# Hepatitis C virus depends on E-cadherin as an entry factor and regulates its expression in epithelial-to-mesenchymal transition

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**Hepatitis C virus (HCV) enters the host cell through interactions with a cascade of cellular factors. Although significant progress has been made in understanding HCV entry, the precise mechanisms by which HCV exploits the receptor complex and host machinery to enter the cell remain unclear. This intricate process of viral entry likely depends on additional yet-to-be-defined cellular molecules. Recently, by applying integrative functional genomics approaches, we identified and interrogated distinct sets of host dependencies in the complete HCV life cycle. Viral entry assays using HCV pseudoparticles (HCVpps) of various genotypes uncovered multiple previously unappreciated host factors, including E-cadherin, that mediate HCV entry. E-cadherin silencing significantly inhibited HCV infection in Huh7.5.1 cells, HepG2/miR122/CD81 cells, and primary human hepatocytes at a postbinding entry step. Knockdown of E-cadherin, however, had no effect on HCV RNA replication or internal ribosomal entry site (IRES)-mediated translation. In addition, an E-cadherin monoclonal antibody effectively blocked HCV entry and infection in hepatocytes. Mechanistic studies demonstrated that E-cadherin is closely associated with claudin-1 (CLDN1) and occludin (OCLN) on the cell membrane. Depletion of E-cadherin drastically diminished the cell-surface distribution of these two tight junction proteins in various hepatic cell lines, indicating that E-cadherin plays an important regulatory role in CLDN1/OCLN localization on the cell surface. Furthermore, loss of E-cadherin expression in hepatocytes is associated with HCV-induced epithelial-to-mesenchymal transition (EMT), providing an important link between HCV infection and liver cancer. Our data indicate that a dynamic interplay among E-cadherin, tight junctions, and EMT exists and mediates an important function in HCV entry.**

hepatitis C virus | viral entry | E-cadherin | tight junction | epithelial–mesenchymal transition

Hepatitis C virus (HCV), a member of the *Hepacivirus* genus in the Flaviviridae family, is an enveloped, single-stranded and positive-sense RNA virus that infects humans and other higher primates, with a selective tropism to the liver. The virus is estimated to infect 2.8% of the world's population (1), and has evolved into a major causative agent of end-stage liver diseases, including cirrhosis and hepatocellular carcinoma (HCC) (2). Chronic hepatitis C is also the leading indication for liver transplantation in the United States (3). To date, a protective vaccine is not available. Current therapeutic regimens applying direct-acting antivirals with or without ribavirin have made it possible to cure the majority of patients with HCV (4).

HCV infection gains chronicity in ∼75–85% of patients, facilitated by various viral mechanisms to evade host immune responses and exploit the cellular machinery (5). The replication cycle of HCV in the host cell consists of multiple sequential steps, beginning with the lipo-viro-particle binding and entry, followed by viral RNA translation and replication, packaging and assembly of the virion, and finally secretion from host cells (6, 7). Each of these steps relies on extensive interactions with cellular factors and molecular pathways (8, 9). Identification and characterization of these HCV host dependencies may provide not only critical insights into mechanisms of HCV-induced disease, but also potential intervention and prophylactic targets.

HCV entry plays a central role in cell tropism and species specificity. The highly coordinated entry process involves a variety of cellular molecules—termed HCV entry factors—including the tetraspanin CD81 (10), scavenger receptor class B type I (SR-BI) (11), the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) (12, 13), the receptor tyrosine kinases EGFR and ephrin receptor A2 (14), the cholesterol transporter Niemann–Pick C1-like 1 (15), and the iron-uptake receptor transferrin receptor 1 (16). These cell-surface molecules have been shown to interact with viral proteins or particles to facilitate HCV entry.

Although great advances have been achieved in elucidating the HCV entry pathway, the precise mechanisms by which HCV exploits the aforementioned cellular signals and gains entry to the host cell remain unclear. Moreover, the highly complex and dynamic entry process most likely requires additional yet-to-be-defined molecules, interacting simultaneously or in sequence, to bind, endocytose, and internalize the virus. Recently, to interrogate global HCV–host interactions in the entire viral life cycle, we conducted an unbiased, genome-wide siRNA screen, followed by targeted screens applying integrative functional genomics and systems virology approaches (8, 17). Five previously unappreciated HCV host dependencies were identified as putative viral entry

## Significance

**Hepatitis C virus (HCV) infects hepatocytes via an intricate series of interactions with the host cell machinery. Recently, we identified E-cadherin as a host dependency factor mediating HCV entry through integrative functional genomics studies. E-cadherin silencing restricted HCV entry and infection in hepatocytes. Mechanistic studies demonstrated that E-cadherin is a prerequisite for the cell-surface localization of claudin-1 and OCLN, two major HCV coreceptors. Moreover, HCV-induced loss of E-cadherin is associated with cancer-related cellular changes. Our study suggests that a dynamic interplay among E-cadherin, tight junction coreceptors, and epithelial-to-mesenchymal transition exists and plays an important role in regulating HCV entry. E-cadherin thereby represents a missing host factor in the comprehensive understanding of the molecular mechanisms and cellular regulatory events underlying HCV entry and pathogenesis.**

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factors, and these include CDH1 (E-cadherin), a major adherens junction protein. In this study, we investigated the precise role and mechanism of action of E-cadherin in modulating HCV infection. We demonstrated that E-cadherin expression is specifically required for HCV entry. Loss of E-cadherin in hepatocytes resulted in aberrant cell surface distribution of the tight junction proteins CLDN1 and OCLN—two well-characterized HCV entry factors. Loss of E-cadherin is also a hallmark of HCV-induced epithelial-to-mesenchymal transition (EMT), a cellular mechanism that presumably restricts HCV entry to inhibit viral superinfection and mediates the progression of HCV-induced liver diseases.

## Results

**E-Cadherin Is Required for HCV Infection.** To validate the function of E-cadherin in modulating HCV infection, we performed various virologic assays. Huh7.5.1 cells were treated with E-cadherin siRNA before infection with HCV. Depletion of E-cadherin expression by siRNA drastically inhibited HCV core protein production (Fig. 1*A*). E-cadherin silencing, like silencing of CLDN1, a previously known HCV entry factor (12), significantly reduced HCV RNA levels in cells and in the medium (Fig. 1*B*). E-cadherin or CLDN1 mRNA and protein levels were substantially repressed by SMARTpool siRNA treatment without inducing apparent cytotoxicity (Fig. 1 *C* and *D* and Fig. S1*A*). The phenotype-specific role of E-cadherin in HCV infection was confirmed by testing four individual siRNAs targeting various regions of the E-cadherin sequence. All four E-cadherin siRNAs inhibited HCV infection to varying extents, which correlated with the knockdown efficiencies at the mRNA and protein levels (Fig. S1 *B–D*). As expected, treatment of cells with four CLDN1 individual siRNAs of the SMARTpool blocked HCV infection in proportion to their silencing potencies (Fig. S1 *E–G*). In primary human hepatocytes (PHHs), E-cadherin or CLDN1 knockdown resulted in marked inhibition of HCV infection (Fig. 1 *E* and *F*). The proviral effect of E-cadherin is pan-genotypic. In Huh7.5.1 cells, depletion of E-cadherin significantly inhibited the infection of all HCV major genotypes (Fig. 1 *G* and *H*).

**E-Cadherin Specifically Modulates HCV Entry at a Postbinding Step.** We next examined the steps of the HCV life cycle that are affected by E-cadherin expression. Viral entry assays were conducted by using HCV pseudoparticles (HCVpps) of various genotypes and

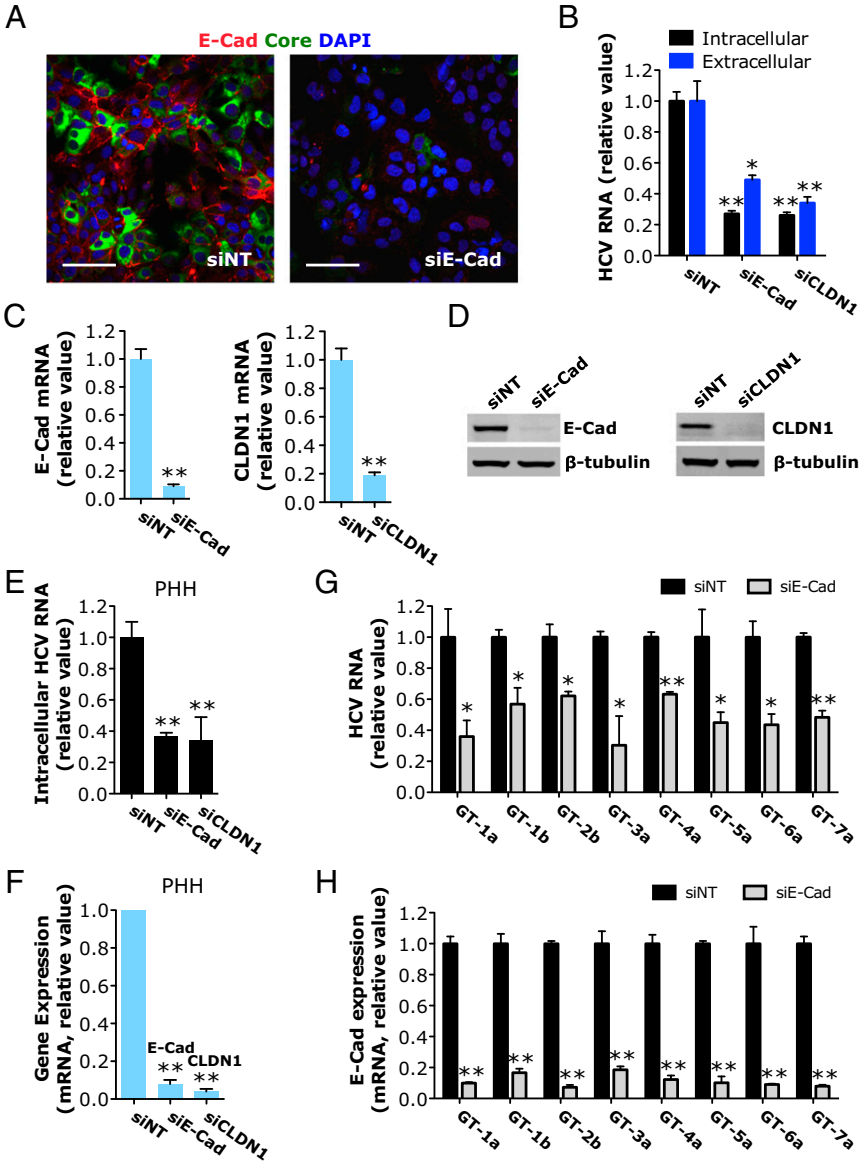


**Fig. 1.** HCV infection is dependent on E-cadherin (E-Cad) expression. (*A*) Confocal microscopic imaging of CDH1 (red) and HCV core (green) expression in the nontargeting control siRNA (siNT)- or siE-cadherin–treated Huh7.5.1 cells 48 h after HCV infection. (Scale bars: 50 μm.) (*B*) Effects of siRNA-mediated E-cadherin or CLDN1 silencing on HCV RNA levels in Huh7.5.1 cells or the culture supernatant. (*C* and *D*) Knockdown efficiency of E-cadherin or CLDN1 siRNA. E-cadherin and CLDN1 mRNA (*C*) or protein (*D*) levels were determined by quantitative RT-PCR (Q-PCR) or Western blot, respectively. β-Tubulin served as a loading control for Western blot. (*E* and *F*) Efficacy of E-cadherin or CLDN1 siRNA in restricting HCV infection (*E*) or silencing target gene (*F*) in PHHs. (*G* and *H*) E-cadherin knockdown inhibits HCV infection of different genotypes. (*G*) Quantification of intracellular HCV RNA levels in siNT- or siE-cadherin–treated Huh7.5.1 cells infected with HCV of various genotypes. (*H*) Knockdown efficiency of E-cadherin siRNA in the aforementioned cells. (*B–H*) All values were normalized to siNT (as 1) and represent the mean ± SD ($n$ = 3). Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$).

other pseudoviruses including VSV-Gpp, a pseudotyped virus bearing the vesicular stomatitis virus glycoprotein, and the MLV pseudoparticle (MLVpp) in Huh7.5.1 cells. Silencing of E-cadherin by siRNA considerably blocked the entry of HCVpps but not that of VSV-Gpps or MLVpps (Fig. 2*A*), suggesting that E-cadherin expression is uniquely required for HCV entry. Similarly, in HepG2/miR122/CD81 cells (18), E-cadherin depletion significantly inhibited HCVpp infection, but did not exert noticeable effects on VSV-Gpp or MLVpp infection (Fig. 2*B*), confirming the specificity of E-cadherin in modulating HCV entry. To examine whether E-cadherin is involved in the initial attachment or downstream steps in the viral entry process, we conducted an HCV infectious cell culture system (HCVcc) binding assay. SiRNA-mediated knockdown of E-cadherin had no effect on HCVcc binding at 4 °C (Fig. S2*A*). In contrast, silencing of SDC1, a previously reported host-dependency factor that mediates HCV attachment (19), significantly inhibited HCV binding (Fig. S2*A*). These data indicate that E-cadherin acts on a postbinding step during HCV entry. Next, we performed transferrin and LDL-uptake assays and demonstrated that E-cadherin is not involved in the much later-stage clathrin-mediated endocytosis of HCV (Fig. S2 *B* and *C*). E-cadherin knockdown by siRNA nevertheless decreased the infection of single-round infectious HCV (HCVsc) in the single cycle infection assay that represents a single-round packaged replicon transduction assay (20, 21), reinforcing the importance of this gene at an early step of the HCV life cycle (Fig. 2*C*). Silencing of E-cadherin, however, had no effect on HCV RNA replication or internal ribosomal entry site (IRES)-mediated translation, as shown by HCV replicon or IRES assays (Fig. 2*D*). In addition, the anti–E-cadherin mAb blocked the infection of HCVpp and HCVcc in a dose-dependent manner (Fig. 2*E*).

**E-Cadherin Regulates CLDN1 and OCLN Cell Surface Distribution.** To explore the mechanism of E-cadherin–mediated HCV entry, we first showed that E-cadherin depletion in Huh7.5.1 cells did not alter the mRNA or protein levels of four major HCV entry factors—CLDN1, OCLN, CD81, and SR-BI (Fig. S3 *A* and *B*)—suggesting that the function of E-cadherin in modulating HCV entry is not directly related to affecting the expression levels of these known entry factors. By confocal microscopy, E-cadherin appeared to be closely associated with CLDN1 and OCLN on the cell membrane (Fig. 3*A*). Strikingly, silencing of E-cadherin in these cells drastically reduced distribution of CLDN1 and OCLN on the cell surface (Fig. 3*A*). In contrast, localization of zonula occludens 1 (ZO-1)—another tight junction protein—and CD81 and SR-BI remained unchanged upon E-cadherin siRNA treatment (Fig. 3*A* and Fig. S4*A*). This effect on CLDN1 localization appeared to be E-cadherin–specific, as knocking down other known (CD81) or recently defined entry factors (CHKA, CYBA, SMAD6, and RAC1) (8) did not decrease CLDN1 cell membrane expression (Fig. S4*B*). Similar E-cadherin–dependent CLDN1/OCLN cell membrane distribution was observed in HepG2/miR122/CD81 cells (Fig. 3*B*), further confirming that E-cadherin plays an important regulatory role in CLDN1/OCLN localization on the cell surface.

The requirement of E-cadherin expression for CLDN1 cell surface distribution occurs only in the context of cell-to-cell contact, as the subcellular localization of CLDN1 is not affected by E-cadherin silencing in sparsely grown cells (Fig. S4*C*). In addition, treating Huh7.5.1 cells with anti–E-cadherin monoclonal antibody did not cause any apparent reduction in CLDN1 cell membrane distribution (Fig. S4*D*), implying that the anti–E-cadherin antibody interferes with the subsequent function of the CDH1–CLDN1–OCLN complex in HCV entry.

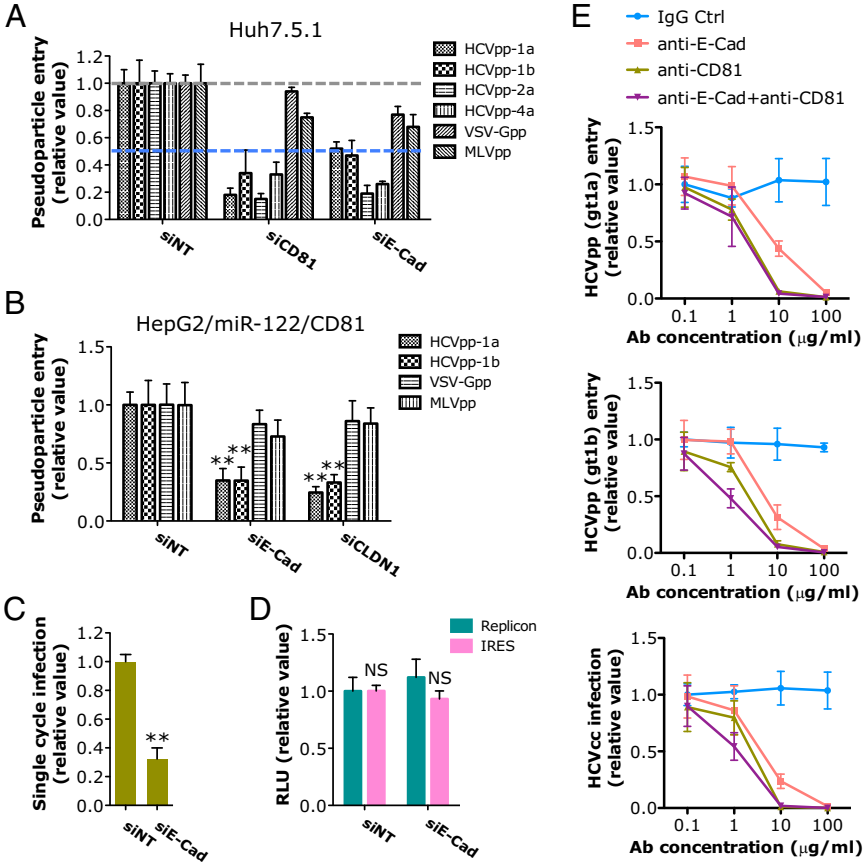


**Fig. 2.** E-cadherin (E-Cad) is specifically required for HCV entry. (*A* and *B*) The effects of siRNA-mediated gene silencing on infection of firefly luciferase-encoded pseudotyped viruses bearing HCV of various genotypes, VSV or MLV envelopes in Huh7.5.1 cells (*A*) or HepG2/miR-122/CD81 cells (*B*). The previously known HCV entry factors CD81 (*A*) and CLDN1 (*B*) were used as positive controls. (*C*) Effect of E-cadherin knockdown on HCVsc infection. (*D*) HCV replicon and IRES assays of Huh7.5.1 cells transfected with nontargeting control or E-cadherin siRNA. (*A–D*) All values, based on luciferase readings, were normalized to siNT (as 1) and represent the mean ± SD ($n = 5$). Asterisks indicate statistically significant differences (**$P < 0.01$); NS, not significant. (*E*) Effects of anti–E-cadherin blocking antibody on HCV infection. Huh7.5.1 cells were preincubated with increasing concentrations of anti–E-cadherin, anti-CD81, or isotype IgG control (Ctrl) mAbs for 2 h at 37 °C before infection with HCVpp-1a (*Upper*), HCVpp-1b (*Middle*), or HCVcc (HCV P7-Luc; *Lower*). After 48 h, viral infection was determined by measuring the luciferase activities. Results are shown as relative values compared with IgG control administered at 0.1 μg/mL (as 1) and represent the mean ± SD ($n = 5$).

CLDN1 and OCLN are involved in the postbinding step of HCV entry, although the mechanism governing this process remains elusive (12, 13, 22). As expected, silencing of CLDN1 by siRNA significantly inhibited HCV infection in Huh7.5.1 cells and PHHs (Fig. 1 *B*–*F* and Fig. S1 *E*–*G*). Whereas silencing of E-cadherin disrupted the localization of CLDN1, silencing of CLDN1, on the contrary, had no effect on the subcellular localization or overall expression level of E-cadherin (Fig. 3*C* and Fig. S3*C*).

**HCV Infection Represses E-Cadherin Expression and Induces EMT.** Aberrant expression of E-cadherin is considered to be a fundamental event in EMT, a process by which epithelial cells lose their cell polarity and adhesions junctions, thereby acquiring more migratory and invasive mesenchymal properties (23). EMT constitutes an important mechanism in wound healing, organ fibrosis, and the initiation of cancer metastasis. To study the effect of HCV infection on E-cadherin expression and EMT, we showed that, in HCV-infected cells positive for core or NS5A, the expression and cell-surface distribution of E-cadherin were considerably diminished (Fig. 4*A* and Fig. S5). As shown by Western blot, E-cadherin expression was also noticeably reduced as the HCV infection progressed (Fig. 4*B*). We then investigated whether the down-regulation of E-cadherin by HCV is indeed associated with EMT in Huh7.5.1 cells. We showed that the expression level of vimentin (VIM), a major mesenchymal marker, was markedly induced after HCV infection, whereas E-cadherin, the epithelial marker, was down-regulated in HCV-infected cells (Fig. 4*C*).

TGF-β signaling is an important and well-recognized cellular inducer of EMT. Interestingly, HCV core and envelope proteins have been shown to trigger EMT via activation of TGF-β (24). We demonstrated that TGF-β treatment of Huh7.5.1 cells resulted in the loss of E-Cad, CLDN1, and OCLN and subsequent induction of EMT, as evidenced by elevated expression of the mesenchymal markers VIM, fibronectin (FN1), and *N*-cadherin in these cells (Fig. 4 *D* and *E* and Fig. S6*A*). These effects of TGF-β can be efficiently blocked by adding SB 431542, a bona fide TGF-β inhibitor (Fig. S6 *B* and *C*). Interestingly, when cells were treated with SB 431542, HCV-triggered loss of E-cadherin and induction of EMT was considerably abrogated (Fig. S6 *D* and *E*), suggesting that the function of HCV in regulating E-cadherin expression and inducing EMT relies, at least partially, on TGF-β activation. The induction of TGF-β by HCV has been previously reported (25). In addition, treatment of cells with E-cadherin blocking antibody had no effect on VIM and FN1 expression (Fig. S7), indicating that the inhibitory effect of the anti–E-cadherin mAb on HCV entry is mediated by another mechanism distinct from EMT induction.

## Discussion

HCV infects hepatocytes through a highly orchestrated cascade of cellular events that engage multiple cellular cofactors: the viral

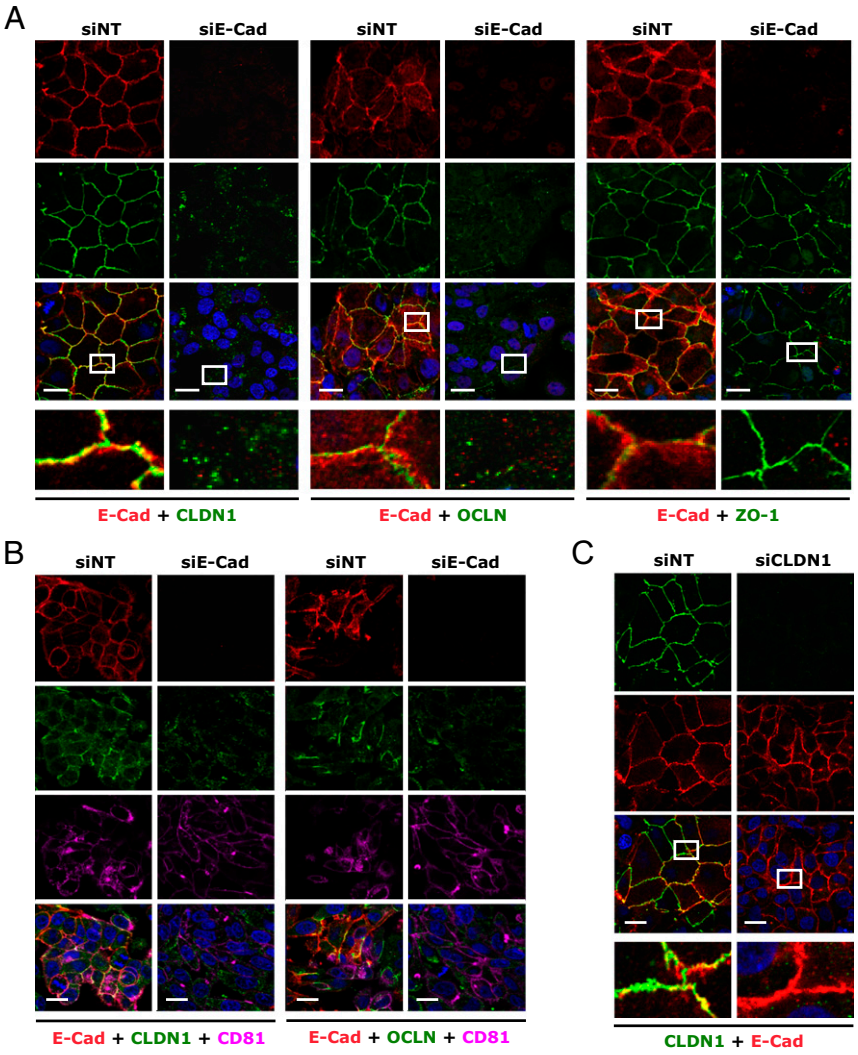


**Fig. 3.** E-cadherin (E-Cad) is required for proper CLDN1 and OCLN cell-surface localization. (*A*) Colocalization analyses of E-cadherin (red) with CLDN1, OCLN, or ZO-1 (green) in Huh7.5.1 cells transfected with siNT or E-cadherin siRNA. Immunostaining and subsequent confocal microscopic imaging were performed at 72 h after siRNA treatment. (*B*) Effect of E-cadherin silencing on cell membrane distribution of CLDN1 and OCLN in HepG2/miR-122/CD81 cells. Red, E-cadherin; green, CLDN1 or OCLN; magenta, CD81. (*C*) Effect of CLDN1 silencing on E-cadherin expression and subcellular localization. Green, CLDN1; red, E-cadherin. (*A* and *C*) Magnified view (white box) of each merged image is shown at the bottom. (Scale bars: 20 μm.)

entry factors. Our recent effort in pursuing novel HCV host dependencies by using functional genomics and systems biology approaches identified E-cadherin as a previously unappreciated host factor for HCV. Subsequent confirmatory assays demonstrated that E-cadherin is specifically required for the entry of HCV and targets a postbinding step of the viral entry process. The dependence of HCV infection on E-cadherin expression was observed in multiple hepatic cell lines, including Huh7.5.1, HepG2, and PHHs, cells either lacking or presenting polarity. Therefore, it is unlikely that changes in polarity would influence the mode of action of E-cadherin. Mechanistic studies suggested that E-cadherin expression regulates the cell-surface distribution of two main tight junction proteins and HCV coreceptors, CLDN1 and OCLN. In addition, HCV infection is able to down-regulate the expression of E-cadherin and induce EMT. This study represents an important step forward in understanding the molecular mechanisms and cellular regulatory events in the process of HCV entry.

E-cadherin, a type I cadherin and a calcium-dependent cell–cell adhesion glycoprotein expressed in the epithelium, constitutes the core component of adherens junctions that are localized at the basolateral surfaces of polarized epithelia to establish cell polarity, enhance intercellular adhesion, and consequently confer and maintain tissue integrity (26). E-cadherin is composed of an extracellular domain that mediates pathogen adhesion to host cells, a transmembrane domain, and a highly conserved cytoplasmic domain that interacts with catenin to form cadherin–catenin complexes that engage actin, microtubules, and endocytic machinery for pathogen internalization (27). Loss of E-cadherin function or its aberrant expression has been shown to disrupt cell adhesion, resulting in enhanced cellular motility and EMT, thus contributing to the metastatic potential of cancerous cells (27). A large number of pathogens, from viruses (herpes simplex viruses) to bacteria (*Listeria monocytogenes*), have developed numerous strategies to specifically target cell adhesion molecules for binding and invading host cells or disrupting epithelial integrity for dissemination (28).

We show here that E-cadherin is crucial for HCV entry, although the mechanism is unrelated to virus binding. Silencing of E-cadherin in hepatocytes disrupts the cell surface distribution of CLDN1 and OCLN, two constituent elements of the tight junctions that are essential for HCV entry (12, 13), without affecting the distribution of another tight junction protein, ZO-1. Cellular defenses can target invading viruses in the tight junction region. In hepatocytes, the tight junction protein IFITM1 interacts with CD81 and OCLN and impedes HCV entry (29). Pathogens, like HCV, preferentially take advantage of tight junctions to invade host cells and spread (12). Here we show that localization of CLDN1 and OCLN to the cell surface is dependent on E-cadherin expression. Interestingly, in E-cadherin–KO mice, tight junctions in the neonatal uterus were disrupted, leading to the loss of epithelial cell–cell interaction (30). We therefore propose that E-cadherin exerts a regulatory role in CLDN1 and OCLN localization, as well as tight junction integrity, in hepatocytes to modulate HCV entry.

HCV-infected cells have been shown to exhibit loss of E-cadherin and induction of EMT (31–33). The mechanisms behind these HCV-triggered effects are not fully known. It has been shown that HCV core up-regulates DNMT1 and 3b expression and thus induces hypermethylation of the E-cadherin gene, leading to its

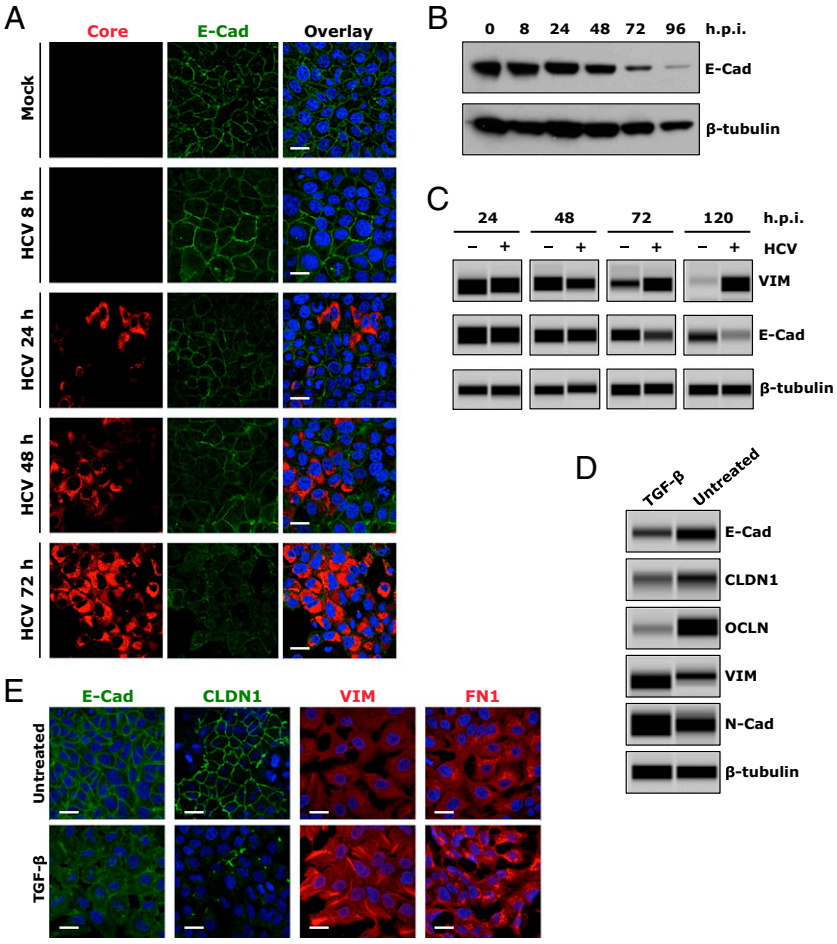


**Fig. 4.** Induction of EMT by HCV infection or TGF-β treatment. (*A* and *B*) Effect of HCV infection on E-cadherin (E-cad) expression in Huh7.5.1 cells at various time points postinfection, examined by immunofluorescence/confocal microscopy (*A*) or Western blot (*B*). (*A*) Levels of HCV infection were determined by core staining (red). (*C*) Effect of HCV infection on expression of VIM, a cellular EMT marker. Cells were mock-infected or infected with HCV, and harvested at various indicated time points, before being examined for E-cadherin or VIM expression by Western blot. (*D* and *E*) TGF-β treatment induces EMT in Huh7.5.1 cells. Cells were incubated in the absence (untreated) or presence of TGF-β (0.1 ng/mL) for 24 h, and expression levels of E-cadherin, CLDN1, OCLN, and multiple EMT markers were determined by Western blot (*E*). E-cadherin, CLDN1, VIM, and FN1 expression levels were also determined by immunofluorescence and confocal microscopy (*E*). β-Tubulin was used as a loading control in *B*–*D*. (Scale bars: 20 μm.)

repression (34, 35). Many other transcription factors such as Snail1, Slug, ZEB1, and Twist may also suppress E-cadherin directly or indirectly (36). It is worth exploring whether HCV exploits these EMT-related transcription factors to down-regulate E-cadherin expression and induce EMT in hepatocytes. In light of the inhibitory effect of E-cadherin depletion on HCV entry, the induction of EMT by HCV may be a mechanism used by the virus to limit the deleterious effect of superinfection of already infected cells (37, 38).

Chronic HCV infection is an important risk factor for the development of HCC (39). However, the mechanisms underlying HCV-induced hepatocarcinogenesis have yet to be defined. Loss or aberrant expression of E-cadherin has been implicated in a number of human malignancies, including HCC (40). Evidence has also suggested that EMT is an important mechanism for HCC metastasis (41). HCV may use distinct mechanisms for induction of EMT and subsequent hepatic carcinogenesis. The identification of cellular or viral factors that initiate, modulate, or sustain EMT signatures in HCV-triggered liver malignancies may provide valuable targets and strategies to prevent or treat HCV-associated HCC.

## Materials and Methods

HCV life-cycle assays were conducted applying various viral pseudoparticles (for entry), subgenomic replicons (for IRES-mediated translation and RNA replication), HCVsc (for single-cycle infection), and HCVcc (for multiple stages) in Huh7.5.1 cells. siRNA treatment (at 50 nM final concentration) was typically allowed for 72 h to achieve maximum knockdown. Blocking antibodies at various concentrations were applied for 2 h before infection with HCVpp or HCVcc, and were present continuously during the infection. Additional information regarding study materials and methods is provided in *SI Materials and Methods*.

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