Infection of Hepatocytes with HCV Increases Cell Surface Levels of Heparan Sulfate Proteoglycans, Uptake of Cholesterol and Lipoprotein, and Virus Entry by Upregulating SMAD6 and SMAD7

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Abstract

BACKGROUND & AIMS—The signaling molecule and transcriptional regulator SMAD6, which inhibits the transforming growth factor beta (TGFB) signaling pathway, is required for infection of hepatocytes by hepatitis C virus (HCV). We investigated the mechanisms by which SMAD6, and another inhibitory SMAD (SMAD7), promote HCV infection in human hepatoma cells and hepatocytes.

METHODS—We infected Huh7 and Huh7.5.1 cells and primary human hepatocytes with JFH1 HCVcc; we measured HCV binding, intracellular levels of HCV RNA, and expression of target genes. HCV entry in HepG2/miR122/CD81 cells, which support entry and replication of HCV, were transfected with small-interfering (si)RNAs and gene expression profiles were analyzed. Uptake of labeled low-density lipoprotein (LDL) and cholesterol were measured. Cell surface proteins were quantified by flow cytometry. We obtained liver biopsy samples from 69 patients with chronic HCV infection and 19 uninfected individuals (controls) and measured levels of syndecan 1 (*SDC1*), *SMAD7*, and *SMAD6* mRNAs.

RESULTS—siRNA knockdown of SMAD6 blocked the binding and infection of cell lines and primary human hepatocytes by HCV, whereas SMAD6 overexpression increased infection by HCV. We found levels of mRNAs encoding heparan sulfate proteoglycans (HSPGs), particularly *SDC1* mRNA, and cell surface levels of heparan sulfate to be reduced in cells following SMAD6 knockdown. SMAD6 knockdown also reduced transcription of gene encoding lipoprotein and

cholesterol uptake receptors, including the LDL receptor (LDLR), the very LDLR (VLDLR), and the scavenger receptor class B member 1 (SCARB1 or SR-BI) in hepatocytes; SMAD6 knockdown also reduced cell uptake of cholesterol and lipoprotein. Overexpression of SMAD6 increased expression of these genes. Similar effects were observed with knockdown and overexpression of SMAD7. HCV infection of cells increased expression of SMAD6, which required the activity of nuclear factor-kB (NF-kB), but not TGFB. Liver tissues from patients with chronic HCV infection had significantly higher levels of SMAD6, SMAD7, and HSPG mRNAs than controls.

Conclusions—In studies of hepatoma cell lines and human primary hepatocytes, we found that infection with HCV leads to activation of NF-κB, leading to increased expression of SMAD6 and SMAD7. Upregulation of SMAD6 and SMAD7 lead to increased expression of HSPGs, such as SDC1, as well as LDLR, VLDLR, and SR-BI, which promote HCV entry and propagation, as well as cell uptake of cholesterol and lipoprotein.

Keywords

I-SMAD; viral entry; BMP; CTBP1

Hepatitis C virus (HCV) is an etiologic agent of chronic hepatitis that can progress to end-stage live disease including fibrosis, cirrhosis and hepatocellular carcinoma[1]. Approximately 3% of the world population are estimated to be infected with HCV, and among them around 170 million people are chronically infected, leading to about 350,000 death each year globally[2]. Current therapeutic regimens consist of peginterferon, ribavirin, and recently approved direct-acting antivirals (DAAs) that are highly effective for most chronic hepatitis C patients[3]. HCV extensively interacts with host factors throughout the viral life cycle and thus trigger various pathological processes in hepatocytes[4, 5]. As such, interrogating these host dependencies of HCV may reveal not only novel targets for development of host-derived antivirals, but also cellular mechanisms underlying HCV-mediated liver disease.

HCV infects hepatocytes through a highly coordinated process that involves complex interactions of its envelope proteins (E1/E2) with cellular co-receptors and a number of other entry factors. The entry pathway consists of multiple sequential steps, starting from viral attachment or binding, to receptor-dependent intake followed by clathrin-mediated endocytosis, and finally fusion in early endosomes[6]. HCV attachment occurs when apolipoproteins present on lipoviral particles (LVPs) bind to heparan sulfate proteoglycans (HSPGs) expressed on cell surface[7]. Concurrently, low-density lipoprotein receptor (LDLR)[8] and scavenger receptor class B1 (SR-BI)[9] also interact with HCV LVPs to initiate HCV entry. SR-BI can also bind to HDL, VLDL and the oxidized forms of LDL to promote cholesterol uptake in hepatocytes[10]. In addition, SR-BI possesses cholesterol-transfer activity that may help unmask virus particles from their associated lipoproteins during the entry process[11].

Through systems biology and integrative functional genomics approaches, we recently identified SMAD6, among a number of other cellular factors, as novel HCV host dependencies[12]. SMAD6 belongs to the inhibitory SMAD (I-SMAD) family that includes SMAD7, both of which negatively regulate TGF-β signaling pathway through feedback loop

effects[13]. Here we explore the intrinsic roles and mechanisms of these I-SMADs in modulating HCV infection. We showed that both I-SMADs are requisite for HCV entry, acting mainly on the viral attachment step *via* novel transcriptional mechanisms that regulate both cell surface HSPG expression and cholesterol uptake. HCV infection, on the other hand, co-opts this proviral pathway for its own advantage through up-regulation of I-SMADs and their downstream signaling pathways.

Materials and Methods

HCV Infection

Huh7 or Huh7.5.1 cells were infected with JFH1 HCVcc at a multiplicity of infection (MOI) of 0.5 and passaged every three days after infection. Intracellular HCV RNA and target gene mRNA levels were measured every day until 7 days post-infection. Primary human hepatocytes were infected at a MOI of 1 for 7 days before harvested for various assays.

HCV Entry and Binding Assays

Huh7.5.1 or HepG2/miR122/CD81 cells were transfected with siRNAs for 72h and then infected with HCVpp, VSV-Gpp or MLVpp for 48h. Firefly luciferase activity, reflecting the entry level of the pseudoparticles, was measured using the Luciferase Assay System (Promega) according the manufacture's protocols. For HCV binding assay, Huh7.5.1 cells were treated with various siRNAs for 72h, and subsequently infected with JFH1 HCVcc at 4°C for 2 h. Afterwards, unbound virus was removed by washing cells extensively with cold PBS, and virus bound to cells were determined by quantifying HCV RNA levels by RT-qPCR.

Flow Cytometry Analysis of Cell Surface HSPGs

Huh7.5.1 cells were seeded onto 12-wells plates and treated with siNT or SMAD6 siRNA as described above. After 72 h, cells were harvested and immunostained with a mouse anti-heparan sulfate delta antibody (3G10 epitope, US biological, 1:100), followed by incubation with a FITC-conjugated goat anti-mouse IgG antibody (Invitrogen, 1:1000). After extensively washing, cells were resuspended in cold PBS and subjected to flow cytometry on a FACS Canto flow cytometer (BD Biosciences) that collects ~40,000 gated events. FlowJo flow cytometry analysis software (Flowjo LLC) was used for analysis of HSPG expression levels under various treatment conditions.

Promoter Reporter Activity Assay

Huh7.5.1 cells were transfected with various siRNAs for 72 h or with Flag-tagged SMAD6 plasmid for 48 h, and then transfected with various indicated promoter constructs that encode a luciferase reporter (SwitchGear Genomics). After 48 h, cells were lysed and luciferase activities were measured with the Lightswitch Luciferase Assay System (SwitchGear Genomics).

Low-density Lipoprotein and Cholesterol Uptake Assay

For LDL uptake assay, Huh7.5.1 were transfected with various siRNAs for 72 h, and subsequently treated with 12.5 μg/mL of BODIPY-labeled LDL (Life Technologies) at 37°C for 12 h. Cells were then fixed with 4% paraformaldehyde before nuclear staining with Hoechst (Invitrogen). Images were acquired using ZEN 2009 software on a Zeiss confocal microscope (Carl Zeiss). For cholesterol uptake assay, cells pre-treated with various indicated siRNAs were incubated with 10 μg/mL of NBD cholesterol (Cayman Chemical Company) for 48 h. Cell based assay buffer was subsequently added and meanwhile cell nuclei were stained with Hoechst. NBD cholesterol uptake in cells was analyzed immediately under a fluorescent microscope (Carl Zeiss).

Microarray analysis

Huh7.5.1 cells were treated with siNT or siSMAD6 for 72 h, and then mock infected or infected with HCV at an MOI of 1. At 48 h post-infection, total cellular RNA was extracted and purified. RNA quality and quantity were analyzed using an Agilent Bioanalyzer. Cellular RNA was then amplified using an Agilent Enzo kit. Amplified complementary RNA was hybridized to an Affymetrix Human 133 Plus 2.0 microarray chip containing 54,675 gene transcripts. For microarray analysis, a >1.5-fold change in expression combining a >95% probability of being differentially expressed ($P < 0.05$) was considered to be biologically significant. Bioinformatics and statistical analysis of microarray data were performed at the NIDDK Genomics Core Facility.

Patient Samples

Sixty-nine patients were provided by the Liver Clinic at the National Institutes of Health (NIH) Clinical Center from a large cohort of CHC patients (n = 712). To exclude other factors, patients with other causes of liver diseases (including HBV or HIV co-infection), excessive alcohol consumption, decompensated liver disease, active substance abuse or severe systemic disease were not chosen. All tested samples were included in this retrospective analysis based on the availability of adequate stored liver biopsy specimen for analysis. All patients gave written informed consent for participation in clinical research and genetic testing. Normal human liver tissues (frozen) were provided by the NIH-supported Liver tissue Cell Distribution system (LTCDS).

Results

SMAD6 Enhances HCV Entry at the Attachment Stage

SMAD6, an I-SMAD and a versatile transcription factor that mediates multiple cellular signaling pathways, was identified as a putative HCV entry factor through our functional genomics screens conducted in the Huh7.5.1 cells[12]. Here we first validated the effect of SMAD6 on HCV entry in HepG2/miR122/CD81 cells that support efficient viral entry and replication[14]. Silencing of SMAD6 by pooled siRNA effectively blocked the infection of HCV pseudoparticle (HCVpp) genotypes 1a and 1b, but not that of VSV-Gpp or MLVpp, two control pseudoviruses (Figure 1A), suggesting that SMAD6 is uniquely exploited by HCV to infect hepatocytes. The function of SMAD6 in mediating HCV infection was further

confirmed by the HCVcc assays. Efficient knockdown of SMAD6 by siRNA drastically reduced HCV RNA and core protein expression in Huh7.5.1 cells (Figures 1B–1C). Similarly, in primary human hepatocytes (PHHs), SMAD6 silencing resulted in marked decrease of HCV propagation (Figure 1D). SMAD6 siRNA robustly depleted target gene expression in both Huh7.5.1 cells and PHHs (Figures 1C and 1D). Treatment of cells with four individual SMAD6 siRNAs within the SMARTpool inhibited HCV infection that was proportionate to their knockdown efficiencies (Figure S1A–1B), revealing its physiological relevance in modulating HCV infection in hepatocytes. We also performed a gain-of-function assay by transfecting a Flag-tagged SMAD6 plasmid into Huh7.5.1 cells, and demonstrated that SMAD6 overexpression significantly enhanced HCV infection (Figure 1E). Furthermore, transfecting a siRNA-resistant mutant form of SMAD6 restored HCV infection in cells treated with SMAD6 siRNA (Figure S1C), supporting the phenotype-specific role of SMAD6 in mediating HCV infection.

Next, to examine whether SMAD6 is involved in the initial attachment step or downstream steps in the viral entry process, we performed HCVcc binding assay. Treatment of cells with SMAD6 siRNA inhibited HCV binding at 4°C to an extent comparable to treatment with heparin (Figure 1F) – a homologue of highly sulfated heparan sulfate that has been shown to selectively block HCV envelope glycoprotein binding[7]. In contrast, knockdown of CLDN1, a post-binding entry factor[15], had no effect on HCV binding (Figure 1F). Collectively, these data suggest that SMAD6 is involved in HCV attachment that initiates viral entry.

SMAD6 Modulates HCV Entry Through Transcriptional Regulation of Cell Surface Heparan Sulfate Expression

To identify the mechanism and cellular signaling pathways that mediate the effect of SMAD6 on cell binding of HCV, we performed microarray gene expression analysis and evaluated global transcriptomic changes in hepatocytes null of SMAD6. Intriguingly, the mRNA levels of multiple HSPG core proteins[16], particularly those of syndecan-1 (SDC1) and syndecan-2 (SDC2) were abundantly reduced upon SMAD6 silencing, either in the absence or in the presence of HCV infection (Figure 2A). Quantitative RT-PCR (qPCR) validated the SMAD6 depletion-based microarray results (Figure 2B), whereas overexpression of the protein significantly upregulated the HSPGs (Figure 2C), indicating a regulatory role of SMAD6 in HSPG expression in hepatocytes. Furthermore, knockdown of SMAD6 by siRNA in Huh7.5.1 cells substantially reduced cell surface heparan sulfate contents, examined by immunofluorescence and flow cytometry using a heparan sulfate-specific antibody (Figures 2D and 2E). Cell surface HSPGs, in particular SDC1, are critical host factors that mediate HCV attachment during viral entry[7, 17, 18]. As expected, SDC1 knockdown in Huh7.5.1 cells significantly inhibited HCV binding (Figure S2). To assess whether the transcription factor SMAD6 directly regulates SDC1 promoter activity, we either silenced or overexpressed SMAD6 in Huh7.5.1 cells, and demonstrated that the SDC1 promoter-driven luciferase reporter activity was significantly inhibited or enhanced, respectively (Figures 2F). Taken together, these results revealed a previously undescribed SMAD6-guided pathway that mediates the initial attachment step of HCV entry through transcriptional regulation of cell surface HSPGs.

Involvement of SMAD6 Co-repressors in HCV Entry

Like SMAD6, SMAD7 is another I-SMAD that acts as an antagonist of the canonical TGF-β pathway and its upstream bone morphogenetic protein (BMP) signaling[19–21]. Interestingly, we found that SMAD7 possesses similar function as SMAD6 in modulating HCV infection. SMAD7 knockdown by siRNA significantly restricted HCV infection and entry in Huh7.5.1 cells (Figures 3A, 3B and S3A), while overexpression of the protein led to a proviral effect (Figure 3C). Interestingly, silencing of various R-SMADs or Co-SMADs including SMAD2, SMAD3, SMAD4 and SMAD5, which are positive regulators of the TGF-β signaling, did not affect HCV entry or production (Figures 3A, S3A and S3B).

Since SMAD6 has been shown to recruit co-repressor C-terminal binding protein (CTBP1) in suppression of BMP-induced transcription[22], we examined the potential role of CTBP1 in HCV infection. SiRNA-mediated knockdown of CTBP1 resulted in a significant reduction of HCV RNA level in Huh7.5.1 cells (Figure 3D), suggesting a similar proviral function of CTBP1 as the I-SMADs. CTBP1 or SMAD7 depletion in cells significantly inhibited HCV binding (Figure 3E), indicating that these I-SMAD co-repressors regulate the initial attachment step of HCV entry. Likewise, silencing of SMAD7 or CTBP1, but not SMAD3 or SMAD4, markedly attenuated SDC1 promoter activity (Figures 3F and S3C). In addition, SMAD7 or CTBP1 silencing, like knockdown of SMAD6, substantially reduced the mRNA levels of various HSPG core proteins in hepatocytes (Figure S3D), whereas overexpression of SMAD7 significantly up-regulated these HSPGs genes expression (Figure S3E). These data further validate the intrinsic role of I-SMADs and co-repressors in regulating HSPG expression and thus affecting HCV entry.

BMP6 and BMP7 Induce I-SMAD and HSPG Expression and Promote HCV Entry

I-SMADs are induced by BMP/TGF-β signaling pathway, while they also function as feedback inhibitors of the BMP/TGF-β signaling[19–21]. HSPGs, shown above to be regulated by I-SMADs and co-repressors (Figures 2 and 3), serve as BMP co-receptors that are associated with TGF-β components to modulate BMP/TGF-β signaling[23–25]. In order to examine whether the TGF-β superfamily members, including the BMPs and TGF-β1, affect HCV entry, we treated cells with these cytokines and performed viral infection assays. We showed that BMP6 and BMP7 treatment, like overexpression of I-SMADs in cells, significantly increased HCV core protein and RNA production (Figures 4A and 4B). This proviral function seems to be BMP6/7-specific, as treatment of cells with other BMPs, including BMP2 and BMP4, had no effect on HCV infection (Figure 4B). By contrast, TGF-β1 treatment markedly reduced HCV infection (Figures 4A and 4B), supporting the previously identified antiviral function of the TGF-β pathway in HCV infection[26]. We further showed that BMP6 and BMP7 facilitate HCV attachment in the early entry process, while other BMPs had no effect (Figure 4C). All cytokines tested are not associated with any significant cytotoxicity at the concentrations used (Figure S4A).

Interestingly, BMP6 or BMP7 treatment drastically induced the expression of I-SMADs in Huh7.5.1 cells (Figure 4D), whereas the effects on the other SMAD family members were none (SMAD3 and SMAD5) or minor (SMAD4) (Figure S4B). In addition, BMP6 and BMP7, but not TGF-β1 or BMP-2/4 treatments, significantly induced the mRNA levels of

various HSPG core proteins in hepatocytes (Figures 4E and S4C), likely reflecting their divergent effects on I-SMAD expression (Figure 4D). It is noteworthy that BMP2, while modestly upregulating SMAD6, did not significantly enhance HSPG core mRNA levels (Figure S4C). It is possible that the modest effect of BMP2 on SMAD6 expression was not sufficient to cause a significant increase in HSPG expression. To summarize, these data demonstrated a BMP6/7-triggered, I-SMAD-mediated, and canonical TGF-β pathway-independent transcriptional mechanism that preferentially acts on the initial attachment step of HCV infection *via* upregulation of HSPG expression on cell surface.

SMAD6 Regulates Hepatocellular Lipoprotein and Cholesterol Uptake

HCV, circulating in the form of LVPs that highly resemble very low-density lipoprotein (VLDL), exploits cell surface receptors that are involved in cholesterol and lipid uptake to gain entry into hepatocytes[27]. Intriguingly, in our microarray analysis as mentioned above, multiple lipoprotein and cholesterol uptake receptors, including LDLR, SR-BI and VLDLR were markedly downregulated at the mRNA levels as a result of SMAD6 knockdown (Figure 5A). Confirmatory qPCR assays validated the SMAD6 siRNA-mediated effects (Figure 5B), while over-expression of SMAD6 up-regulated these genes (Figure 5C). These data indicate that SMAD6 may also transcriptionally regulate hepatic cholesterol and lipid uptake through its effect on the expression of various lipo-receptors. Indeed, silencing of SMAD6 by siRNA in Huh7.5.1 cells markedly reduced cellular uptake of NBD cholesterol and BODIPY-labeled LDL as measured by fluorescent microscopy and subsequent quantitative analysis (Figure 5D and 5E). Interestingly, cell surface HSPGs have also been attributed to hepatic lipoprotein uptake[28, 29]. As expected, depletion of SDC1 in hepatocytes reduced cellular uptake of cholesterol and LDL which is similar to the effects of SR-BI, LDLR or VLDLR silencing (Figure 5D and 5E). These data collectively suggest that SMAD6 directs a transcriptional mechanism in regulating hepatic lipoprotein and cholesterol uptake that is important in HCV entry. As such, we conclude that SMAD6 mediates HCV attachment and entry *via* two concurrent pathways that both involve its transcriptional activities.

HCV Infection Induces I-SMAD and HSPG Expression

Viruses like HCV employ various strategies, such as inducing or activating host proviral machineries or subverting host antiviral responses to achieve productive infection. Here we examined whether HCV infection manipulates the I-SMAD–HSPG pathway for its own benefit. First, we infected Huh7 cells with the JFH1 strain of HCV, and evaluated viral replication and relevant expression levels of I-SMADs and HSPGs (Figure S5A). We found that I-SMAD mRNA levels were substantially elevated from day 5 of the infection, and increased by over 3.5-fold on day 7, the longest duration of HCV infection we can keep *in vitro* without cell death (Figure 6A). HCV infection also markedly increased SMAD6 protein level in Huh7 cells (Figure 6B). Similarly, in PHHs infected with HCV, I-SMAD mRNA levels were increased by over 2.5-fold on day 7 (Figure 6C).

In addition, SMAD6 protein level was increased in HCV-infected PHHs examined by Western blot (Figure 6D). Likewise, the expression levels of SDC1 and various other HSPG core proteins were significantly elevated during HCV infection in both Huh7 cells and PHHs

(Figures 6A, 6C and S5A, S5B), indicating that the I-SMAD–HSPG pathway is induced upon HCV infection.

We then investigated the mediator of I-SMAD upregulation in the setting of HCV infection. Expression of I-SMADs can be regulated by multiple extracellular signals, including EGF/MAPK, TNF-α/NF-κB, IFN-γ/JAK1/STAT1, and TGF-β/BMP[30]. Genes in these pathways (MAPK1, NF-κB1, NF-κB2, STAT1, TGF-β1, TGF-β2, TGF-β3, BMP6 and BMP7) were studied in HCV-infected cells (Figure S5C). Among them, TGF-β and NF-κB components were shown to be upregulated by HCV infection. Indeed, both pathways have previously been shown to be activated by HCV[4, 31–33]. To explore whether these signaling pathways are involved in the HCV-triggered induction of I-SMADs, we first treated Huh7 cells with SB-431542, a potent and specific inhibitor of TGF-β signaling. We showed that SB-431542 treatment did not diminish the induction of SMAD6 expression by HCV infection (Figure S5D), implying that the TGF-β pathway is not involved. We then studied the NF-κB pathway. Cells were depleted of NEMO – a crucial NF-κB-activating kinase, or NF-κB1/2 by siRNA treatment to silence the NF-κB signaling (Figure S5E). We also treated cells with a NF-κB specific inhibitor – caffeic acid phenethyl este (CAPE) (Figure S5F). Interestingly, HCV-triggered induction of I-SMAD and HSPG expression was substantially abrogated in the NF-κB-deficient cells (Figures 6E, S5F and S5G), suggesting that NF-κB activation is required for HCV-mediated upregulation of I-SMAD and HSPG expression in hepatocytes.

Finally, we asked whether the upregulation of I-SMADs and HSPGs is clinically relevant to HCV infection. We examined the expression levels of these genes in liver biopsy samples of HCV-naïve (n = 19) or chronic hepatitis C (n = 69) patients. Significantly higher mRNA levels of I-SMADs and SDC1 were observed in the liver tissues infected with HCV (Figure 6F). These results support that chronic HCV infection leads to the induction of a specific proviral host pathway involving the I-SMADs and HSPGs, which may in turn contribute to viral persistence.

Discussion

HCV enters host cells through a coordinated cascade of pathways engaging a number of entry factors, including CD81[34], tight junction proteins CLDN1[15] and OCLN[35], the receptor tyrosine kinases epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2)[36], Niemann-Pick C1-like 1 (NPC1L1)[37] and iron uptake receptor transferrin receptor 1 (TRF1)[38]. Indeed, how this complexed chain of cell signals and virus-host interactions is orchestrated and regulated to the advantage of the virus is not yet clear. Applying integrative functional genomics and systems biology approaches, we recently dissected global HCV–host interactions and uncovered SMAD6 as a novel host proviral factor for HCV[39]. In the present study, we interrogated the precise functions and mechanisms of action of SMAD6 in modulating HCV infection. We found that SMAD6 and the other I-SMAD, SMAD7, are both required for HCV entry. Present as important transcription factors in cells, these I-SMADs were shown to regulate the expression of HSPGs (particularly syndecans) on cell surface, thereby affecting the initial attachment step of the viral entry process. I-SMADs were also shown to manipulate cholesterol uptake *via* multiple pathways (mediated by SR-BI, VLDLR or LDLR) that are closely associated with

HCV entry. Intriguingly, HCV infection, through enhancing I-SMAD and HSPG expression in hepatocytes, co-opts this intrinsic proviral pathway for its own advantage. A working model of these novel interactions is illustrated in Figure 7.

Here we identified I-SMADs as novel HCV entry factors acting at the early viral attachment stage. The I-SMADs belong to the SMAD family of proteins that are important signal transducers and key regulators of proliferation, differentiation, migration, apoptosis and immune response[40]. The SMAD family consists of eight members, and among them, five (SMAD1, 2, 3, 5 and 8) are R-SMADs, all containing a phosphorylation domain that can be activated by type I receptor of the TGF-β receptor family. Specifically, SMAD2 and 3 mediate the signaling through TGF-β, whereas SMAD1, 5 and 8 mediate the BMP signaling. The R-SMADs, once phosphorylated through receptor binding, complex with SMAD4, which plays a role in recruiting the downstream co-regulators for transcriptional activation of various cellular processes. SMAD6 and 7 are both I-SMADs and are induced by the TGF-β and BMP signaling while also act as TGF-β/BMP feedback inhibitors. The main function of the I-SMADs is to prevent ligand-induced activation of SMAD proteins in the TGF-β signaling pathway[19–21]. In fact, these diverse properties and functions of SMAD family members may account for their divergent effects on HCV entry and infection observed in this study.

Accumulating evidence has demonstrated that I-SMADs may act as transcriptional co-activators that are independent of the R-SMAD signaling[41, 42]. The C-terminus of the I-SMADs has a MH2 domain that is indeed conserved among all SMAD proteins. Domain mapping studies and relative structure-based data suggested that MH2 domains, in response to TGF-β or BMP stimulation, form a complex with transcription activation potential[43]. Surprisingly, our data indicate that only the I-SMADs but not the R-SMADs or SMAD4 possess transcription regulatory activities on the HSPG expression in hepatocytes that is essential for HCV attachment and entry, implicating a previously unidentified and unique function of I-SMADs that is distinct from the other SMAD proteins.

In our study, two secreted signaling molecules and TGF-β superfamily members, BMP6 and BMP7, were shown to enhance HCV attachment and entry by inducing I-SMAD and HSPG expression. Interestingly, BMP6 was found to facilitate SMAD phosphorylation, a process that can be inhibited by adding exogenous heparan sulfate. Therefore, BMP6 may functionally interact with HSPGs[44]. Indeed, HSPGs can act as BMP co-receptors, bind to TGF-β components and play an important role in regulating BMP/TGF-β signaling[23, 24]. We showed that BMP6/7 but not BMP2/4 affected HCV binding and I-SMAD and HSPG expression. Mechanistically, BMP6/7 interact with type II receptors Act RII and Act RIIB on the cell surface, and recruit type I receptors Alk2, Alk3 and Alk6, while the receptor-binding affinity is reversed for BMP2/4, which preferentially bind to the type I receptors Alk3 and Alk6 and recruit type II receptors to the heteromeric signaling complexes[45]. The difference in receptor binding activities between two BMP sets may explain the BMP6/7-specific function in modulating I-SMADs. It is interesting that BMP6, which regulates iron uptake via hepcidin[46], appears to have a previously unrecognized function in regulating cellular cholesterol uptake via induction of I-SMADs, linking iron homeostasis and lipid metabolism[47, 48].

It is known that I-SMADs are regulated by TGF-β/BMP and other signaling, including EGF/ MAPK, IFN-γ/STAT and TNF-α/NF-κB pathways[30]. We found that only NF-κB signaling but not TGF-β/BMP. STAT1 or MAPK1 is responsible for the I-SMAD induction upon HCV infection. Indeed, the NF-κB pathway has been implicated in multiple viral infections[49]. The correlation between NF-κB and I-SMAD-mediated pathway identified in this study suggests that HCV, by activating the innate NF-κB pathway - probably via engaging pattern recognition receptors (RIG-I for example), upregulates I-SMADs to facilitate viral propagation.

Heparan sulfate is expressed on most mammalian cell surface as proteoglycan and consists of a core and highly sulfated glycosaminoglycan (GAG) side chains. HSPGs bind to various extracellular proteins, adhesion molecules and growth factors and have been shown to mediate the binding and entry of a variety of viruses including HCV[7,50]. The role of cell surface HSPGs in HCV binding appears to be mediated by their specific interaction with ApoE present on viral LVPs[18, 28]. HSPG core proteins consist of membrane-bound syndecans (SDC1-4) and glypicans (GPC1-6), serglycin expressed in secretory vesicles, and perlecan (also known as HSPG2)[50]. The majority of these HSPG core proteins were shown to be regulated by I-SMADs in our study. A previous report demonstrated that multiple SDCs mediate HCV attachment to hepatocytes[4], further supporting our notion that I-SMADs modulate HCV binding and entry through up-regulation of HSPGs.

Collectively, our study, for the first time, reveals a transcription regulatory mechanism that mediates viral entry. The pathways underlying the regulation of HCV entry process involve I-SMADs, cell surface HSPGs and cholesterol uptake receptors. In addition, this specific I-SMAD–HSPG axis is induced by HCV infection, likely *via* the activation of NF-κB, either in cell culture models or in liver biopsies of chronic hepatitis C patients. Apart from the two I-SMADs, other members of SMAD proteins, including SMAD2/3/4/5 – positive regulators of the TGF-β signaling pathway – are dispensable for HCV entry and HSPG regulation, indicating that the functions of I-SMADs observed in this study are unique and independent of the canonical TGF-β pathway. Our study not only provides crucial insight into the complex mechanism of HCV entry but also reveals previously undescribed biological pathways in lipid and cholesterol metabolism that may link inflammation, immune regulation and metabolic dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

BMP	bone morphogenetic protein
CD81	cluster of differentiation 81
CLDN1	claudin-1
CTBP1	co-repressor C-terminal binding protein
HCVpp	HCV pseudoparticle
LDLR	low density lipoprotein receptor
VLDLR	very low density lipoprotein receptor
NEMO	NF-kappa-B essential modulator
NF-κB	nuclear factor-kappa B
PHHs	primary human hepatocytes
SDC	syndecan
SR-BI	scavenger receptor class B member 1
TGF-β	Transforming growth factor beta

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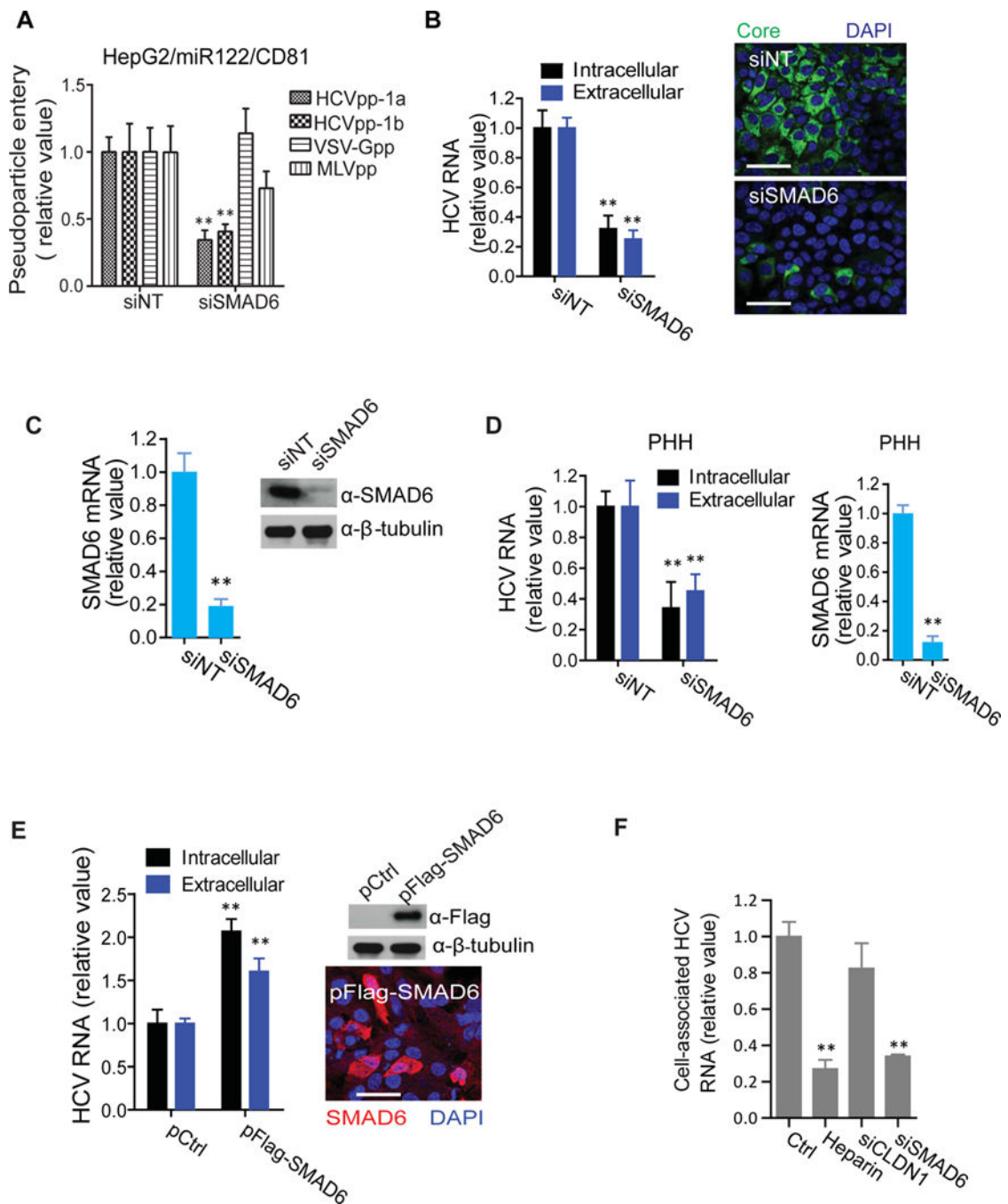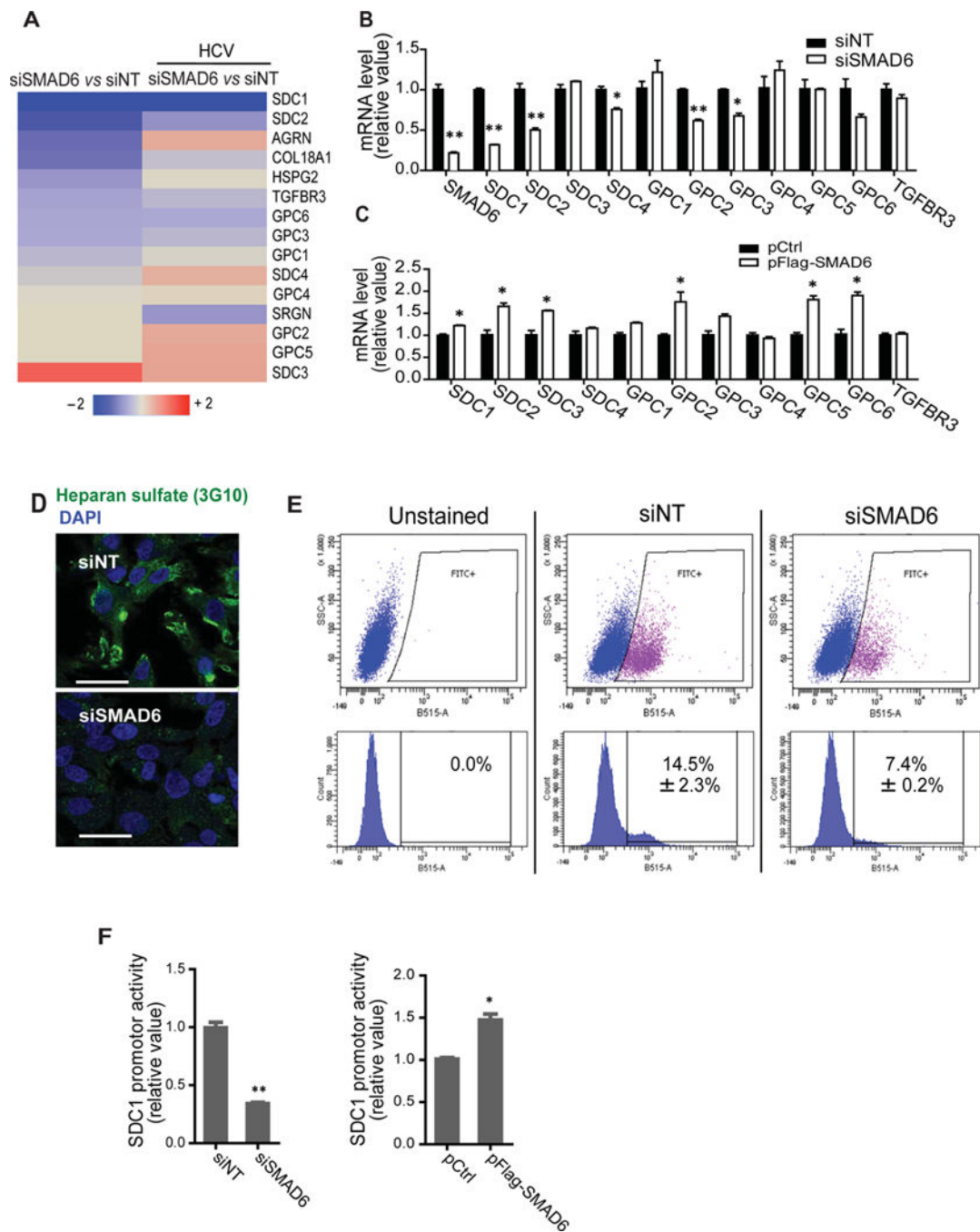
Figure 1.
SMAD6 modulates HCV attachment, entry and infection. (*A*) Effect of SMAD6 knockdown on viral entry in HepG2/miR-122/CD81 cells. Cells were treated with non-targeting control (NT) or SMAD6 siRNA, and then infected with various pseudoviruses. Two days after infection, firefly luciferase activity was measured. (*B*) Effect of SMAD6 silencing on HCV RNA abundance (right panel) and core protein expression (left panel) in Huh7.5.1 cells. Intracellular and extracellular HCV RNA levels were determined by qPCR, and HCV core protein was immunostained with an anti-core monoclonal antibody. Representative images

are shown. Green: core; blue: nuclei. Scale bar, 50 μm. (*C*) Knockdown efficiency of siSMAD6 in Huh7.5.1 cells. SMAD6 mRNA or protein levels were determined by qPCR or Western blot, respectively. β–tubulin was used as a loading control for Western blot. (*D*) SMAD6 silencing in PHHs inhibits HCV infection. HCV RNA and SMAD6 mRNA levels were quantified by qPCR. (*E*) SMAD6 overexpression enhances HCV infection. Huh7.5.1 cells were transfected with control (Ctrl) or Flag-tagged SMAD6 plasmid for 24 h, and then infected with HCV. At 48 h post-infection, HCV RNA levels was measured by qPCR, and SMAD6 protein level was determined by both Western blot and immunofluorescence using an anti-Flag antibody. For Western blot, β–tubulin serves as a loading control. A representative microscopic image is shown in the lower right panel. Red: SMAD6; blue: nuclei. Scale bar, 50 μm. (*F*) Effect of SMAD6 silencing on HCV binding. Huh7.5.1 cells were treated with SMAD6 or CLDN1 siRNA for 72 h or with heparin (200 g/mL) for 2 h and then incubated with HCV at 4°C for 2 h. Unbound virus was removed by extensive washes with cold PBS. Intracellular HCV RNA levels were determined by qPCR. All values were normalized as relative to siNT or Ctrl (set as 1), and represent the mean ± SD, n = 3 (*B–F*) or 5 (*A*). The asterisks indicate statistically significant differences (**$p < 0.01$).

Figure 2.

Regulation of cell surface heparan sulfate contents by SMAD6. (*A*) Heatmap illustrating siSMAD6-mediated regulation of HSPGs either in the absence or in the presence of HCV infection. Fold changes of mRNA levels of all known HSPG core proteins in Huh7.5.1 cells relative to siNT are shown. Values in the heatmap are depicted in a continuum of blue (downregulation) to red (upregulation). (*B, C*) Effects of SMAD6 knockdown (*B*) or overexpression (*C*) on mRNA levels of various HSPG core protein in Huh 7.5.1 cells, determined by qPCR. (*D, E*) Reduction of cell surface heparan sulfate contents upon

SMAD6 silencing. Huh7.5.1 cells were depleted of SMAD6 expression *via* siRNA treatment for 72 h, and then immunostained with an anti-heparan sulfate delta (3G10 epitope) antibody for either immunofluorescence/confocal microscopy (*D*) or flow cytometry (*E*) that analyzes cell surface HSPG levels. Images shown are representatives of triplicated experiments. Scale bars represent 50 μM. (*F*) Effects of SMAD6 knockdown or overexpression on SDC1 promoter activity. Huh7.5.1 cells were treated with control or SMAD6 siRNA (left panel) or transfected with Flag-tagged SMAD6 (right panel) before transfection with a SDC1 promoter-driven luciferase reporter construct. Luciferase activities were measured after 48 h and are normalized to control cells. (*B, C, F*) Values were normalized as relative to siNT or Ctrl (set as 1), and represent the mean ± SD, n = 3 (B, C) or 5 (F). The asterisks indicate statistically significant differences (*$p < 0.05$; **$p < 0.01$).
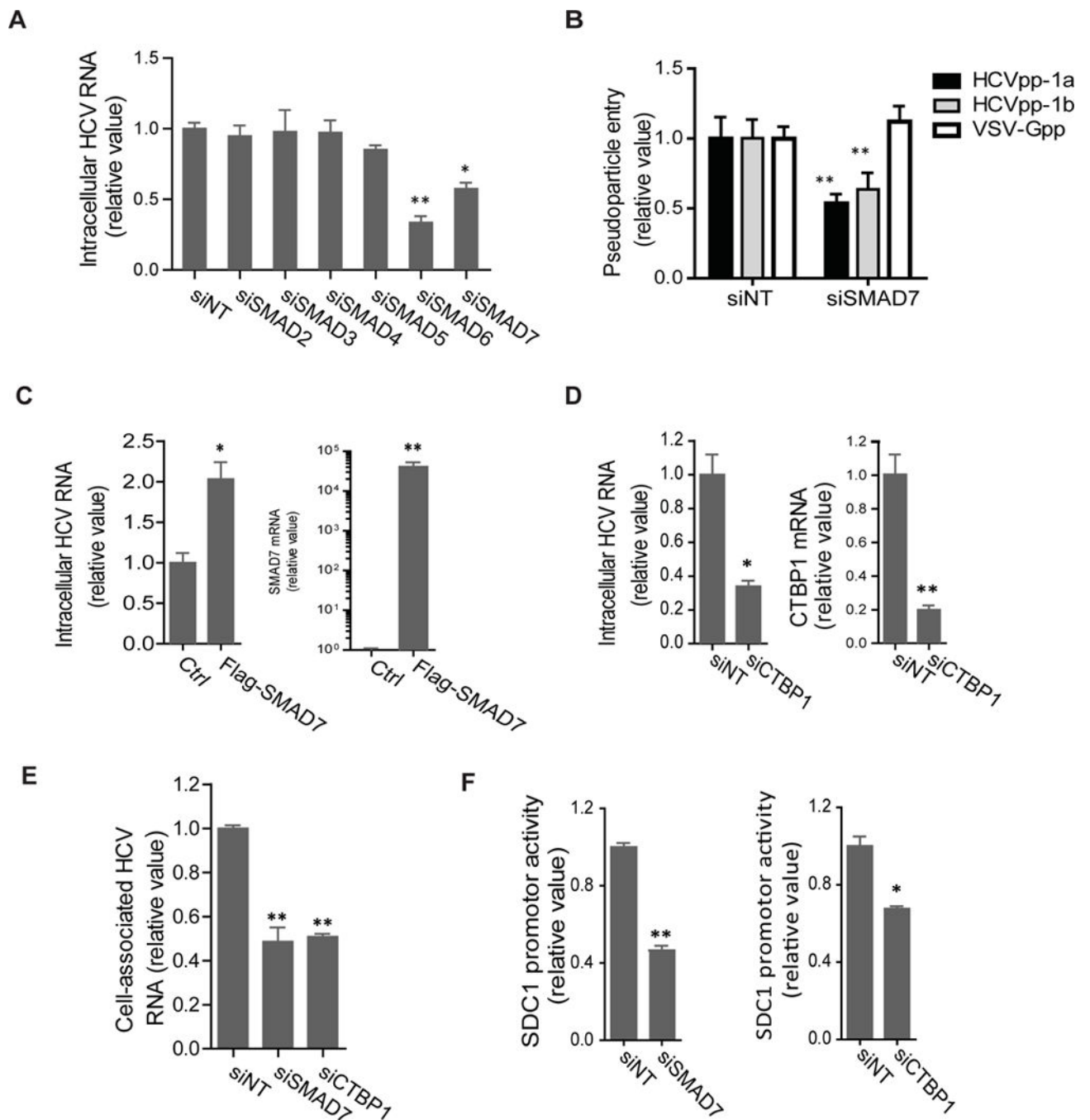
Figure 3.

SMAD6 co-repressors regulate HSPG expression and HCV entry. (*A*) Silencing of various SMAD family members exerts varying effects on HCV infection. Intracellular HCV RNA levels were quantified at 48 h post-infection in Huh7.5.1 cells pre-treated with various indicated siRNAs. (*B*) Effect of SMAD7 knockdown on HCV genotypes 1a and 1b entry in Huh 7.5.1 cells. (*C*) SMAD7 overexpression enhances HCV infection. Intracellular HCV RNA and SMAD7 mRNA levels in Ctrl or Flag-SMAD7-transfected cells were determined by qPCR. (*D*) CTBP1 knockdown restricts HCV infection. Intracellular HCV RNA and

CTBP1 mRNA levels in siNT or siCTBP1-treated cells were measured by qPCR. (*E*) Effect of SMAD7 or CTBP1 silencing on HCV attachment. SMAD7 or CTBP1 were depleted in Huh7.5.1 cells by siRNA treatment, and HCV binding ability at 4°C was evaluated by quantifying levels of cell-associated HCV RNA. (*F*) Silencing of SMAD7 or CTBP1 by siRNAs decreases SDC1 promoter activity in Huh7.5.1 cells. All values were normalized as relative to siNT or Ctrl (set as 1), and represent the mean $\pm$ SD, n = 5 (*A, F*) or 3 (*B–E*). The asterisks indicate statistically significant differences (*$p < 0.05$; **$p < 0.01$).
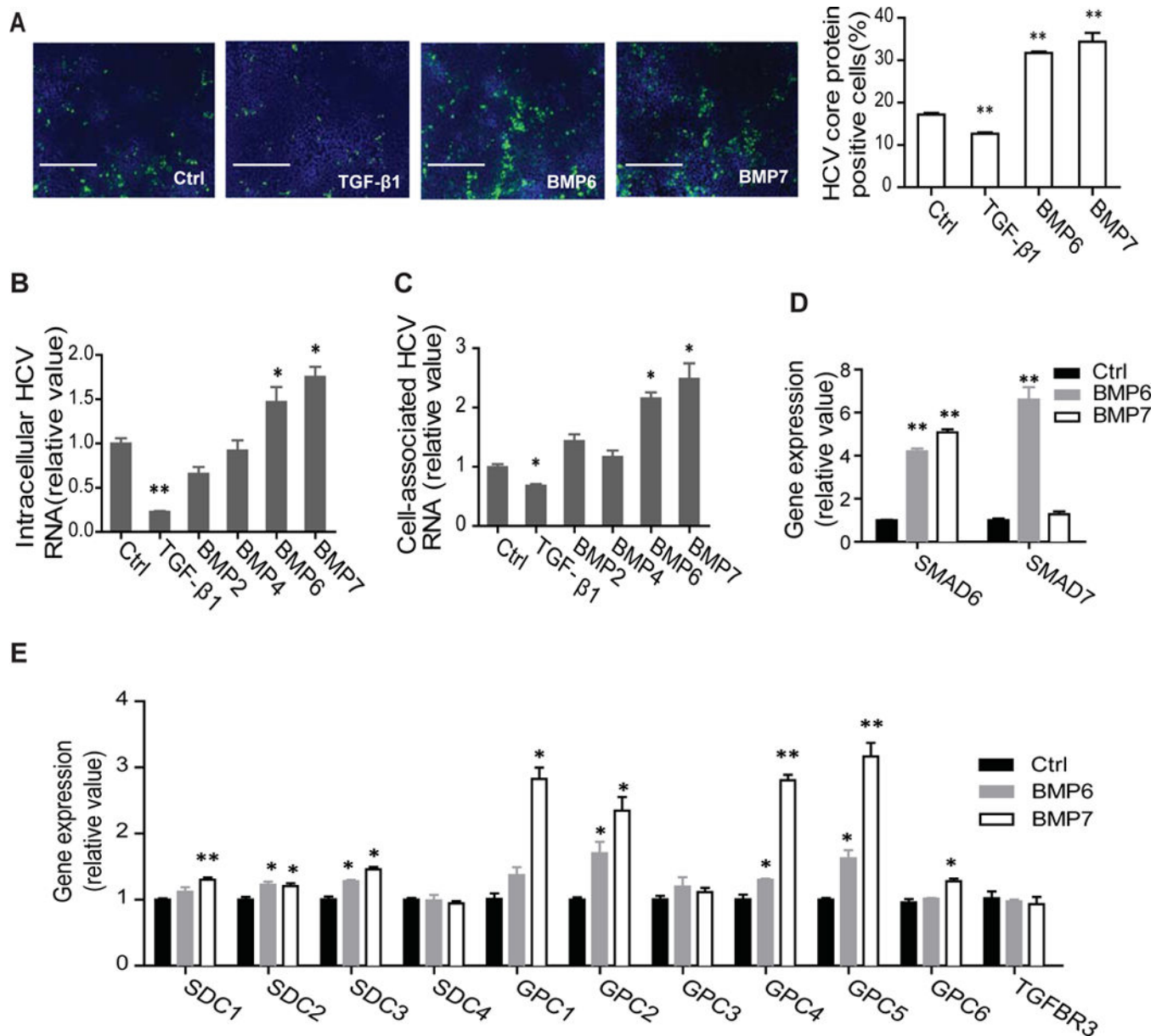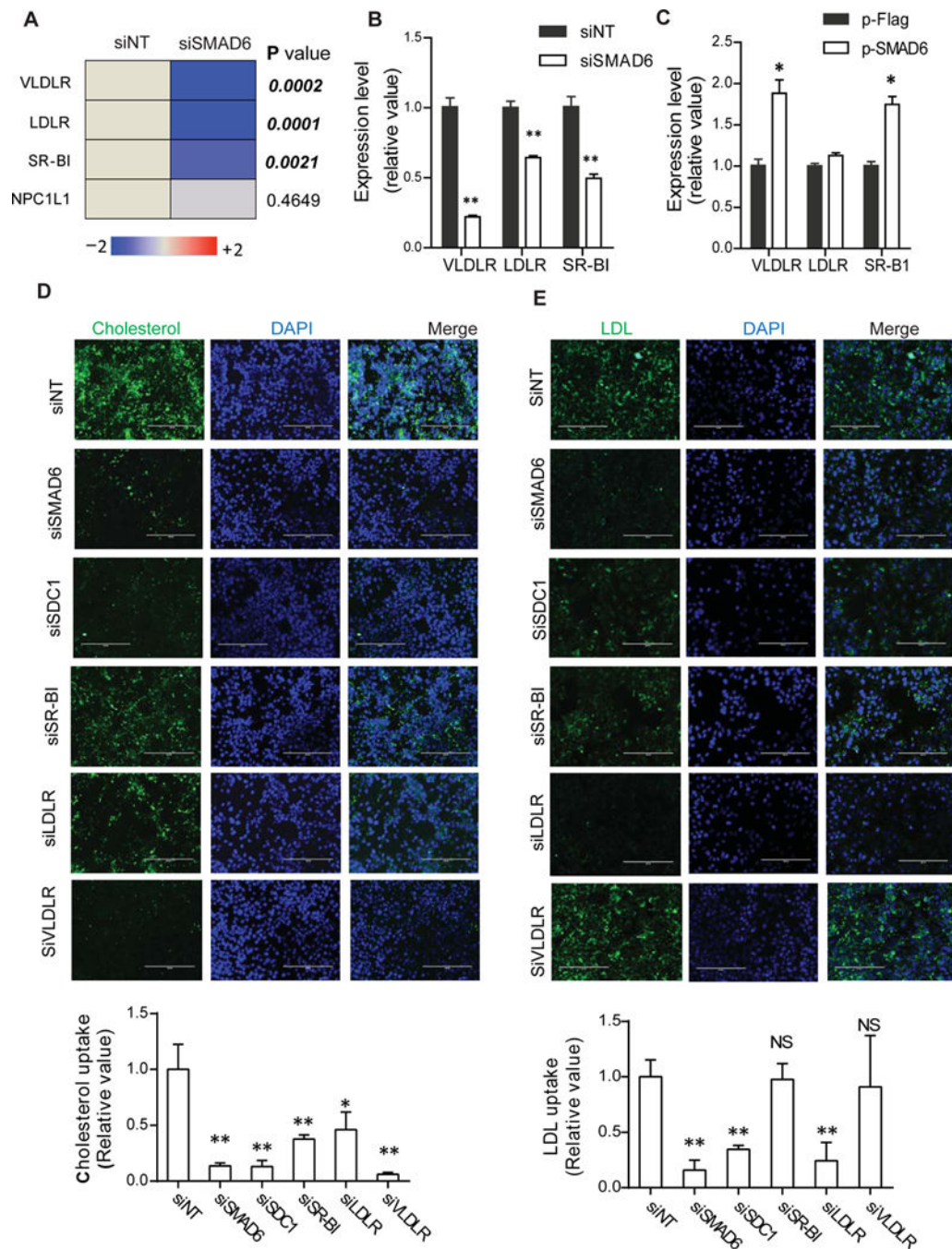
Figure 4.
BMP6/7 enhance I-SMAD expression and HCV binding. (*A–C*) TGF-β or BMP treatment executes varying effects on HCV infection. Huh7.5.1 cells were treated with TGF-β1 (0.1 ng/mL), BMP2 (0.1 g/mL), BMP4 (20 ng/mL), BMP6 (0.1 μg/mL) or BMP7 (0.5 μg/mL) for 24 h, and then infected with HCV at 37°C for 48 h (*A*, *B*) or 4°C for 2 h (*C*). HCV core staining (*A*), viral RNA quantification (*B*) and HCV binding assay (*C*) were subsequently performed. (A) Representative immunofluorescence images are shown. Green: core; blue: nuclei. Scale bar, 200 μm. The right panel shows quantification of core staining results. (*D*, *E*) BMP6/7 treatment induces I-SMAD (*D*) and HSPG (*E*) expression in Huh7.5.1 cells. All values were normalized as relative to Ctrl (set as 1), and represent the mean ± SD, n = 3. The asterisks indicate statistically significant differences (*p < 0.05; **p < 0.01).

Figure 5.
SMAD6 regulates cholesterol and lipid uptake in hepatocytes. (*A*) Heatmap eliciting the suppression of various lipoprotein and cholesterol uptake receptors by SMAD6 silencing in Huh7.5.1 cells. Reduction of gene expression in the heatmap is illustrated as blue comparing with siNT. (*B*) Validation of the microarray results on siSMAD6-medaited reduction of VLDLR, LDLR and SR-BI expression by qPCR. (*C*) Effect of SMAD6 overexpression on VLDLR, LDLR and SR-BI expression. (*B*, *C*) Values were normalized as relative to siNT (set as 1), and represent the mean ± SD, n = 3. The asterisks indicate statistically significant

differences (*p < 0.05; **p < 0.01). (*D, E*) Effects of SMAD6, SDC1, SR-BI, LDLR or VLDLR silencing on uptake of NBD cholesterol (*D*) or BODIPY-labeled LDL (*E*) into Huh7.5.1 cells, examined by fluorescent microscopy. Representative immunofluorescence images are shown. Green: cholesterol (*D*) or LDL (*E*); blue: nuclei. Scale bar, 200 μm. Quantitative analyses of cholesterol and LDL up taken from above shown images were performed using Image J by counting various florescent spots, and the results are shown in the bottom panels. Values were normalized as relative to siNT (set as 1), and the asterisks indicate statistically significant differences (**p < 0.01; NS, not significant).
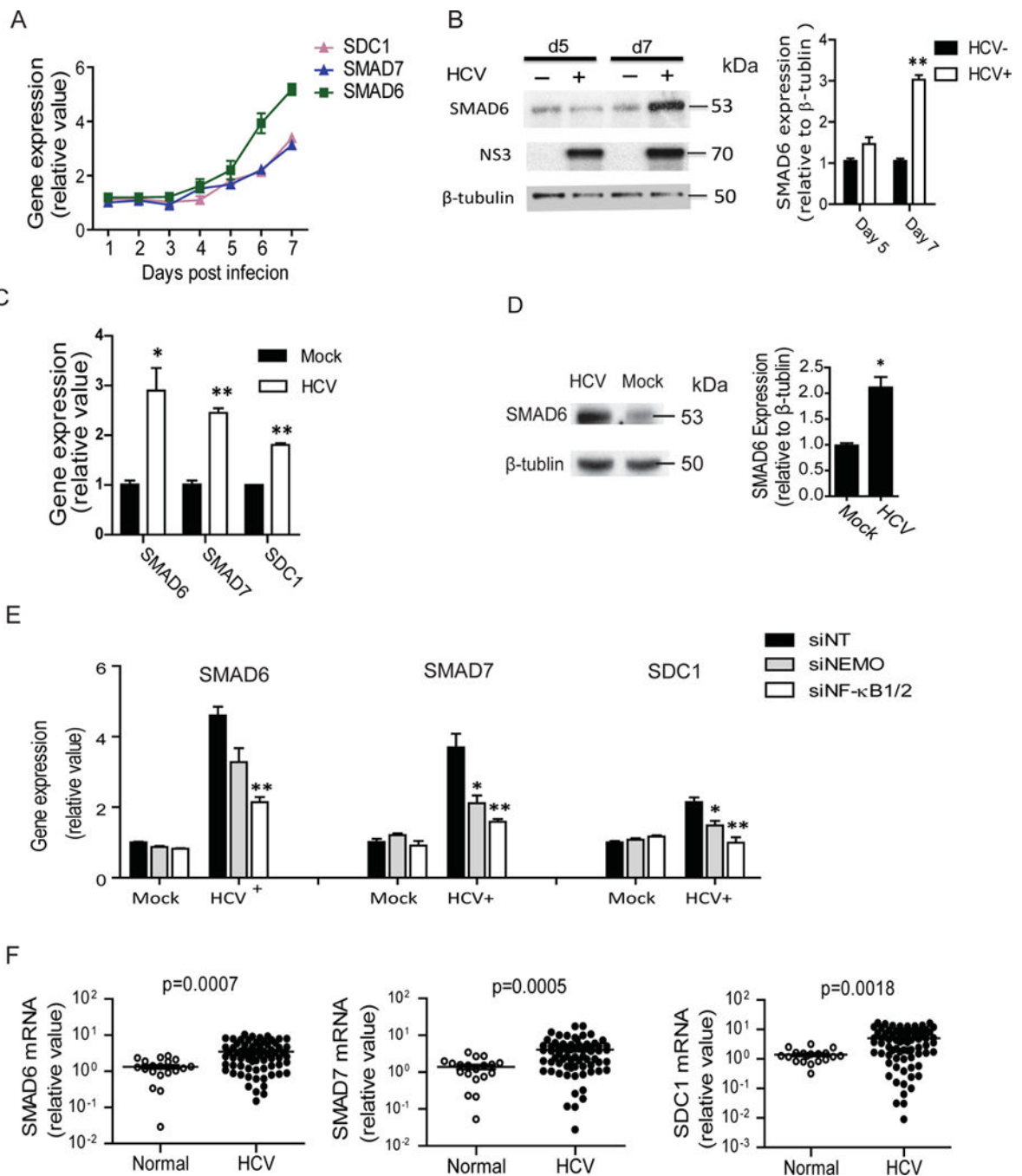
Figure 6.
HCV infection upregulates I-SMAD and HSPG expression. (*A*) I-SMAD and SDC1 mRNA levels increased over time upon HCV infection in Huh7 cells. HCV infection was maintained for 7 days. (*B*) SMAD6 protein level also increased at 5 and 7 days post-infection in Huh7 cells. (*C*) The expression of I-SMAD and SDC1 was induced by HCV in PHHs. Various indicated mRNA levels were quantified at 7 days post-infection. (*D*) HCV infection increases SMAD6 protein level in PHHs as determined by Western blot. β–tubulin was used as a loading control. Representative Western blot gels and quantification of band

intensities are shown. (*E*) HCV infection induces I-SMAD and SDC1 expression *via* the NF-κB pathway. Huh7 cells were infected with HCV JFH1 strain for 24 h, and then treated with siRNAs targeting NF-κB1/2 or NEMO. The expression of I-SMADs and SDC1 were subsequently assessed by RT-qPCR. (*C–E*) Values were normalized as relative to mock infection (set as 1), and represent the mean ± SD, n = 3. The asterisks indicate statistically significant differences (*$p < 0.05$; **$p < 0.01$). (*F*) Enhanced I-SMADs and SDC1 expression in the liver biopsy samples of chronic hepatitis C patients (n=69), comparing with those of HCV-naïve patients (n=19).
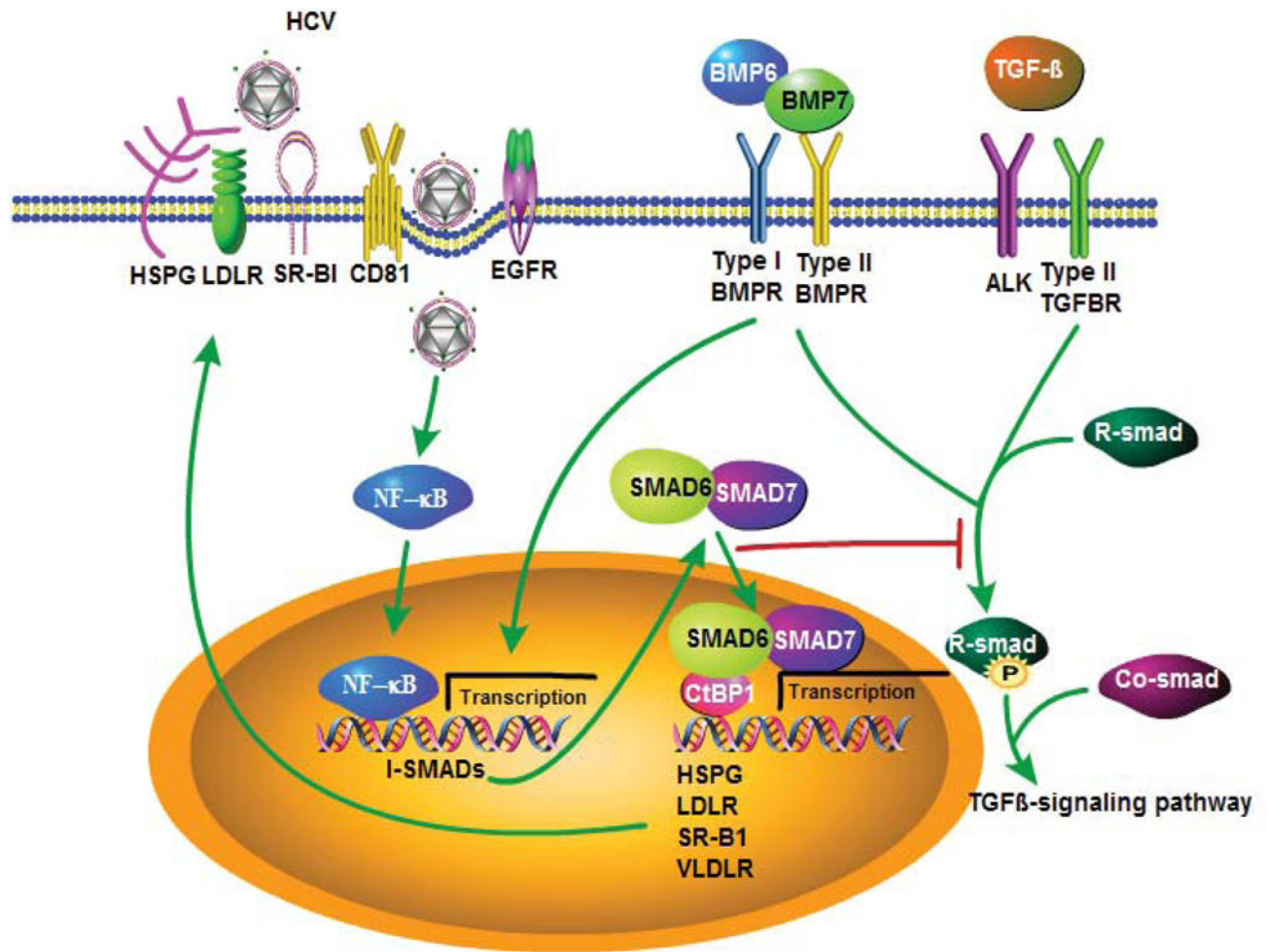
Figure 7.
A proposed model for I-SMAD-regulated signaling that enhances HSPG expression, cholesterol uptake and HCV entry. HCV entry is mediated by viral binding to HSPGs, LDLR and SR-BI (and other entry factors, not shown) on the host cell surface, thereby triggering a cassette of signaling to facilitate the internalization of HCV virions. HCV infection also induces the expression of SMAD6 and SMAD7, two I-SMADs of the TGF-β signaling pathway *via* NF-κB regulation. The I-SMADs translocate to the nucleus and transcriptionally activate the expression HSPG core protein and other cholesterol uptake receptors. Increased expression level of heparin sulfate on the cell surface in turn enhances HCV binding. Additionally, the I-SMADs and HSPGs can be induced by BMP6 and BMP7, the ligands in the BMP/TGF-β pathway.