Hepatitis C Virus Cell-Cell Transmission in Hepatoma Cells in the Presence of Neutralizing Antibodies

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Hepatitis C virus (HCV) infection of Huh-7.5 hepatoma cells results in focal areas of infection where transmission is potentiated by cell-cell contact. To define route(s) of transmission, HCV was allowed to infect hepatoma cells in the presence or absence of antibodies that neutralize cell-free virus infectivity. Neutralizing antibodies (nAbs) reduced cell-free virus infectivity by >95% and had minimal effect(s) on the frequency of infected cells in the culture. To assess whether cell-cell transfer of viral infectivity occurs, HCV-infected cells were cocultured with fluorescently labeled naïve cells in the presence or absence of nAbs. Enumeration by flow cytometry demonstrated cell-cell transfer of infectivity in the presence or absence of nAbs and immunoglobulins from HCV$^+$ patients. The host cell molecule CD81 and the tight junction protein Claudin 1 (CLDN1) are critical factors defining HCV entry. Soluble CD81 and anti-CD81 abrogated cell-free infection of Huh-7.5 and partially inhibited cell-cell transfer of infection. CD81-negative HepG2 hepatoma cells were resistant to cell-free virus infection but became infected after coculturing with JFH-infected cells in the presence of nAb, confirming that CD81-independent routes of cell-cell transmission exist. Further experiments with 293T and 293T-CLDN1 targets suggested that cell-cell transmission is dependent on CLDN1 expression. *Conclusion:* These data suggest that HCV can transmit *in vitro* by at least two routes, cell-free virus infection and direct transfer between cells, with the latter offering a novel route for evading nAbs. (HEPATOLOGY 2008;47:17-24.)

Hepatitis C virus (HCV) has emerged as the major etiological agent of liver disease. Approximately 170 million individuals are infected worldwide, and the majority are at risk for developing serious progressive liver disease, with HCV being the leading indication for liver transplantation. The HCV single-stranded RNA genome encodes a single polyprotein, which is cleaved by viral and cellular proteases to produce the structural proteins; core E1 and E2 and nonstructural proteins; p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The only approved treatment for HCV infection is interferon-α in combination with ribavirin, which is toxic and only effective in 50% of individuals with genotype I infections. Clearly, there is a need for more effective therapies and for the development of prophylactic and/or therapeutic vaccines.

Cellular and humoral responses are generated during acute infection, but they are insufficient to achieve viral clearance in the majority of individuals, with approximately 60%-80% of new infections becoming persistent.[1,2] Neutralizing antibody (nAb) responses often provide the first-line adaptive defense against infection by limiting virus spread. However, little is known about the impact of the humoral immune response on HCV pathobiology. Serum antibodies (Abs) from chronically HCV-infected individuals demonstrate broadly reactive neutralizing properties *in vitro* and yet fail to control viral infection *in vivo*.[3-5] The reasons for their lack of effect are poorly understood. HCV may escape neutralization by

Abbreviations: Ab, antibody; CFSE, carboxyfluorescein diacetate succinimidyl ester; CLDN1, Claudin 1; CMFDA, 5-chloromethylfluorescein diacetate; DEN3, dengue virus type-3; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HCV, hepatitis C virus; HCVcc, cell culture–grown hepatitis C virus; HCVpp, hepatitis C virus pseudotype; HTLV-1, human T cell leukemia virus type I; IU, infectious unit; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MLVpp, murine leukemia virus pseudoparticle; nAb, neutralizing antibody; pi., post infection; sCD81, soluble CD81; SEM, standard error of the mean; SI, specific infectivity.

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conventional genetic mutation[6] or other less direct evasion strategies.

Viruses can disseminate within a host by two mechanisms: release of cell-free virions or direct passage between infected and uninfected cells. In general, direct cell-cell transfer is considered more rapid and efficient than cell-free spread because it obviates rate-limiting early steps in the virus life cycle, such as virion attachment.[7] Moreover, cell-cell transfer of viral infectivity may allow viruses to evade elements of the immune response, such as nAbs and complement. For viruses such as human T cell leukemia virus type I (HTLV-I), cell-cell infection appears to be the principal mode of dissemination both within and between hosts.[8]

Recent developments have allowed HCV to be propagated in cell cultures [cell culture–grown hepatitis C virus (HCVcc)],[9-11] allowing studies on viral transmission. HCV infection of hepatoma cells results in focal areas of infection that are potentiated by cell-cell contact, and this suggests localized transmission between adjacent cells. The ability of HCV to transmit to naïve target cells when cultured in the presence of an agarose overlay or nAbs suggests that direct cell-cell routes of HCV transmission exist. The host molecule CD81 and the tight junction protein Claudin 1 (CLDN1) are reported to be critical factors defining HCV entry.[12,13] Experiments to address the receptor dependency of cell-cell transmission suggest that CLDN1 is required but that CD81-dependent and independent routes exist. These data support cell-free and cell-cell routes of HCV transmission *in vitro* and raise questions on the mechanism of HCV spread *in vivo* and the effectiveness of prophylactic Abs and agents targeting the entry step of the life cycle.

Materials and Methods

Cells and Reagents. HeLa, HepG2, and 293T cells were obtained from the American Type Culture Collection and propagated in 10% fetal bovine serum (FBS)/Dulbecco's modified Eagle's medium (DMEM). Huh-7.5 cells were provided by Dr. Rice (Rockefeller University)[14] and propagated in 10% FBS/DMEM/1% nonessential amino acids. Abs and recombinant proteins were provided as follows: anti-NS5A 9E10[9] (Dr. Rice, Rockefeller University), anti-core JM122 (Dr. McLauchlan, Medical Research Council (MRC) Institute for Virology), anti-E2 C1 and dengue virus type-3 (DEN3) monoclonal antibodies (mAbs; Dr. Burton, Scripps Research Institute),[9] anti-CD81 M38 (Dr. Berditchevski, Birmingham University), and anti-CLDN1 JAY.8 (Zymed) and soluble CD81 (sCD81; Dr. Liu, Massachusetts Institute of Technology).[9] Immunoglobulin was purified from two HCV-infected patients (P68 and P70, genotype 1a) and a noninfected individual (NC2) by protein A chromatography (Amersham).

HCV Genesis and Infection. Retroviral pseudotypes bearing HCV glycoproteins [hepatitis C virus pseudotype (HCVpp)] were generated by transfection of plasmids encoding human immunodeficiency virus provirus expressing luciferase and E1E2 glycoproteins as previously described.[15] HCVcc strains J6/JFH[9] and JFH[10] were generated by electroporation of transcribed RNA from full-length genomes (Megascript-T7 kit, Ambion) into Huh-7.5 cells as previously described.[9] Supernatants collected 72-96 hours post electroporation were stored at −80°C.

Huh-7.5 cells were seeded at 0.75×10^4 (low density), 1.5×10^4 (standard density), or 3×10^4 (high density) cells/cm^2. After 24 hours, cells were infected for 1 hour with J6/JFH or JFH diluted in 3% FBS/DMEM at a multiplicity of infection of 0.01. Unbound virus was removed, and fresh 3% FBS/DMEM ± 1.5% Seaplaque agarose was added.[16] Infection was detected by staining for NS5A as previously described.[9] To monitor viral transmission in the presence of nAbs, anti-E2 C1 mAb was added at a concentration (10 μg/mL) able to neutralize >90% infectivity, and media were replenished with 3% FBS/DMEM containing Ab every 24 hours.[9] To quantify the infectivity of cell-free virus, extracellular media were collected and allowed to infect naïve Huh-7.5 cells. Infection was quantified by the enumeration of NS5A$^+$ cells 48 hours post infection (pi.) and was defined as the number of infected cells or infectious units per milliliter (IU/mL).

Proliferation Assay. Cells were labeled with 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE; CellTrace, Invitrogen) and seeded at the densities listed previously. Labeled cells were infected with J6/JFH or JFH and 24, 48, or 72 hours pi. were collected by trypsinization and stained for NS5A, and the doubling time for infected and noninfected cells was determined by flow cytometry.

Infectious Center Assay. Huh-7.5 cells were infected with J6/JFH or JFH at a multiplicity of infection of 0.01 and 96 hours pi. (producer cells) were mixed with naïve target cells labeled with 5μM 5-chloromethylfluorescein diacetate (CMFDA; CellTracker, Invitrogen). Producers were incubated for 15 minutes at 37°C with Abs and seeded with targets at a defined ratio (see the legends) at high density. nAbs were added at a minimum concentration 10 times greater than the defined concentration that inhibited 90% of infection (C1 and DEN3 at 10 μg/mL; NC2, P68, and P70 at 100 μg/mL). After 24 hours, extracellular media were collected and quantified for infectious virus as detailed previously. The cells were

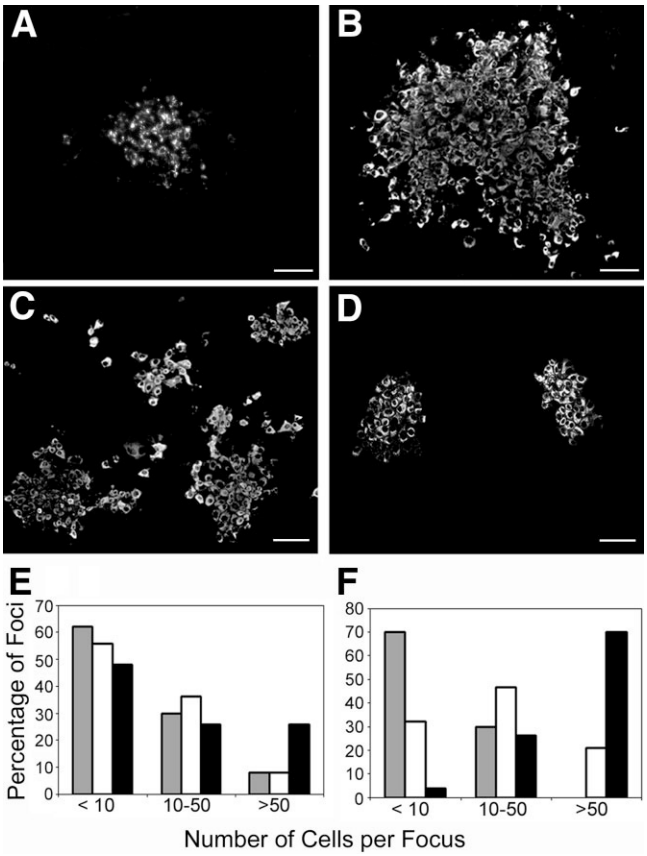

Fig. 1. Formation of HCV foci: effects of cell density and agarose overlay. Huh-7.5 cells were plated at low (gray), standard (white), or high (black) seeding densities and infected with JFH in the presence or absence of a 1.5% agarose overlay. At 72 hours pi., infected foci were visualized by the detection of (A) core or (B,C) NS5A in cultures maintained in media alone or (D) agarose overlay. The scale bar is 100 μm. The number of infected cells within a focus were enumerated and classified into 3 groups: fewer than 10, 10-50, and more than 50 cells per focus. Fifty foci were counted at each seeding density. The percentage of the size of foci at different cell seeding densities in (E) the absence of agarose and (F) the presence of agarose is shown. Cell density ($P < 0.0085$, χ^2 correction for multiple testing) and agarose significantly increase the JFH focal size ($P < 0.001$, χ^2).

permeabilized with 1% paraformaldehyde/0.1% saponin, stained for NS5A, and analyzed by flow cytometry or indirect immunofluorescence.

Results

Routes of HCV Transmission. HCV infection of the human hepatoma Huh-7.5 cell line results in focal areas of infected cells expressing virally encoded structural (core) and nonstructural (NS5A) proteins (Fig. 1), which suggest localized viral transmission. A high degree of variability in the size of infected foci was noted, ranging from 1 to >200 infected cells. Because increased contact between target cells may potentiate viral transmission, we examined the relationship between cell seeding density and focal size. Infected cells were visualized by expression of NS5A, and foci were partitioned into three groups on the basis of size: fewer than 10, 10-50, and more than 50 infected cells. The proportion of large foci increased with cell seeding density for both JFH and J6/JFH, and this suggests that a high cell density facilitates viral transmission to neighboring cells. All subsequent assays to monitor virus transmission were conducted at a high cell seeding density.

To assess the role of cell division in viral transmission, we quantified the proliferation time of naïve and JFH-infected Huh-7.5 by labeling cells with CFSE and measuring proliferation over 72 hours. Under high-density culture conditions, the mean doubling time for uninfected cells was 32 hours versus 34 hours for infected cells. It is therefore unlikely that the large foci observed after 72 hours could derive simply from the division of infected cells.

To address whether the development of foci requires cell-free virus infection of naïve cells, JFH-infected cells were cultured in a semisolid medium containing agarose, which limits the diffusion of virus particles. Inclusion of an agarose overlay did not abrogate viral transmission but resulted in compact foci reminiscent of plaques (Fig. 1D). In contrast, infection of cells cultured in media alone resulted in the development of medium to large foci surrounded by smaller foci, or satellites (Fig. 1C). Inclusion of an agarose overlay increased the proportion of large foci at a high cell density from 26% to 70% for JFH, with a concomitant decrease in small foci (<10; Fig. 1F). Parallel assays studying J6/JFH-infected cells gave comparable results (not shown). Thus, inclusion of an agarose overlay to restrict cell-free virus transmission promotes localized viral spread at high cell density, resulting in a greater proportion of large foci.

The agarose overlay method has been used by virologists for many years. However, it is difficult to assess its effectiveness in limiting cell-free virus spread. In contrast, inclusion of Abs in the extracellular media to inhibit viral infectivity can be readily quantified. To monitor the level of infectious virus released from JFH-infected cells, the number of infected cells and the infectivity of cell-free virus were quantified over a 72-hour period. Infectious virus was readily detected between 48 and 72 hours pi. However, the level of infectious virus released per infected cell was low (approximately 1.0 IU per 5 infected cells; Fig. 2B). JFH transmission in the presence or absence of extracellular nAbs was assessed by the determination of the frequency of infected cells and the infectivity of cell-free virus at 72 hours pi. A concentration of anti-E2 (C1, 10 μg/mL) capable of neutralizing JFH infectivity (Supplementary Fig. 1) or a control anti-dengue (DEN3) mAb

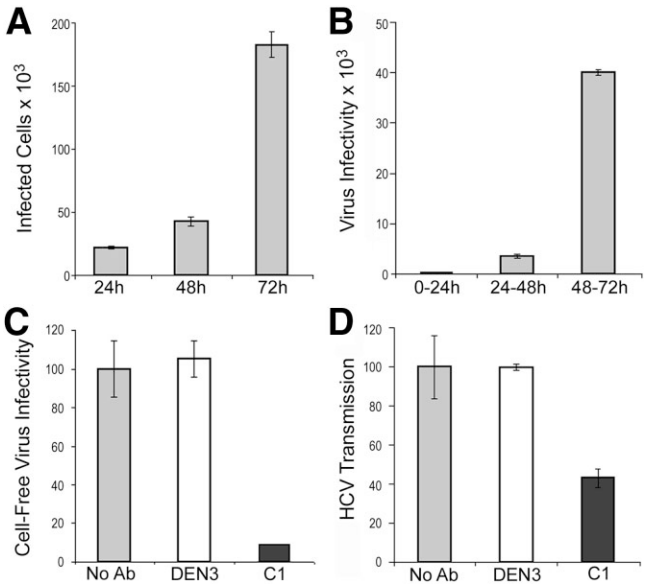

Fig. 2. HCV JFH transmission occurs in the presence of an nAb. Huh-7.5 cells were seeded at high density and infected for 1 hour at 37°C, and unbound virus was removed. (A) Cells were fixed at 24, 48, and 72 hours pi., and NS5A$^+$ cells were quantified. (B) The infectivity of cell-free virus is expressed as the number of NS5A-positive cells or infected units per milliliter. (C) The infectivity of cell-free JFH released from control Ab DEN3–treated (white) or nAb C1–treated (black) cultures is expressed as a percentage of the untreated culture (gray). (D) The relative number of JFH-infected cells at 72 hours pi. in DEN3-treated (white) or C1-treated (black) cultures is expressed as a percentage of the untreated culture (gray). Infectivity for Ab-treated cells is expressed with respect to untreated cells. Error bars represent the standard error of the mean of 4 replicates and reflect the results from 4 independent experiments.

was added to the infected cultures at 8 hours pi. and replenished every 24 hours to maintain bioactivity. C1 neutralized extracellular virus infectivity by 95% in comparison with untreated or DEN3-treated cells (Fig. 2C), yet the relative frequency of infected cells in the C1-treated culture was 43% versus 99% in the presence of DEN3 (Fig. 2D). Infected foci in the presence of nAbs exhibited fewer satellite colonies than untreated cultures, and this is consistent with the effect(s) of agarose. In summary, these results show that HCV can transmit to naïve target cells in the presence of an agarose overlay or nAb, suggesting that cell-cell routes of HCV transmission exist.

Cell-Cell HCV Transmission. To study cell-cell transmission of infectivity, we developed an assay where HCV-infected (producer) cells are cultured with fluorescently labeled naïve (target) cells in the presence of Abs that neutralize the infectivity of cell-free virus. Labeling the target cells allows one to discriminate between infection of naïve cells and division of infected producers. JFH-infected cells were seeded at high density in the presence or absence of nAbs for 15 minutes prior to the addition of labeled targets. Twenty-four hours later, the cells were assayed for NS5A expression by indirect immunofluorescence, and extracellular media were quantified for infectivity. Cell-free infectivity was reduced by >95% in the presence of Abs. Figure 3 depicts the green-labeled target cells and infected NS5A$^+$ producers indirectly labeled red. Viral transmission from a producer to a target cell results in a CMFDA-labeled green cell expressing NS5A and costaining orange. HCV transmits to target cells in the presence of C1 or purified Abs from two HCV-infected patients (P68 and P70) in a comparable fashion to untreated or control Ab–treated cultures (Fig. 3). Thus, a >95% reduction in the infectivity of cell-free virus has minimal effect(s) on the localized spread of HCV infection.

To obtain a quantitative analysis of viral transmission in the presence of nAbs, cocultured cells were stained for NS5A and analyzed by flow cytometry. The upper left quadrant shows the frequency of infected producers (red), the upper right shows the frequency of virus-infected naïve targets (orange), and the lower right shows the frequency of uninfected targets (green). JFH-infected targets were detected at frequencies between 27% and 31% of untreated or DEN3/NC2 control Ab–treated cultures (Fig. 4A). In the presence of C1, P68, or p70 nAbs (Fig. 4B), the frequency of infected target cells ranged from 18% to 24%. The mean frequency of JFH-infected tar-

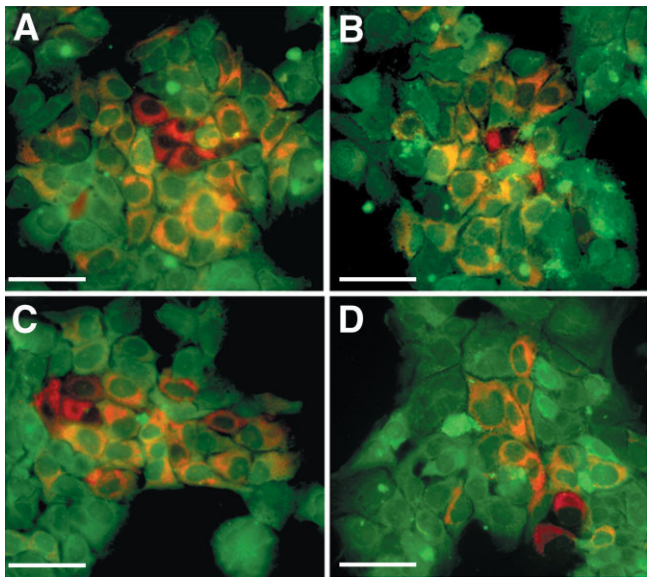

Fig. 3. HCV JFH transmits cell to cell. JFH-infected Huh-7.5 producers were incubated for 15 minutes with (A) DMEM, (B) DMEM plus 10 μg/mL DEN3, (C) DMEM plus 10 μg/mL C1, or (D) DMEM plus 100 μg/mL P70. The producers were cultured with naïve CMFDA-labeled Huh-7.5 targets (green). After 24 hours, infected cells were detected by staining for NS5A (Alexa 594, red). Newly infected target cells, positive for both NS5A and CMFDA, appear orange. The scale bar is 50 μm. The infectivity of cell-free virus in the presence of C1 or P70 was reduced by >95% in comparison with DEN3 or untreated cultures.

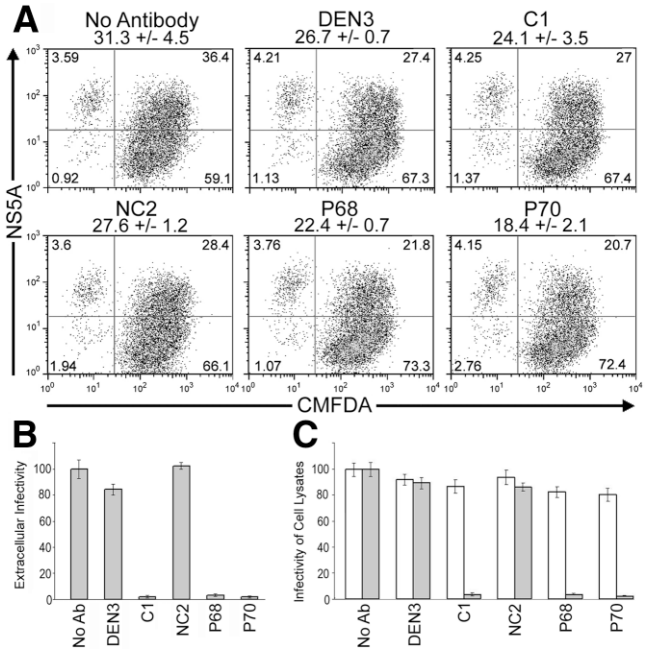

Fig. 4. Quantitation of HCV cell-cell transmission. (A) JFH-infected Huh-7.5 producers were cocultured with CMFDA-labeled targets at a ratio of 1:4 in the presence of the listed Abs. After 24 hours, the cells were fixed, stained for NS5A (R-phycoerythrin) and infected cells quantified by flow cytometry. In each panel, the lower quadrants contain uninfected cells; the upper left represents infected producers, and the upper right represents newly infected targets. The mean number of infected targets [± standard error of the mean (SEM)] from three independent experiments is shown above each panel. (B) The infectivity of cell-free JFH released from Ab-treated cultures is expressed as a percentage of the untreated culture. The mean infection of triplicate wells (±SEM) with respect to the untreated control is shown. (C) To confirm that JFH in the infected cultures was sensitive to nAbs, cell lysates were generated by three freeze/thaw cycles and used to inoculate Huh-7.5 cells in the presence (gray) or absence (white) of the appropriate nAb. The infectivity of three replicates (±SEM) was measured and expressed with respect to untreated cells.

gets from three independent experiments is annotated above each plot. In all cases, the nAbs (C1, P68, and P70) reduced infectivity of cell-free virus by >95% (Fig. 4B) with minimal effect(s) on cell-cell transfer of viral infectivity. Increasing the concentration of C1 in the extracellular media had no additional effect on the efficiency of viral transfer (Supplementary Fig. 2). To ascertain that cell-cell transmitted virus was not a variant resistant to the nAbs under testing, infected cells were collected, and intracellular virus was released by three rounds of rapid freezing and thawing and tested for infectivity in the presence and absence of the selecting Ab. In all cases, the intracellular virus remained sensitive to the neutralizing effects of the Abs (Fig. 4C), and this indicated that viral spread was not mediated by Ab-resistant variants. In summary, these data suggest that direct cell-cell transfer is an efficient route for HCV transmission between cells cultured at high density.

Receptor Dependency of HCV Cell-Cell Transmission. To ascertain the role of CD81 in cell-free and cell-cell infection, we evaluated the ability of an anti-CD81 mAb, M38 or sCD81, to inhibit HCV infection via these two routes. M38 and sCD81 inhibited >90% of cell-free infection of Huh-7.5 and reduced cell-cell transmission by 43% and 15%, respectively (Fig. 5A,B). We investigated whether the CD81-negative hepatoma cell line HepG2 could be infected via cell-free or cell-cell infection routes. HepG2 cells failed to support cell-free HCVcc infection (Fig. 5D). However, coculturing of JFH-infected producers with labeled HepG2 for 48 hours resulted in 5.5% of HepG2 becoming infected versus 0.3% for the nonpermissive Hela cell line (Fig. 5E). These data confirm that CD81-independent routes of cell-cell transmission exist.

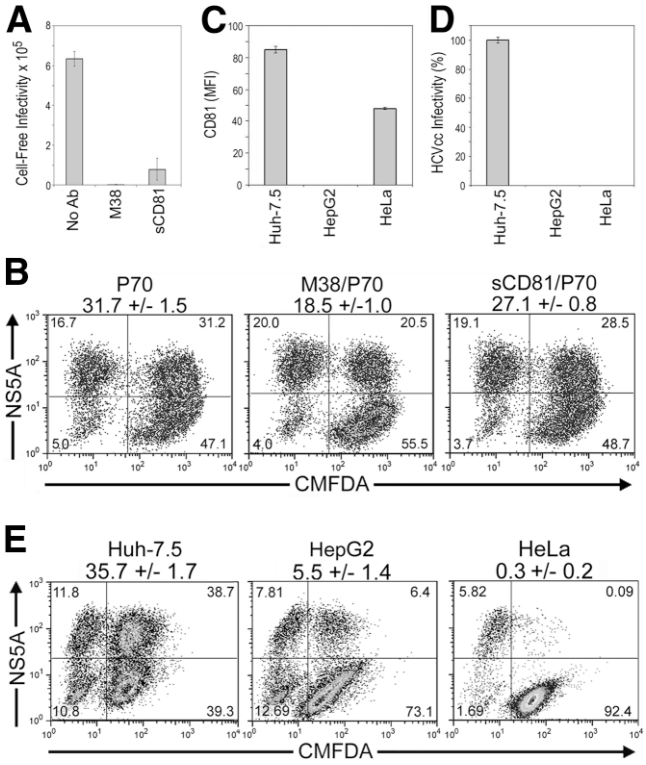

Fig. 5. The role of CD81 in HCV cell-cell transmission. Anti-CD81 M38 (10 μg/mL) and sCD81 (10 μg/mL) inhibition of (A) cell-free JFH infection, where infectivity is expressed as the number of NS5A-positive cells or infected units per milliliter, and (B) cell-cell JFH infection, where producer and targets were mixed in a 1:4 ratio for 24 hours. The mean number of infected Huh-7.5 targets [± standard error of the mean (SEM)] in the presence of p70, plus or minus M38 and sCD81, is shown and is representative of 3 independent experiments. (C) CD81 cell surface expression was monitored by flow cytometry, and the data are expressed as the mean fluorescence intensity (MFI). (D) JFH cell-free infection of targets. Infectivity is shown with respect to Huh-7.5, where the error bars represent the SEM of three replicates. (E) JFH cell-cell infection: producer and Huh-7.5, HepG2, and HeLa target cells were mixed in a 1:4 ratio for 48 hours in the presence of p70. The mean number of infected targets (±SEM) is shown and is representative of 3 independent experiments.

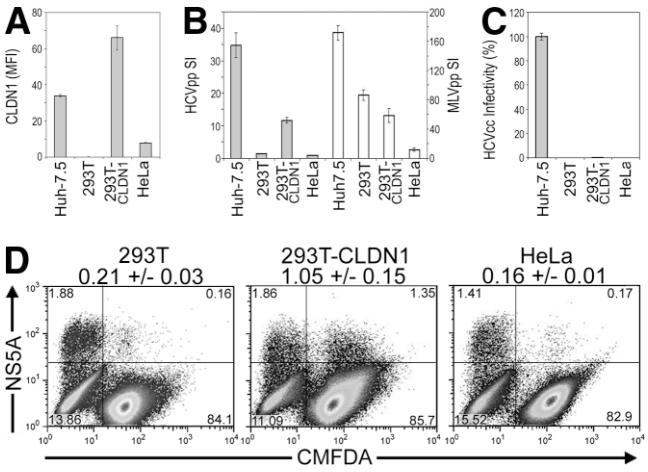

Fig. 6. The role of CLDN1 in HCV cell-cell transmission. (A) Target cell expression of CLDN1 was monitored by flow cytometry, and data are expressed as the mean fluorescence intensity (MFI). (B) HCVpp and murine leukemia virus pseudoparticle (MLVpp) infection of targets. Pseudoparticles encode a luciferase reporter, and data are presented as the specific infectivity (SI), where the HCVpp (gray) or MLVpp (white) signal with respect to a pseudoparticle lacking envelope is determined from three replicate infections [± standard error of the mean (SEM)]. (C) JFH cell-free infection of targets. Infectivity is shown with respect to Huh-7.5, and the error bars represent the SEM of three replicates. (D) JFH cell-cell infection: producer and 293T, 293T-CLDN1, and HeLa target cells were mixed in a 1:1 ratio for 48 hours in the presence of p70. The mean number of infected targets (±SEM) is shown and is representative of 3 independent experiments.

To assess the role of CLDN1 in cell-cell transmission, we studied cell-free and cell-cell infection of the human embryonal 293T kidney cell line before ($CD81^+$/$CLDN1^-$) and after transduction ($CD81^+$/$CLDN1^+$) to express CLDN1. CLDN1 expression levels were confirmed by flow cytometry and receptor activity by HCVpp and HCVcc infection (Fig. 6A-C). Expression of CLDN1 in 293T cells increased cell-cell transmission 5-fold in comparison with 293T, which gave background levels of infection comparable to that seen with Hela cells (Fig. 6D). In contrast, 293T-CLDN1 cells were poor targets for JFH cell-free virus infection (Fig. 6C). In summary, these data support a model in which cell-cell transmission is dependent on CLDN1 expression and CD81-dependent and independent routes exist.

Discussion

In this study, we demonstrate that HCV can transmit to naïve cells in the presence of agarose or Abs that limit or neutralize cell-free virus infectivity. Intracellular sources of virus remain sensitive to the neutralizing activity of Abs, and this confirms that the transmitting viruses are not resistant to the nAbs used. The frequency of infected naïve target cells in the infectious center assay was minimally affected by the inhibition of extracellular routes of virus transmission. These data suggest that HCV can transmit by at least two routes *in vitro*: cell-free virus infection of naïve targets and direct transfer between cells. The latter route offers a mechanism to evade nAbs that may partially explain the ineffectiveness of Abs in controlling HCV replication during the chronic phase of disease[3-5] in addition to more conventional genetic escape mechanisms.[6] If such routes of transmission occur *in vivo*, one may question whether therapeutic vaccination to elicit nAbs[17] or immunoprophylaxis will control persistent HCV replication.

To address the receptor dependency of HCV transmission between infected and naïve cells, we used cell lines lacking CD81 or CLDN1 expression as targets for cell-free and cell-cell transfer of infection. CD81-negative HepG2 cells failed to support cell-free HCVcc infection but were infected after coculturing with JFH-infected producers (Fig. 5). This observation, alongside the partial inhibition of JFH cell-cell transmission by sCD81 and anti-CD81 to Huh-7.5 targets, suggests that CD81-dependent and CD81-independent routes of cell-cell transmission occur. The recent discovery that the tight junction protein CLDN1 is an essential factor allowing HCV entry into cells prompted us to study its role in cell-cell transmission.[13] Expression of CLDN1 in 293T target cells conferred JFH infection via cell-cell transmission (Fig. 6), and this suggests that CLDN1 expression in the target cell is essential for cell-cell transfer of infectivity. The lower level of JFH cell-cell transmission to HepG2 and 293T-CLDN1 compared to Huh-7.5 cells most likely reflects the reduced ability of HepG2 and 293T cells to support HCV RNA replication.[13,18]

We noted considerable variability in the size of infected foci, with some producer cells failing to transmit virus to naïve target cells (Fig. 3). One explanation may reflect the heterogeneous expression of CD81 and CLDN1 in Huh-7.5 targets. An alternative explanation may be the inherent variability of Huh-7 in supporting HCV RNA replication.[19,20] Indeed, close inspection of foci in the infectious center assay allows one to track the transfer of HCV infectivity from producer to labeled targets. NS5A or core expression in the target cell is variable and does not associate with proximity to producer cells, which most likely reflects variation in the target's ability to support HCV RNA replication (Fig. 3).

Target cell density affects HCV transmission, with an increased proportion of large foci at high density (Fig. 1). HCVpp entry into Huh-7.5 cells is enhanced when cells are seeded at higher density, and this supports a model in which cell-cell contact facilitates the expression or complex formation of host cell molecules required for HCV entry (A.S., unpublished observations, 2007). It is inter-

esting to speculate on the physiological significance of these results. The principal site of HCV replication *in vivo* is thought to be hepatocytes within the liver, which form polarized sheets where the cells are at high density (2-3.0 $\times$ 10^5 hepatocytes/cm^2 of liver tissue).[21] In contrast, HCV RNA has been reported to replicate more efficiently in actively proliferating cells, demonstrating reduced replication in cells at high density.[22,23] Because the majority of hepatocytes within the liver are not proliferating and are arrested in G_0, these data suggest a delicate balance between the cell requirements that are optimal for HCV entry and transmission versus those required for efficient viral RNA replication.

Cell-cell spread of viruses in solid tissues is a complex and poorly understood process.[7] The alphaherpes viruses replicate in polarized cells, epithelial cells, and neurons and mimic intracellular sorting pathways to promote infection of adjacent cells.[24,25] Human immunodeficiency virus and HTLV-I infect T cells that form immunological synapses with cells of the immune system. Both viruses appear to use these pathways to form viral synapses to facilitate the transfer of infectivity between immune cell conjugates.[8,26]

It is noteworthy that JFH-infected Huh-7.5 cells release low levels of infectious virus (Fig. 2C) that may favor cell-cell transfer of viral infectivity *in vitro*. At the present time, we can only speculate on the mechanism(s) of HCV transmission *in vivo* both within and between hosts. Identification of HCV-infected cells within the liver has been difficult to demonstrate with conflicting reports, and this makes viral production rates difficult to ascertain.[27-29] A recent report by Gale and colleagues[30] demonstrated focal areas of infected cells within the liver that were consistent with our *in vitro* observations.[30] Plasma from HCV-infected individuals has been reported to infect chimpanzees[31] and mice bearing chimeric human livers,[32] and this suggests that transmission between hosts is most likely mediated via cell-free virus. Our data support a model in which HCV may transmit within the liver by multiple routes, including cell-free virus and direct cell-cell transfer, with the latter offering a novel route for evading nAb responses. These data may partly explain the inability of nAbs to control HCV replication during the chronic phase of disease and raise concerns over the effectiveness of therapeutic vaccination targeting the humoral immune response and of antiviral agents targeting CD81-dependent routes of infection.

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