infection (35–37). We focused on clones 1-3 (recognized by EL2) and 37-5 (recognized by EL1) in the *in vivo* HCV infection analysis, because these two MAbs recognize different epitopes of OCLN, are of the same IgG2b subclass, and sufficiently inhibit HCV infection *in vitro*. Chimeric mice were intraperitoneally administered clone 1-3 or 37-5 at concentrations of 50, 30, 20, and 10 mg/kg of body weight on days 0, 3, 7, and 10, respectively. On day 0, 6 h after MAb administration, the mice were challenged with HCV (genotype 1b) (Fig. 11A). All mice that received the control rat IgG were HCV positive at 14 dpi. Serum HCV RNA levels in all mice plateaued at $>5 \times 10^6$ copies/ml at 21 dpi. Meanwhile, the serum of all mice that received anti-OCLN MAbs (clone 1-3 or 37-5) was HCV negative even after 35 dpi (Fig. 11B). We also determined serum anti-OCLN MAb levels at 0 to 35 dpi using cell-based ELISA. The serum MAbs in clone 1-3-treated mice and clone 37-5-treated mice were detectable ($>0.3 \mu\text{g/ml}$) until 28 dpi, and those at 35 dpi were under the limit of detection (Fig. 11C and D). These results indicated that anti-OCLN MAb clones 1-3 and 37-5 completely prevented HCV infection *in vivo*. To monitor the toxic effects of anti-OCLN MAbs, we measured the body weights and analyzed human albumin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels in the sera of MAb-treated mice. As shown in Fig. 11E to H, there were no significant changes in body weight or human albumin, AST, and ALT levels in mice that received control rat IgG or anti-OCLN MAbs. These data suggested that anti-OCLN MAb clones 1-3 and 37-5 had no significant cytotoxic effect on human hepatocytes at the administered doses.

DISCUSSION

HCV entry into host cells is a complex process involving multiple host factors, including the TJ protein OCLN. Because OCLN is essential for HCV infection (11), it may be a promising target for novel host-targeting anti-HCV agents. However, to date, there are no OCLN-binding probes that can inhibit HCV infection, because OCLN is a multiple transmembrane protein and MAbs against such proteins are very difficult to produce. Therefore, MAbs recognizing the extracellular domains of intact hOCLN have not yet been developed. However, we successfully developed MAbs against the extracellular

FIG 10 Anti-OCN MAb prevented cell-to-cell HCV transmission. Huh7.5.1-8/mCherry-NLS-MAVS cells (A) or Δ CD81-defective 751r/mCherry-NLS-MAVS cells (C) were infected with HCV-JFH1 or left uninfected. (B) 751r/mCherry-NLS-MAVS cells were left untransfected or were transfected with HCV subgenomic replicon (Continued on next page)

loops of the TJ transmembrane proteins CLDN1, CLDN2, CLDN3/4, and CLDN4 (12, 38–41) using DNA immunization methods. Anti-CLDN1 MABs, which we developed, had a particularly high affinity for the intact extracellular domains of CLDN1. Using a similar strategy, we successfully generated four MABs (clones 1-3, 32-1, 37-5, and 44-10) that recognize the intact extracellular loop domains of OCLN with very high affinity and specificity (Fig. 1B and C, 2, and 4).

All of these MABs can bind to intact OCLN but were not available for immunoblot analysis, suggesting that all MABs recognize conformational epitopes rather than linear epitopes. Among the four anti-OCLN MABs created, clone 1-3 recognized the second loop of human and mouse OCLN, whereas the three other MABs recognized the first loop of human OCLN. Consistent with this, the complementarity-determining region sequences of clone 1-3 MAB have a very low homology with other sequences. However, further epitope analyses using OCLN constructs with each point mutation are needed.

All anti-OCLN MABs prevented infection of Huh7.5.1-8 cells with HCVcc and HCVpp in a dose-dependent manner without apparent cytotoxicity (Fig. 6D, 7, and 8). In particular, the MAB clone 1-3 showed much stronger HCV inhibition. Consistent with this observation, previous reports have suggested that this second extracellular loop of OCLN is critical for HCV infection (10, 11, 42, 43). Conversely, the first extracellular loop of OCLN is nonessential for HCV entry into hepatic cells, as we showed previously (11). Therefore, the inhibitory effects of MABs that recognize the first loop of OCLN may be due to steric effects.

To investigate the antiviral effects of the anti-OCLN MABs *in vivo*, anti-OCLN MAB clones 1-3 and 37-5 or a control antibody were administered to human liver chimeric uPA-SCID mice. As shown in Fig. 11, each anti-OCLN MAB completely blocked HCV infection in human liver chimeric mice without apparent adverse effects and could be detected in the sera until 28 dpi. It is well known that HCV is quickly inactivated (44) compared with other viruses, which may be the reason why the anti-OCLN MABs completely blocked HCV invasion into hepatocytes of chimeric mice. Furthermore, we demonstrated that the anti-OCLN MABs completely blocked cell-to-cell transmission as well as cell-free infection (Fig. 7 and 10F). The ability of these anti-OCLN MABs to inhibit both modes of infection may result in efficient blockage of HCV transmission among hepatocytes *in vivo*, suggesting that the anti-OCLN MABs convey antiviral effects in the hepatocytes of patients with persistent HCV infection.

Hopcraft and Evans recently reported that an HCV variant with a single-amino-acid mutation could infect cells via a CLDN1-independent cell entry mechanism (45). Meanwhile, Mailly et al. demonstrated that a single-dose treatment with anti-CLDN1 MAB could cure persistent HCV infection in a humanized mouse model (15). Furthermore, no escape viruses were observed in the mouse model or on primary human hepatocytes (20). These results indicated that escape via CLDN6 and CLDN9 is a cell line-specific observation. Based on these studies, CLDN1 is considered a promising anti-HCV target (12, 19, 20, 46), although *cldn1* knockout mice (23) had wrinkled skin and died within 1 day of birth. *Ocln* knockout mice were born with no gross phenotypes (24). We also demonstrated, using a transwell culture system, that the anti-OCLN MABs were not cytotoxic to cultured cells (Fig. 6B and D) and did not disrupt the epithelial TJ barrier in polarized Caco-2 cells (Fig. 6C). The administration of the second loop-

FIG 10 Legend (Continued)

RNA. (D) An overview of the detection of cell-to-cell HCV transmission. (E) Huh7.5.1-8 cells were preinfected with HCV-JFH1 and cultured for 1 day, mixed with 751r/mCherry-NLS-MAVS cells (at a cell ratio of 1:1), and plated in a 24-well plate at 1×10^5 cells/well. After 4 days, the cells were fixed, stained with anti-HCV core protein MAB (green) and DAPI (blue), and then visualized by confocal microscopy. Arrows, donor Huh7.5.1-8 cells preinfected with HCV. Arrowheads, acceptor 751r/mCherry-NLS-MAVS cells in which nucleus was stained red. Solid line, HCV core-positive Huh7.5.1-8 cells. Broken line, HCV core-positive 751r/mCherry-NLS-MAVS. (F) Huh7.5.1-8 cells were preinfected with HCV-JFH1, or not, and cultured for 1 day, mixed with 751r/mCherry-NLS-MAVS cells (at a cell ratio of 1:1), and plated in a 24-well plate at 1×10^5 cells/well in the presence of control rat IgG or anti-OCLN MAB (clone 1-3 or 37-5). (A to C and F) After 4 days, the cells were visualized by confocal microscopy. (F) Arrows indicate HCV-infected 751r/mCherry-NLS-MAVS cells by cell-to-cell transmission. Scale bars, 20 μ m (A to C), 50 μ m (E), and 200 μ m (F).

FIG 11 Anti-OCN MAbs completely blocked *in vivo* HCV infection. (A) Experimental procedure of *in vivo* HCV infection using human liver chimeric mice. i.p., intraperitoneal; i.v., intravenous. (B) Human liver chimeric mice were treated with control rat IgG ($n = 4$) or anti-OCN MAb (clone 1-3 or 37-5; $n = 3$), as described in Materials and Methods. HCV (genotype 1b) was used for infections in these experiments. At the indicated time points, serum HCV RNA levels were determined by qRT-PCR. The dotted line indicates the limit of detection (2×10^4 copies/ml). Arrows show the times of injection of MAbs. (C and D) Anti-OCN MAb contents in sera were determined using a cell ELISA-based assay, as described in Materials and Methods (clone 1-3-treated mice [C] and clone 37-5-treated mice [D]). Data are presented as means $\pm$ SD ($n = 3$). The limit of detection was 0.27 ± 0.012 μ g/ml for the clone 1-3-treated mice and 0.33 ± 0.010 μ g/ml for the clone 37-5-treated mice. Characteristics of Ab-treated chimeric mice, including body weight (E) and human albumin (F), AST (G), and ALT (H) levels in sera.

recognizing MAb clone 1-3, which reacts with both human and mouse OCLN, showed no systemic toxicity in human liver chimeric mice (Fig. 11E to H). Furthermore, there have been no reports of HCV infection via OCLN-independent routes. The attempted genetic approaches with OCLN knockout cells showed that multiple serial passages of HCV-JFH-1 with OCLN knockout cells resulted in no isolation of viruses. Taken together, we believe that OCLN, in addition to CLDN1, is a promising anti-HCV target.

In conclusion, we developed four MAbs against the intact extracellular domains of OCLN that strongly prevented HCV infection both *in vitro* and *in vivo* and demonstrated

that OCLN is one of the best host target molecules of HCV entry inhibitors. These anti-OCLN MABs, especially clone 1-3, that recognized the second extracellular loop would be a promising lead for the development of OCLN-targeting anti-HCV agents. We also propose that these anti-OCLN MABs would be a powerful tool to understand the OCLN-mediated HCV entry mechanism as well as to evaluate the cell biological roles of OCLN in TJs and plasma membrane.

MATERIALS AND METHODS

Plasmid construction. Retroviral pCX4-bleo and pCX4-pur (47) vectors were obtained from the Osaka Bioscience Institute (Suita, Japan). The mammalian expression vector pcDNA3.1(+) was purchased from Invitrogen Corporation (Carlsbad, CA). *hOCLN* and *mOCLN* cDNAs were generated via PCR using primer pairs specific to each *OCLN* gene. The resultant cDNA was inserted into the *MluI*/*SacI* sites of the pCX4-bleo vector or the *EcoRV*/*XhoI* sites of the pcDNA3.1(+) vector. The constructed vectors are referred to as pCX4-bleo-*hOCLN*, pCX4-bleo-*mOCLN*, and pcDNA3.1/*hOCLN*. Retroviral expression vectors for *hOCLN* deletion mutants of the first or second extracellular loop (referred to as Δ EL1 and Δ EL2, respectively) were constructed by inverse PCR from pCX4-bleo-*hOCLN*, as described previously (11). The pCX4-bleo-*hTricellulin* and pCX4-bleo-*hMARVELD3-1* vectors were kindly provided by Akihiro Watari (Osaka University, Japan).

The *mCherry-NLS-MAVS* gene, which contains amino acids (aa) 1 to 262 of the *mCherry* gene, the nuclear localization signal sequence of SV40 (corresponding to aa 2 to 8), the spacer nucleotides GGT, and the C-terminal region of the *MAVS* gene (corresponding to aa 503 to 540), provided by Yoshiharu Matsuura (Osaka University, Japan) (48), was cloned into the *Bam*HI/*Eco*RI sites of the pCX4-pur vector. The constructed vector was referred to as pCX4-pur-*mCherry-NLS-MAVS*.

Cell lines. We used human hepatoma cells derived from Huh7.5.1 cells, kindly provided by Francis V. Chisari (The Scripps Research Institute, La Jolla, CA, USA) (49), including Huh7.5.1-8 cells (25), which are highly permissive to HCV infection, and *CD81*-defective 751r (25) and *OCLN*-defective OKH-4 (11) cells, which are nonpermissive to HCV infection. Human fibrosarcoma HT1080 cells and human hepatoma HepG2 cells were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). Human embryonic kidney 293T (HEK 293T), human colorectal adenocarcinoma Caco-2, and CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Huh7.5.1-derived, HepG2, HEK 293T, and HT1080 cells were cultured at 37°C under an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 0.1 mM nonessential amino acids (normal medium). Caco-2 cells were maintained in Eagle's minimum essential medium supplemented with 60 µg/ml kanamycin (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and 10% FBS. CHO-K1 cells were maintained in Ham's F-12 medium supplemented with 10% newborn calf serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate (50).

Various stable transfectants (HT1080/mock, HT1080/*hOCLN*, HT1080/*hOCLN*- Δ EL1, HT1080/*hOCLN*- Δ EL2, HT1080/*mOCLN*, HepG2/*hCD81*, Huh7.5.1-8/*mCherry-NLS-MAVS*, and 751r/*mCherry-NLS-MAVS* cells) were established by retroviral-mediated gene transfer and drug selection, as described previously (11). HepG2/*hCD81*-derived *OCLN* knockout cells, referred to as HG2-OK/*hCD81*, were established by the same method as that used for the generation of OKH-4 cells (11).

Isolation of MABs against the extracellular domains of OCLN. Six-week-old male Wistar rats were subcutaneously immunized with a mammalian expression vector encoding *hOCLN* every 2 weeks for 2 months according to proprietary GENOVAC technology (GENOVAC GmbH, Freiburg, Germany) (40). Plasma cells were removed 7 days after the last immunization and fused with mouse myeloma P3U1 cells in the presence of polyethylene glycol 1000 to generate hybridomas. Hybridomas producing anti-OCLN MABs were initially screened for the ability of their culture supernatants to bind to CHO-K1 cells transiently expressing *hOCLN*, but not to wild-type CHO-K1 cells, by cell ELISA and flow cytometry. The hybridomas were then further selected by flow cytometry based on their ability to bind Huh7.5.1-8 cells but not to *OCLN* knockout OKH-4 cells. After cloning by limiting dilution, four stable hybridomas that produced MABs binding to the extracellular domains of *hOCLN* (clones 1-3, 32-1, 37-5, and 44-10) were obtained. Immunoglobulin classes and subclasses of the clones were determined using a rat immunoglobulin isotyping ELISA kit (BD Biosciences, Woburn, MA, USA).

Flow-cytometric analysis. Cells were detached from culture dishes with 0.05% (wt/vol) Trypsin–0.5 mM EDTA and then treated with antibodies (5 µg/ml) in PBS containing 2% FBS for 60 min at 4°C. After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rat IgG (Thermo Fisher Scientific, Waltham, MA, USA) at 2 µg/ml in PBS containing 2% FBS for 30 min at 4°C. After washing with PBS, the cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Cell ELISA. Cell ELISA was performed as described previously (12), with slight modifications. In brief, confluent HT1080/*hOCLN* or HT1080/mock cells in 96-well plates, fixed with 3.7% formaldehyde, were incubated with serial dilutions of 100 µl of each anti-OCLN MAB or control IgG (5 µg/ml) for 2 h, followed by a 1:5,000 dilution of 100 µl of horseradish peroxidase-conjugated goat anti-rat IgG (GE Healthcare, Waukesha, WI, USA) for 1 h at room temperature. To detect antibody binding to the cells, α -phenylenediamine dihydrochloride and hydrogen peroxide were used as substrates. The absorbance at 492 nm for each well was determined on an OpsysMR plate reader (Dynex Technologies GmbH, Denkendorf, Germany). The half-saturating concentrations (apparent K_d [antibody dissociation constants] values) were determined using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA) as described previously (51).

TJ barrier function assay. To determine the TJ barrier function of cell monolayers, we measured the electrical resistance across the cell monolayers (TEER) as described previously (52–54). In brief, Caco-2 cells were seeded in 24-well Transwell filters (Corning, NY) at a subconfluent density. TEER of the cell sheets on the filter was monitored using a Millicell electrical resistance system with a dual electrode (Millipore, Billerica, MA). After TEER values reached a plateau, showing that TJs were well developed in the cell sheets, the cell monolayers were treated with 5.0 μg of TJ barrier breaker C-CPE (55) or 50 μg of each anti-OCN MAb on the basal side of the filter. The TEER values on the filter were then monitored.

Cell viability assay. To determine the cytotoxicity of anti-OCN MAbs, a colorimetric XTT assay was performed with cell proliferation kit II (XTT) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Briefly, after treatment of the cells with the XTT reagent for 2 h, the supernatants were transferred to a 96-well plate, and the absorbance at 450 nm was measured using an OpsysMR plate reader (Dynex Technologies GmbH).

In vitro HCV infection. The infectious source of HCV-JFH1 (49, 56) was prepared from culture supernatants of Huh7.5.1-8 cells that had been transfected with *in vitro*-transcribed HCV-JFH1 RNA at a multiplicity of infection (MOI) of 0.1 for 2 h and passaged a few times on Huh7.5.1-8 cells (12). Two other infectious HCV-JFH1 chimera strains, Jc1 (genotype 2a/2a) (57) and TNS2J1 (genotype 1b/2a) (58), were prepared as described previously (11, 59). HCV infection was detected using qRT-PCR and immunohistochemical analysis as described previously (12, 25, 60).

HCVpp infection. HCVpps were generated as described previously (11). In brief, a *gag-pol* packaging construct (Gag-Pol 5349), a transfer vector construct (Luc 126), and an envelope glycoprotein expression vector (H77c [genotype 1a], TH [genotype 1b], Con1 [genotype 1b; GenBank accession number AJ238799], JFH1 [genotype 2a], J6 [genotype 2a], and VSV-G) were transfected into HEK 293T cells. The medium from the transfected cells was collected and used as the HCVpp source. HCVpp infection and a luciferase reporter assay were performed as described previously (12, 25).

Cell-to-cell HCV transmission. Huh7.5.1-8 cells were seeded at a density of 2×10^6 cells in a 100-mm dish, cultured for 1 day, and infected with HCV-JFH1 at an MOI of 0.1. The HCV-infected Huh7.5.1-8 cells and naive 751r/mCherry-NLS-MAVS cells were mixed at a 1:1 ratio and plated in each well of a 24-well plate at a density of 8×10^4 cells. Control rat IgG or anti-OCN MAb (clone 1-3 or 37-5; 10 $\mu\text{g}/\text{ml}$) was immediately added to each well. Cells were cultured for 4 days and then observed under a confocal microscope (LSM700; Carl Zeiss, Jena, Germany).

In vivo HCV infection. Human liver chimeric mice (PXB mice) were prepared as described previously (61). PXB mice (16 or 17 weeks old, male) were intraperitoneally injected with anti-OCN MAbs (clone 1-3 or 37-5) or control rat IgG at 50 mg/kg (three anti-OCN-treated mice/group and four control Ab-treated mice/group). At 8 h after Ab administration, the mice were intravenously inoculated with HCV-PBC001 (10^4 copies; genotype 1b; lot 0080811). The total blood volume of a mouse is approximately 80 ml/kg (17); hence, 10^4 HCV RNA copies/mouse corresponds to $\sim 1.7 \times 10^4$ HCV RNA copies/ml in serum. Mice were intraperitoneally injected with anti-OCN MAbs (clone 1-3 or 37-5) or control Ab at 30, 20, and 10 mg/kg on days 3, 7, and 10, respectively. Sera were collected from mice on days 0, 3, 7, 10, 14, 21, 28, and 35 for analyses. HCV RNA in mouse serum was quantified using TaqMan EZ RT-PCR core reagent (Life Technologies Corp., Carlsbad, CA, USA) and an ABI Prism 7500 sequence detector system (Life Technologies Corp.). The lower quantification limit of the assay was 2.0×10^4 copies/ml.

Ethics statement. All procedures using PXB mice were approved by the Animal Ethics Committee of PhoenixBio Co., Ltd. (Hiroshima, Japan) (permission number 1564), and all animal experiments were performed after approval by the Committee on the Ethics of Animal Experiments of the National Institute of Infectious Diseases (permission number 815005) under the guidelines of the National Institute of Infectious Diseases, 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>).

Measurements of anti-OCN MAbs in mouse serum. Anti-OCN MAb (clones 1-3 and 37-5) levels in mouse serum were determined by cell ELISA. In brief, confluent HT1080/hOCN cells in 96-well plates, fixed with 3.7% formaldehyde, were incubated with 100 μl of each serum sample diluted with PBS containing 5% (wt/vol) skim milk for 2 h, followed by a 1:5,000 dilution of 100 μl of horseradish peroxidase-conjugated goat anti-rat IgG (GE Healthcare) for 1 h at room temperature. To detect cellular binding of MAbs, 1-Step Ultra TMB-ELISA substrate solution (Thermo Fischer Scientific) was used as the substrate. For each well, the absorbance at 450 nm was determined on an OpsysMR plate reader (Dynex Technologies GmbH). Each purified MAb was used as a standard.

Other methods. Immunohistochemical analysis was performed as described previously (25). Analysis of the timing of entry factor usage during HCV infection was performed as described previously (29). For *in vivo* experiments, human albumin, AST, and ALT levels in mouse sera were measured as described previously (12).

Statistical analysis. The data for statistical analyses are presented as the averages from three independent experiments $\pm$ standard deviations (SD). The significance of differences in the means was determined by Student's *t* test. A probability (*P*) value of <0.05 was considered statistically significant.

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REFERENCES

- Messina JP, Humphreys I, Flaxman A, Brown A, Cooke GS, Pybus OG, Barnes E. 2015. Global distribution and prevalence of hepatitis C virus genotypes. *Hepatology* 61:77–87. <https://doi.org/10.1002/hep.27259>.
- Petruzziello A, Marigliano S, Loquercio G, Cozzolino A, Cacciapuoti C. 2016. Global epidemiology of hepatitis C virus infection: an up-date of the distribution and circulation of hepatitis C virus genotypes. *World J Gastroenterol* 22:7824–7840. <https://doi.org/10.3748/wjg.v22.i34.7824>.
- Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, Roitelman J, Barbaras R, Graber P, Ghersa P, Smolarsky M, Funaro A, Malavasi F, Larrey D, Coste J, Fabre JM, Sa-Cunha A, Maurel P. 2007. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J Hepatol* 46:411–419. <https://doi.org/10.1016/j.jhep.2006.09.024>.
- Barth H, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, Von Weizsacker F, Blum HE, Baumert TF. 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* 278:41003–41012. <https://doi.org/10.1074/jbc.M302267200>.
- Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* 21:5017–5025. <https://doi.org/10.1093/emboj/cdf529>.
- Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. 1998. Binding of hepatitis C virus to CD81. *Science* 282:938–941. <https://doi.org/10.1126/science.282.5390.938>.
- Sainz B, Jr, Barretto N, Martin DN, Hiraga N, Imamura M, Hussain S, Marsh KA, Yu X, Chayama K, Alrefai WA, Uprichard SL. 2012. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nat Med* 18:281–285. <https://doi.org/10.1038/nm.2581>.
- Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J, Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Doffoel M, Raffelsberger W, Poch O, McKeating JA, Brino L, Baumert TF. 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat Med* 17:589–595. <https://doi.org/10.1038/nm.2341>.
- Evans MJ, von Hahn T, Tschernie DM, Syder AJ, Panis M, Wolk B, Hatzioannou T, McKeating JA, Bieniasz PD, Rice CM. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 446:801–805. <https://doi.org/10.1038/nature05654>.
- Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457:882–886. <https://doi.org/10.1038/nature07684>.
- Shirasago Y, Shimizu Y, Tanida I, Suzuki T, Suzuki R, Sugiyama K, Wakita T, Hanada K, Yagi K, Kondoh M, Fukasawa M. 2016. Occludin-knockout human hepatic Huh7.5.1-8-derived cells are completely resistant to hepatitis C virus infection. *Biol Pharm Bull* 39:839–848. <https://doi.org/10.1248/bpb.b15-01023>.
- Fukasawa M, Nagase S, Shirasago Y, Iida M, Yamashita M, Endo K, Yagi K, Suzuki T, Wakita T, Hanada K, Kuniyasu H, Kondoh M. 2015. Monoclonal antibodies against extracellular domains of claudin-1 block hepatitis C virus infection in a mouse model. *J Virol* 89:4866–4879. <https://doi.org/10.1128/JVI.03676-14>.
- Marceau CD, Puschnik AS, Majzoub K, Ooi YS, Brewer SM, Fuchs G, Swaminathan K, Mata MA, Elias JE, Sarnow P, Carette JE. 2016. Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. *Nature* 535:159–163. <https://doi.org/10.1038/nature18631>.
- Vercauteren K, Van Den Eede N, Mesalam AA, Belouard S, Catanese MT, Bankwitz D, Wong-Staal F, Cortese R, Dubuisson J, Rice CM, Pietschmann T, Leroux-Roels G, Nicosia A, Meuleman P. 2014. Successful anti-scavenger receptor class B type I (SR-BI) monoclonal antibody therapy in humanized mice after challenge with HCV variants with in vitro resistance to SR-BI-targeting agents. *Hepatology* 60:1508–1518. <https://doi.org/10.1002/hep.27196>.
- Maily L, Xiao F, Lupberger J, Wilson GK, Aubert P, Duong FHT, Calabrese D, Leboeuf C, Fofana I, Thumann C, Bandiera S, Lutgehetmann M, Volz T, Davis C, Harris HJ, Mee CJ, Girardi E, Chane-Woon-Ming B, Ericsson M, Fletcher N, Bartenschlager R, Pessaux P, Vercauteren K, Meuleman P, Villa P, Kaderali L, Pfeffer S, Heim MH, Neunlist M, Zeisel MB, Dandri M, McKeating JA, Robinet E, Baumert TF. 2015. Clearance of persistent hepatitis C virus infection in humanized mice using a claudin-1-targeting monoclonal antibody. *Nat Biotechnol* 33:549–554. <https://doi.org/10.1038/nbt.3179>.
- Zahid MN, Turek M, Xiao F, Thi VL, Guerin M, Fofana I, Bachellier P, Thompson J, Delang L, Neyts J, Bankwitz D, Pietschmann T, Dreux M, Cosset FL, Grunert F, Baumert TF, Zeisel MB. 2013. The postbinding activity of scavenger receptor class B type I mediates initiation of hepatitis C virus infection and viral dissemination. *Hepatology* 57:492–504. <https://doi.org/10.1002/hep.26097>.
- Meuleman P, Hesselgesser J, Paulson M, Vanwolleghem T, Desombere I, Reiser H, Leroux-Roels G. 2008. Anti-CD81 antibodies can prevent a hepatitis C virus infection in vivo. *Hepatology* 48:1761–1768. <https://doi.org/10.1002/hep.22547>.
- Syder AJ, Lee H, Zeisel MB, Grove J, Soulier E, Macdonald J, Chow S, Chang J, Baumert TF, McKeating JA, McKelvey J, Wong-Staal F. 2011. Small molecule scavenger receptor BI antagonists are potent HCV entry inhibitors. *J Hepatol* 54:48–55. <https://doi.org/10.1016/j.jhep.2010.06.024>.
- Fofana I, Krieger SE, Grunert F, Glauben S, Xiao F, Fafi-Kremer S, Soulier E, Royer C, Thumann C, Mee CJ, McKeating JA, Dragic T, Pessaux P, Stoll-Keller F, Schuster C, Thompson J, Baumert TF. 2010. Monoclonal anti-claudin 1 antibodies prevent hepatitis C virus infection of primary human hepatocytes. *Gastroenterology* 139:953–964. <https://doi.org/10.1053/j.gastro.2010.05.073>.
- Colpitts CC, Tawar RG, Maily L, Thumann C, Heydmann L, Durand SC, Xiao F, Robinet E, Pessaux P, Zeisel MB, Baumert TF. 30 March 2017. Humanisation of a claudin-1-specific monoclonal antibody for clinical prevention and cure of HCV infection without escape. *Gut* <https://doi.org/10.1136/gutjnl-2016-312577>.
- Rubinstein E, Ziyat A, Prenant M, Wrobel E, Wolf JP, Levy S, Le Naour F, Boucheix C. 2006. Reduced fertility of female mice lacking CD81. *Dev Biol* 290:351–358. <https://doi.org/10.1016/j.ydbio.2005.11.031>.
- Mordica WJ, Gallagher RJ, Kennedy JL, Chapes SK. 2010. Male CD81 knockout genotype disrupts Mendelian distribution of offspring. *Comp Med* 60:196–199.
- Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S. 2002. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* 156:1099–1111. <https://doi.org/10.1083/jcb.200110122>.
- Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T, Tsukita S. 2000. Complex phenotype of mice lacking occludin, a com-

- ponent of tight junction strands. *Mol Biol Cell* 11:4131–4142. <https://doi.org/10.1091/mbc.11.12.4131>.
25. Shirasago Y, Sekizuka T, Saito K, Suzuki T, Wakita T, Hanada K, Kuroda M, Abe R, Fukasawa M. 2015. Isolation and characterization of an Huh.7.5.1-derived cell clone highly permissive to hepatitis C virus. *Jpn J Infect Dis* 68:81–88. <https://doi.org/10.7883/yoken.JJID.2014.231>.
 26. Paris L, Tonutti L, Vannini C, Bazzoni G. 2008. Structural organization of the tight junctions. *Biochim Biophys Acta* 1778:646–659. <https://doi.org/10.1016/j.bbame.2007.08.004>.
 27. Liu S, Yang W, Shen L, Turner JR, Coyne CB, Wang T. 2009. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J Virol* 83: 2011–2014. <https://doi.org/10.1128/JVI.01888-08>.
 28. Douam F, Lavillette D, Cosset FL. 2015. The mechanism of HCV entry into host cells. *Prog Mol Biol Transl Sci* 129:63–107. <https://doi.org/10.1016/b.pmbts.2014.10.003>.
 29. Sourisseau M, Michta ML, Zony C, Israelow B, Hopcraft SE, Narbus CM, Parra Martin A, Evans MJ. 2013. Temporal analysis of hepatitis C virus cell entry with occludin directed blocking antibodies. *PLoS Pathog* 9:e1003244. <https://doi.org/10.1371/journal.ppat.1003244>.
 30. Timpe JM, Stamatakis Z, Jennings A, Hu K, Farquhar MJ, Harris HJ, Schwarz A, Desombere I, Roels GL, Balfe P, McKeating JA. 2008. Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. *Hepatology* 47:17–24. <https://doi.org/10.1002/hep.21959>.
 31. Liu Z, He JJ. 2013. Cell-cell contact-mediated hepatitis C virus (HCV) transfer, productive infection, and replication and their requirement for HCV receptors. *J Virol* 87:8545–8558. <https://doi.org/10.1128/JVI.01062-13>.
 32. Ciesek S, Westhaus S, Wicht M, Wappler I, Henschen S, Sarrazin C, Hamdi N, Abdelaziz AI, Strassburg CP, Wedemeyer H, Manns MP, Pietschmann T, von Hahn T. 2011. Impact of intra- and interspecies variation of occludin on its function as coreceptor for authentic hepatitis C virus particles. *J Virol* 85:7613–7621. <https://doi.org/10.1128/JVI.00212-11>.
 33. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 6:981–988. <https://doi.org/10.1038/ni1243>.
 34. Jones CT, Catanese MT, Law LM, Khetani SR, Syder AJ, Ploss A, Oh TS, Schoggins JW, MacDonald MR, Bhatia SN, Rice CM. 2010. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. *Nat Biotechnol* 28:167–171. <https://doi.org/10.1038/nbt.1604>.
 35. Umehara T, Sudoh M, Yasui F, Matsuda C, Hayashi Y, Chayama K, Kohara M. 2006. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. *Biochem Biophys Res Commun* 346:67–73. <https://doi.org/10.1016/j.bbrc.2006.05.085>.
 36. Hiraga N, Imamura M, Tsuge M, Noguchi C, Takahashi S, Iwao E, Fujimoto Y, Abe H, Maekawa T, Ochi H, Tateno C, Yoshizato K, Sakai A, Sakai Y, Honda M, Kaneko S, Wakita T, Chayama K. 2007. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Lett* 581:1983–1987. <https://doi.org/10.1016/j.febslet.2007.04.021>.
 37. Inoue K, Umehara T, Ruegg UT, Yasui F, Watanabe T, Yasuda H, Dumont JM, Scalfaro P, Yoshida M, Kohara M. 2007. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. *Hepatology* 45:921–928. <https://doi.org/10.1002/hep.21587>.
 38. Hashimoto Y, Kawahigashi Y, Hata T, Li X, Watari A, Tada M, Ishii-Watabe A, Okada Y, Doi T, Fukasawa M, Kuniyasu H, Yagi K, Kondoh M. 2016. Efficacy and safety evaluation of claudin-4-targeted antitumor therapy using a human and mouse cross-reactive monoclonal antibody. *Pharmacol Res Perspect* 4:e00266. <https://doi.org/10.1002/prp2.266>.
 39. Hashimoto Y, Fukasawa M, Kuniyasu H, Yagi K, Kondoh M. 2017. Claudin-targeted drug development using anti-claudin monoclonal antibodies to treat hepatitis and cancer. *Ann N Y Acad Sci* 1397:5–16. <https://doi.org/10.1111/nyas.13337>.
 40. Li X, Iida M, Tada M, Watari A, Kawahigashi Y, Kimura Y, Yamashita T, Ishii-Watabe A, Uno T, Fukasawa M, Kuniyasu H, Yagi K, Kondoh M. 2014. Development of an anti-claudin-3 and -4 bispecific monoclonal antibody for cancer diagnosis and therapy. *J Pharmacol Exp Ther* 351:206–213. <https://doi.org/10.1124/jpet.114.216911>.
 41. Takigawa M, Iida M, Nagase S, Suzuki H, Watari A, Tada M, Okada Y, Doi T, Fukasawa M, Yagi K, Kuniyasu J, Kondoh M. 2017. Creation of a Claudin-2 binder and its tight junction-modulating activity in a human intestinal model. *J Pharmacol Exp Ther* 363:444–451. <https://doi.org/10.1124/jpet.117.242214>.
 42. Liu S, Kuo W, Yang W, Gibson GA, Dorko K, Watkins SC, Strom SC, Wang T. 2010. The second extracellular loop dictates occludin-mediated HCV entry. *Virology* 407:160–170. <https://doi.org/10.1016/j.virol.2010.08.009>.
 43. Michta ML, Hopcraft SE, Narbus CM, Kratovac Z, Israelow B, Sourisseau M, Evans MJ. 2010. Species-specific regions of occludin required by hepatitis C virus for cell entry. *J Virol* 84:11696–11708. <https://doi.org/10.1128/JVI.01555-10>.
 44. Song H, Li J, Shi S, Yan L, Zhuang H, Li K. 2010. Thermal stability and inactivation of hepatitis C virus grown in cell culture. *Virol J* 7:40. <https://doi.org/10.1186/1743-422X-7-40>.
 45. Hopcraft SE, Evans MJ. 2015. Selection of a hepatitis C virus with altered entry factor requirements reveals a genetic interaction between the E1 glycoprotein and claudins. *Hepatology* 62:1059–1069. <https://doi.org/10.1002/hep.27815>.
 46. Krieger SE, Zeisel MB, Davis C, Thumann C, Harris HJ, Schnober EK, Mee C, Soulier E, Royer C, Lambotin M, Grunert F, Dao Thi VL, Dreux M, Cosset FL, McKeating JA, Schuster C, Baumert TF. 2010. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. *Hepatology* 51:1144–1157. <https://doi.org/10.1002/hep.23445>.
 47. Akagi T, Sasai K, Hanafusa H. 2003. Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation. *Proc Natl Acad Sci U S A* 100:13567–13572. <https://doi.org/10.1073/pnas.1834876100>.
 48. Tanaka Y, Mori Y, Tani H, Abe T, Moriishi K, Kojima H, Nagano T, Okabe T, Suzuki T, Tatsumi M, Matsuura Y. 2010. Establishment of an indicator cell system for hepatitis C virus. *Microbiol Immunol* 54:206–220. <https://doi.org/10.1111/j.1348-0421.2010.00209.x>.
 49. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. 2005. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* 102:9294–9299. <https://doi.org/10.1073/pnas.0503596102>.
 50. Fukasawa M, Nishijima M, Hanada K. 1999. Genetic evidence for ATP-dependent endoplasmic reticulum-to-Golgi apparatus trafficking of ceramide for sphingomyelin synthesis in Chinese hamster ovary cells. *J Cell Biol* 144:673–685. <https://doi.org/10.1083/jcb.144.4.673>.
 51. Catanese MT, Graziani R, von Hahn T, Moreau M, Huby T, Paonessa G, Santini C, Luzzago A, Rice CM, Cortese R, Vitelli A, Nicosia A. 2007. High-avidity monoclonal antibodies against the human scavenger class B type I receptor efficiently block hepatitis C virus infection in the presence of high-density lipoprotein. *J Virol* 81:8063–8071. <https://doi.org/10.1128/JVI.00193-07>.
 52. Weiss MD, DeMarco V, Strauss DM, Samuelson DA, Lane ME, Neu J. 1999. Glutamine synthetase: a key enzyme for intestinal epithelial differentiation? *J Parenter Enteral Nutr* 23:140–146. <https://doi.org/10.1177/0148607199023003140>.
 53. Kakutani H, Takahashi A, Kondoh M, Saito Y, Yamaura T, Sakihama T, Hamakubo T, Yagi K. 2011. A novel screening system for claudin binder using baculoviral display. *PLoS One* 6:e16611. <https://doi.org/10.1371/journal.pone.0016611>.
 54. Nakajima M, Nagase S, Iida M, Takeda S, Yamashita M, Watari A, Shirasago Y, Fukasawa M, Takeda H, Sawasaki T, Yagi K, Kondoh M. 2015. Claudin-1 binder enhances epidermal permeability in a human keratinocyte model. *J Pharmacol Exp Ther* 354:440–447. <https://doi.org/10.1124/jpet.115.225391>.
 55. Kondoh M, Masuyama A, Takahashi A, Asano N, Mizuguchi H, Koizumi N, Fujii M, Hayakawa T, Horiguchi Y, Watanabe Y. 2005. A novel strategy for the enhancement of drug absorption using a claudin modulator. *Mol Pharmacol* 67:749–756. <https://doi.org/10.1124/mol.104.008375>.
 56. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 11:791–796. <https://doi.org/10.1038/nm1268>.
 57. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R. 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci U S A* 103:7408–7413. <https://doi.org/10.1073/pnas.0504877103>.
 58. Sugiyama K, Suzuki K, Nakazawa T, Funami K, Hishiki T, Ogawa K, Saito S, Shimotohno KW, Suzuki T, Shimizu Y, Tobita R, Hijikata M, Takaku H,

- Shimotohno K. 2009. Genetic analysis of hepatitis C virus with defective genome and its infectivity in vitro. *J Virol* 83:6922–6928. <https://doi.org/10.1128/JVI.02674-08>.
59. Sugiyama K, Ebinuma H, Nakamoto N, Sakasegawa N, Murakami Y, Chu PS, Usui S, Ishibashi Y, Wakayama Y, Taniki N, Murata H, Saito Y, Fukasawa M, Saito K, Yamagishi Y, Wakita T, Takaku H, Hibi T, Saito H, Kanai T. 2014. Prominent steatosis with hypermetabolism of the cell line permissive for years of infection with hepatitis C virus. *PLoS One* 9:e94460. <https://doi.org/10.1371/journal.pone.0094460>.
60. Saito K, Shirasago Y, Suzuki T, Aizaki H, Hanada K, Wakita T, Nishijima M, Fukasawa M. 2015. Targeting cellular squalene synthase, an enzyme essential for cholesterol biosynthesis, is a potential antiviral strategy against hepatitis C virus. *J Virol* 89:2220–2232. <https://doi.org/10.1128/JVI.03385-14>.
61. Tatenos C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, Tachibana A, Soeno Y, Asahina K, Hino H, Asahara T, Yokoi T, Furukawa T, Yoshizato K. 2004. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 165:901–912. [https://doi.org/10.1016/S0002-9440\(10\)63352-4](https://doi.org/10.1016/S0002-9440(10)63352-4).