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A Targeted Functional RNA Interference Screen Uncovers Glypican 5 as an Entry Factor for Hepatitis B and D Viruses

Eloi R. Verrier,[1,2] Che C. Colpitts,[1,2] Charlotte Bach,[1,2] Laura Heydmann,[1,2] Amélie Weiss,[3] Mickaël Renaud,[3] Sarah C. Durand,[1,2] François Habersetzer,[4] David Durantel,[5] Georges Abou-Jaoudé,[6] Maria M. López Ledesma,[7] Daniel J. Felmlee,[1,2] Magali Soumillon,[8] Tom Croonenborghs,[9,10] Nathalie Pochet,[9] Michael Nassal,[11] Catherine Schuster,[1,2] Laurent Brino,[3] Camille Sureau,[6] Mirjam B. Zeisel,[1,2] and Thomas F. Baumert[1,2,4]

Chronic hepatitis B and D infections are major causes of liver disease and hepatocellular carcinoma worldwide. Efficient therapeutic approaches for cure are absent. Sharing the same envelope proteins, hepatitis B virus and hepatitis delta virus use the sodium/taurocholate cotransporting polypeptide (a bile acid transporter) as a receptor to enter hepatocytes. However, the detailed mechanisms of the viral entry process are still poorly understood. Here, we established a high-throughput infectious cell culture model enabling functional genomics of hepatitis delta virus entry and infection. Using a targeted RNA interference entry screen, we identified glypican 5 as a common host cell entry factor for hepatitis B and delta viruses. *Conclusion*: These findings advance our understanding of virus cell entry and open new avenues for curative therapies. As glypicans have been shown to play a role in the control of cell division and growth regulation, virus–glypican 5 interactions may also play a role in the pathogenesis of virus-induced liver disease and cancer. (HEPATOLOGY 2015; 00:000-000)

Hepatitis B virus (HBV) is a small, enveloped DNA virus[1] and a leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. More than 350 million individuals are chronically infected with HBV.[2] Among these, 5%-10% are likely coinfected with hepatitis delta virus (HDV) and exhibit an increased HCC risk.[3] HDV is a small RNA satellite virus of HBV that uses the HBV envelope proteins to assemble into infectious particles and enter its target cell.[4] Nucleos(t)ide analogues and interferon-based treatment can control HBV infection, but virus eradication and cure remain largely unattainable.[5] While

Abbreviations: CsA, cyclosporin A; DMSO, dimethylsulfoxide; dpi, day postinoculation; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPC, glypican; HBeAg, hepatitis B e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDAg, hepatitis delta antigen; HDV, hepatitis delta virus; HSPG, heparan sulfate proteoglycan; IF, immunofluorescence; IgG, immunoglobulin G; mRNA, messenger RNA; NTCP, sodium/taurocholate cotransporting polypeptide; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PFA, paraformaldehyde; pgRNA, pregenomic RNA; PHH, primary human hepatocyte; pp, pseudoparticle; qRT-PCR, quantitative real-time polymerase chain reaction; RNAi, RNA interference; SDC, syndecan; siRNA, small interfering RNA.

From [1]Inserm, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Strasbourg, France; [2]Université de Strasbourg, Strasbourg, France; [3]IGBMC, Plateforme de Criblage Haut-débit, Illkirch, France; [4]Institut Hospitalo-Universitaire, Pôle Hépato-digestif, Nouvel Hôpital Civil, Strasbourg, France; [5]Inserm, U1052, CNRS UMR 5286, Cancer Research Center of Lyon, Université de Lyon, Lyon, France; [6]INTS, Laboratoire de Virologie Moléculaire, Paris, France; [7]Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina; [8]Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA; [9]Program in Translational NeuroPsychiatric Genomics, Brigham and Women's Hospital, Harvard Medical School, Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA; [10]KU Leuven Technology Campus Geel, AdvISe, Geel, Belgium; [11]Department of Internal Medicine II/Molecular Biology, University Hospital Freiburg, Freiburg, Germany.

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HDV can partially respond to interferon-based treatment,[6] long-term response is marginal.[6,7]

Although many aspects of the HBV and HDV life cycles have been characterized in great detail, others, including viral entry, are still unexplored due to the lack of robust infectious tissue culture systems. Recently, the human sodium/taurocholate cotransporting polypeptide (hNTCP), a bile acid transporter expressed at the basolateral membrane of human hepatocytes, has been identified as a functional receptor of HBV and HDV.[8,9] Exogenous expression of hNTCP in permissive, nonsusceptible human hepatoma cells, such as HepG2 or Huh7, confers susceptibility to HBV and/or HDV infection, thereby constituting cell culture models for HBV/HDV entry.[8-10] However, their limited robustness and the requirement of dimethylsulfoxide (DMSO) and polyethylene glycol (PEG) preclude their use in a high-throughput format and for functional genomics.

Heparan sulfate proteoglycans (HSPGs), composed of a protein core carrying heparan sulfate glycosaminoglycan chains, are widely expressed at the cell surface of mammalian cells in a cell-specific and tissue-specific manner.[11] Many viruses use HSPGs for cell surface attachment and entry.[11,12] HSPGs contribute to HBV/HDV entry, as treatment of target cells or virions with heparinase or soluble heparin (a homologue of a highly sulfated prototype member of the glycosaminoglycan family), respectively, inhibits viral infection.[13,14] HBV is believed to first attach to HSPGs at the hepatocyte surface, through the antigenic loop of the HBV envelope protein[15] and possibly a contribution of the preS1 domain of the large HBV envelope protein.[13] Subsequently, HBV binding to NTCP is engaged by the preS1 domain, which interacts with a discrete domain of NTCP.[8,9] However, the detailed HBV/HDV entry mechanisms are still poorly understood.[10,16,17] Particularly, the HSPG core protein responsible for HBV/HDV attachment remains to be identified.

Viral entry is an important antiviral target complementing direct-acting antivirals targeting the viral polymerase. Indeed, a myristoylated HBV preS1-derived peptide (Myrcludex B) and cyclosporin A (CsA)[18,19] potently inhibit HBV and HDV entry,[17] and Myrcludex B has shown antiviral efficacy in humans.[20] Unraveling the HBV/HDV entry process may thus uncover further antiviral targets.

In this study, we established a robust HDV infection assay based on a novel NTCP-expressing Huh7 cell line that does not require the use of infection-enhancing treatments with DMSO and PEG. Using an RNA interference (RNAi)–based loss-of-function screen, we showed that this model system is amenable to functional genomics and identified glypican 5 (GPC5) as a previously undiscovered HBV and HDV entry factor and antiviral target.

Materials and Methods

Patient Samples. Human material including serum from patients with chronic HBV/HDV infection followed at the Strasbourg University Hospitals, Strasbourg, France, was obtained with informed consent. Primary human hepatocytes (PHHs) were obtained from liver tissue from patients undergoing liver resection for liver metastasis at the Strasbourg University Hospitals with informed consent. Protocols were approved by the local ethics committee of the Strasbourg University Hospitals (CPP 10-17).

Cell Lines and Human Hepatocytes. Huh7[21] and HepG2[22] cells have been described. PHHs were isolated and cultured as described.[22] The HepAD38 cell line is an inducible human hepatoblastoma cell line harboring an integrated tetracycline-responsive 1.2-fold HBV genome (serotype ayw, genotype D) and has been described.[23]

Reagents. DMSO and PEG 8000 were from Sigma-Aldrich; CsA was from Sandimmun (Novartis, Switzerland). HBV preS1-derived peptide, HBV preS1-derived peptide labeled with Alexa Fluor 647 fluorophore, and scrambled peptide control were synthesized by Bachem (Switzerland). The enhanced chemiluminescence reagent and Hyperfilms were from GE Healthcare.

Small Interfering RNAs Used for Functional Studies. ON-TARGETplus small interfering RNA (siRNA) pools (Dharmacon) targeting the transcripts of SLC10A1 (NTCP), syndecan (SDC) family genes, GPC family genes, HSPG2 (perlecan), and agrin were reverse-transfected into cells with Lipofectamine RNAiMAX (Invitrogen) as described.[22] Subsequently, an individual siRNA of the pool (Dharmacon) targeting GPC5

Address reprint requests to: Thomas F. Baumert, M.D., Inserm U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, 3 Rue Koeberlé, 67000 Strasbourg, France. E-mail: thomas.baumert@unistra.fr; tel: +33 3 68 85 37 03; fax: +33 3 68 85 37 24. Or: Camille Sureau, Ph.D., Laboratoire de Virologie Moléculaire, Institut National de la Transfusion Sanguine, 6 rue Alexandre-Cabanel, 75739 Paris, France. E-mail: csureau@ints.fr; fax: +33 1 44 49 30 56.

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was used: GPC5 no. 3, targeted sequence CUUCAAAC GUCCAGCUCUA.

Establishment of Stable NTCP-Overexpressing Hepatoma Cell Lines. The NTCP expression vector was generated containing the full open reading frame of human NTCP complementary DNA (SLC10A1; GenBank L21893.1) inserted between the *Hind*III and *Xba*I restriction sites of the pRc-CMV plasmid (Invitrogen). Huh7 cells were cultured in William's medium E supplemented with 10% fetal bovine serum (Fetaclone-II; Thermo Scientific) and gentamicin. After transfection of Huh7 cells with pRc-CMV-NTCP plasmid DNA, transfected cells were selected for neomycin (G418) resistance, and isolated clones were tested for susceptibility to HDV infection as described.[24] One clone (referred to here as Huh-106) was selected based on the high level of intracellular HDV RNA that accumulated 9 days postinoculation (dpi) with HDV virions. Huh-106 cells were maintained at a concentration of 250 μg/mL G418. HepG2 cells were seeded in six-well plates at 50% confluence 1 day prior to transduction with human NTCP-expressing vesicular stomatitis virus pseudoparticles (pp) (GeneCopoeia). After 3 days, cells were expanded and selected for NTCP expression with 0.9 μg/mL puromycin. HepG2-NTCP cells were maintained at a concentration of 0.9 μg/mL puromycin.

Analysis of Messenger RNA Expression by Quantitative Real-Time Polymerase Chain Reaction. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). Gene expression in the total RNA extracts was assessed using quantitative real-time polymerase chain reaction (qRT-PCR). Reverse-transcription of total RNA extracts was performed using MAXIMA reverse-transcriptase (Thermo Scientific). Quantitative PCR was performed using a Corbett rotor gene 6000 (Qiagen) following the manufacturer's instructions. Primers and TaqMan probes for NTCP, GPC5, SDC4, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) detection were from Applied Biosystems. All values were normalized to GAPDH expression.

Protein Expression Analysis. Immunoblots of cell lysates using protein–specific antibodies (rabbit polyclonal anti-NTCP antibody [Sigma; HPA042727], rabbit anti-GPC5 monoclonal antibody [Abcam; EPR6756(B)], and mouse anti-β-actin monoclonal antibody [Sigma; A5441]) were performed following GE Healthcare protocols using Hybond-P membranes and visualized using enhanced chemiluminescence western blotting detection reagents following the manufacturer's instructions. For western blot detection of NTCP, cell lysates were pretreated with peptide-N-glycosidase (New England Biolabs, Evry, France) following the manufacturer's instructions. For fluorescent detection of NTCP protein in cells, an HBV preS1-derived peptide labeled with Alexa Fluor 647 fluorophore (Bachem, Switzerland) was used as described.[8,9] Cells were treated with the peptide for 1 hour at 37°C and then fixed with 4% paraformaldehyde (PFA). Fluorescent imaging was performed using an Axio Observer Z1 microscope (Carl Zeiss, Germany). NTCP expression at the cell surface was quantified by flow cytometry as described.[8,22]

HDV Production and Infection. The HDV recombinant plasmid pSVLD3 and the HBV expression vector pT7HB2.7 were used for production of HDV ribonucleoprotein and of L, M, and S HBV surface antigen (HBsAg) proteins.[25] Huh7.5.1 cells were transfected with the two plasmids using FuGENE HD Transfection Reagent. HDV-containing supernatants were harvested 9 days after transfection and subsequently used for infection experiments. For HDV infection, Huh-106 cells were plated in 96-well or 384-well plates and maintained in culture for 1 day without DMSO. HBV preS1-derived peptide or scrambled peptide control (control), CsA, rabbit anti-GPC5 antibody, rabbit anti-SDC4 antibody (Life Technologies; 36-3100), and rabbit control immunoglobulin G (IgG; Invitrogen) were added to the cell medium 1 hour before infection with recombinant HDV with or without PEG at the indicated concentrations. Cells were cultured in primary hepatocyte maintenance medium[8] containing 2% DMSO following infection to slow cell growth. HDV infection was assessed 7 dpi by immunofluorescence (IF) or qRT-PCR.

Detection of Hepatitis Delta Antigen and HDV RNA in Infected Cells. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% PFA. Nonspecific binding sites were saturated using 0.5% bovine serum albumin, and cells were permeabilized using 0.05% saponin. Cells were stained with an antibody targeting the hepatitis delta antigen (HDAg) purified from serum of an HBV/HDV coinfected patient[26] and Alexa Fluor 647-labeled secondary antibody targeting human IgGs (Jackson Research). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole. Fluorescent imaging was performed using an Axio Observer Z1 microscope (Carl Zeiss, Germany). Total RNA extraction and reverse-transcription were performed as described above. Quantitative PCR was performed using the SensiFAST Probe No-ROX Kit (Bioline) and Corbett rotor gene 6000 (Qiagen) following the manufacturer's instructions. The following specific primers for HDV RNA quantification were used: forward primer 5′-GCATGGTCCCAGCCT CC-3′, reverse primer 5′-CTTCGGGTCGGCATGG-3′; TaqMan probe 5′-[fluorescein amidite]-ATGCCCAGGT

CGGAC-[Black Hole Quencher-1]-3′. All values were normalized to GAPDH (Applied Biosystems) expression.

HBV Production and Infection. HBV infectious particles from sera of HBV carriers (genotype D) were concentrated with a 30% sucrose cushion and purified using a 10%-45% iodixanol density gradient. Recombinant HBV (strain ayw, genotype D) was obtained by 100-fold concentration of supernatant of HepAD38 cells using 8% PEG.[23] HepG2-NTCP cells were plated in 96-well plates and maintained in culture for 1 day without DMSO prior to HBV infection in the presence of 4% PEG. Cells were cultured in 2% DMSO primary hepatocyte maintenance medium following infection to slow cell growth. HBV infection was assessed 10 dpi by immunodetection of HBsAg using an HBsAg-specific monoclonal antibody (NCL-HBsAg-2, clone 1044/341; Leica Biosystems) and by qRT-PCR quantification of HBV pregenomic RNA (pgRNA) using the following primers and probe[27]: forward primer 5′-GGTCCCCT AGAAGAAGAACTCCCT-3′, reverse primer 5′-CATT GAGATTCCCGAGAT TGAGAT-3′; TaqMan probe 5′-[6-fluorescein amidite]-TCTCAATCGCCGCGTC GCAGA-[carboxytetramethylrhodamine]-3′. All values were normalized to GAPDH (Applied Biosystems) expression.

Analysis of HBV and HDV Binding to Liver Cells. Huh-106 and HepG2-NTCP cells were incubated for 1 hour at 37°C with a GPC5-specific or control antibody at various time points before and after virus inoculation. Recombinant HBV was pretreated with PBS, heparin (30 μg/mL), rGPC5 (5 μg/mL and 30 μg/mL; R&D Systems), or rSDC4 (30 μg/mL; R&D Systems) for 30 minutes at 37°C. Cells were washed twice with cold PBS and then incubated with pretreated HBV or HDV in the presence of 4% PEG for 24 hours at 16°C. Unbound virions were removed by three washes with PBS, and cells and bound virions were lysed. A 24-hour incubation period was chosen based on previous observations showing the requirement of a >16-hour virus/cell exposure[28,29] and given that transcription from incoming viral DNA and *de novo* production of virus are not detectable prior to 2 and 3 dpi, respectively.[29] Therefore, the HBV DNA that is associated with cells at 24 hours postinoculation represents HBV particles that have attached to the cells during this time period and potentially virions that have been internalized following attachment, but does not include progeny virions. DNA was extracted from HepG2-NTCP cells according to the QiaAMP DNA MiniKit protocol (Qiagen). HBV total DNA was detected by qPCR using the following primers and probe[27]: forward primer 5′-CA CCTCGCCTAATCATC-3′, reverse primer 5′-GGAAA GAAGTCAGAAGGCA-3′; TaqMan probe 5′-[fluorescein amidite]-TGGAGGCTTCAACAGTAGGACATGAA C-[carboxytetramethylrhodamine]-3′. A standard curve generated from known HBV genome copies was used for quantification. Values were normalized to initial DNA concentration. Total RNA was extracted from Huh-106 cells and HDV RNA was quantified as described above.

Analysis of HBV Replication in Huh-106 Cells With Silenced GPC5 Expression. Huh-106 cells were reverse-cotransfected with the replication-competent HBV expression plasmid adwR9[30] and GPC5-targeting or control siRNAs using Lipofectamine RNAiMAX (Invitrogen). Three days after transfection, HBV DNA was purified and quantified from lysates by qPCR as described above. Hepatitis B e antigen (HBeAg) and HBsAg in cell culture supernatants were quantified using enzyme-linked immunosorbent assays (ELISAs) N0140 and N0019 (Diasorin). Cytoplasmic HBV core antigen was quantified using a sandwich ELISA as described.[31]

Statistical Analysis. All experiments were performed at least three times in an independent manner. Statistical analyses were performed using a Student *t* test or Mann-Whitney test; $P < 0.01$ was considered statistically significant. Significant *P* values are indicated by asterisks in the individual figures.

Results

A Novel hNTCP-Overexpressing Huh7 Cell Line Readily Susceptible for HDV Infection. Aiming to develop a high-throughput system for functional genomics, we established a Huh7-NTCP cell line in which constitutive hNTCP expression is under the control of a cytomegalovirus promoter. Out of 120 single-cell clones, one, termed Huh-106, was found reproducibly susceptible to HDV infection irrespective of the culture conditions. Huh-106 cells express significantly higher levels of NTCP (mRNA and protein) than the parental Huh7 cells (Fig. 1A,B). Moreover, an AF647-labeled peptide derived from the HBV preS1 domain known to bind NTCP,[8,9] specifically bound to Huh-106 cells, demonstrating high levels of cell surface NTCP expression (Fig. 1C,D). To confirm NTCP function, Huh-106 cells were inoculated with recombinant HDV. Indeed, Huh-106 cells were susceptible to HDV entry, as demonstrated by detectable intracellular HDAg and HDV RNA (Fig. 1E,F) at 7 dpi.

Most current *in vitro* HBV/HDV infection systems require the addition of PEG to the inoculum to increase infection.[8,9,29] PEG may modulate viral entry by bypassing virus–host factor interactions. To assess whether PEG increases viral entry in our model, we inoculated Huh-106 cells with HDV in the presence of increasing concentrations

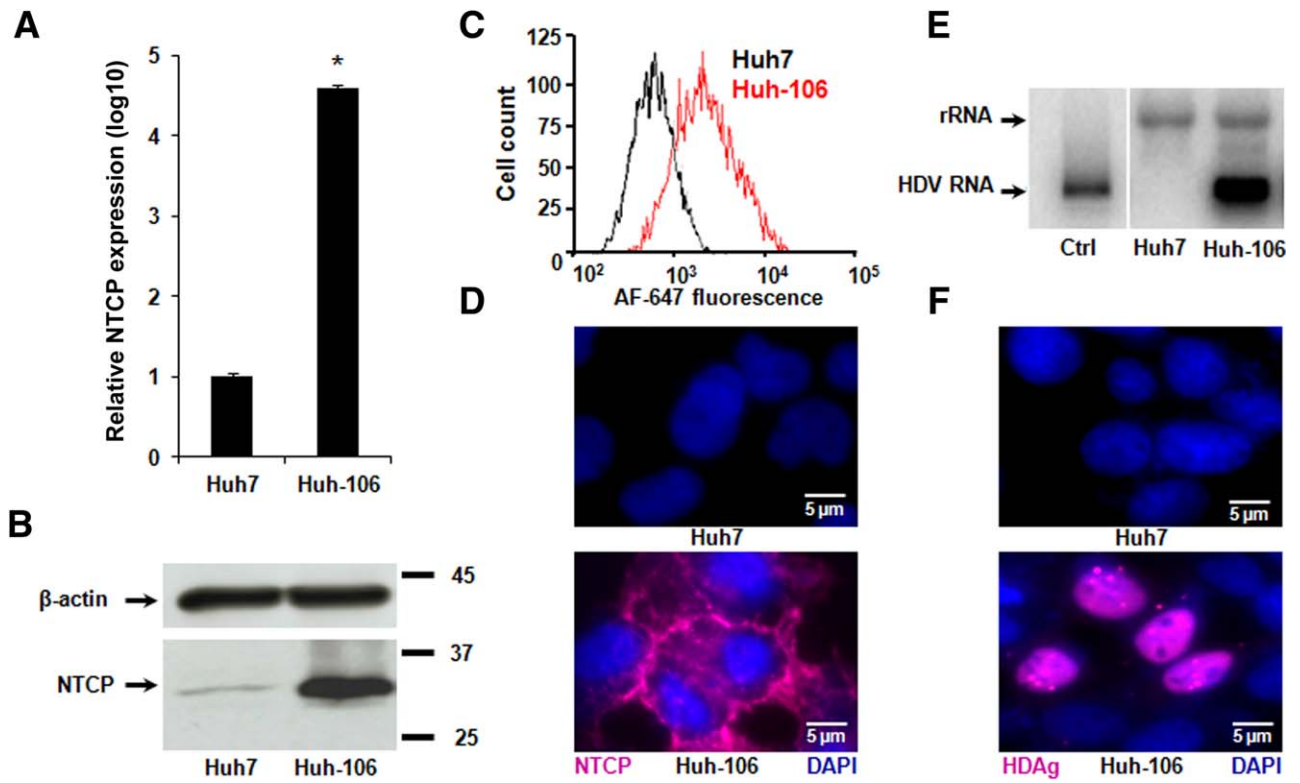


Fig. 1. Production of HDV permissive cell lines overexpressing human NTCP. (A) Relative NTCP expression in Huh-106 cells compared to parental Huh7 cells using qRT-PCR. Results are expressed as means ± standard deviation showing NTCP mRNA expression normalized to GAPDH mRNA expression from three experiments performed in duplicate. (B) Detection of NTCP protein by western blot. Cell lysates were treated with peptide-N-glycosidase and subsequently subjected to immunoblotting using a mouse monoclonal anti-NTCP antibody (Sigma). One representative experiment is shown. (C) Quantification of NTCP at the cell surface by flow cytometry using a labeled HBV preS1-derived peptide. Cells were treated for 1 hour with Alexa Fluor 674–labeled HBV preS1-derived peptide (200 nM) and fixed with 4% PFA. NTCP expression was assessed by flow-cytometric quantification of bound preS1 peptide. One representative experiment is shown. (D) Fluorescence microscopic analysis of NTCP expression in Huh7 and Huh-106 cells. Cells were treated with Alexa Fluor 674–labeled HBV preS1-derived peptide (200 nM) and fixed with 4% PFA before visualization using fluorescence microscopy. HBV preS1-derived peptide binding is shown in red. One representative experiment is shown. (E,F) Functional evaluation of NTCP cell lines using HDV infection. Huh-106 and Huh7 cells were infected with recombinant HDV for 7 days. (E) Total RNA was purified and HDV RNA was detected by northern blot. Control corresponds to approximately 2×10^7 HDV RNA genome equivalents extracted from HDV particles produced in Huh7 cells. One representative experiment is shown. (F) HDV-positive cells were visualized in red after HDAg immunodetection with an anti-HDAg antibody. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). One representative experiment is shown. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; rRNA, ribosomal RNA.

of PEG. Approximately 15%-25% of Huh-106 cells were infected in the absence of PEG treatment compared to 30% of HDAg-positive cells after 6% PEG treatment (Fig. 2A,B). Notably, a two-fold increase of HDV-positive cells is observed in the presence of 8% PEG but with significant toxicity (Fig. 2B). Silencing of NTCP expression with siRNA prior to inoculation (Fig. 2C) led to a significant decrease (from 22% to 7%) of HDV-positive cells when cells were inoculated in the absence of PEG. In contrast, in the presence of 6% PEG, only a slight but insignificant decrease of infection was observed (Fig. 2D), suggesting that HDV infection in the absence of PEG is more susceptible to RNAi-based perturbation studies. These data indicate that our model efficiently supports HDV infection in the absence of PEG.

Treatment of cell cultures with DMSO prior to inoculation has also been shown to improve HBV and HDV infection, possibly by modifying NTCP trafficking.[8,9,32] When Huh-106 cells were treated for 2 days with 2% DMSO prior to inoculation (Fig. 2E), no change in NTCP localization at the cell surface or in HDV infection was observed. Taken together, our data show that the Huh-106 cells are readily susceptible to HDV infection in the absence of PEG and DMSO and, hence, in conditions amenable to functional genomics and RNAi-based loss of function studies.

Development of a High-Throughput System for Functional Perturbations of the HDV Life Cycle. To establish a high-throughput model enabling perturbation studies of the HDV life cycle, we applied an automated imaging system for detection of viral infection using a 96-well or a 384-well plate format and immunodetection of intracellular HDAg. As HDAg localizes in Huh7 cells predominantly to the nucleus (Fig. 1F), a

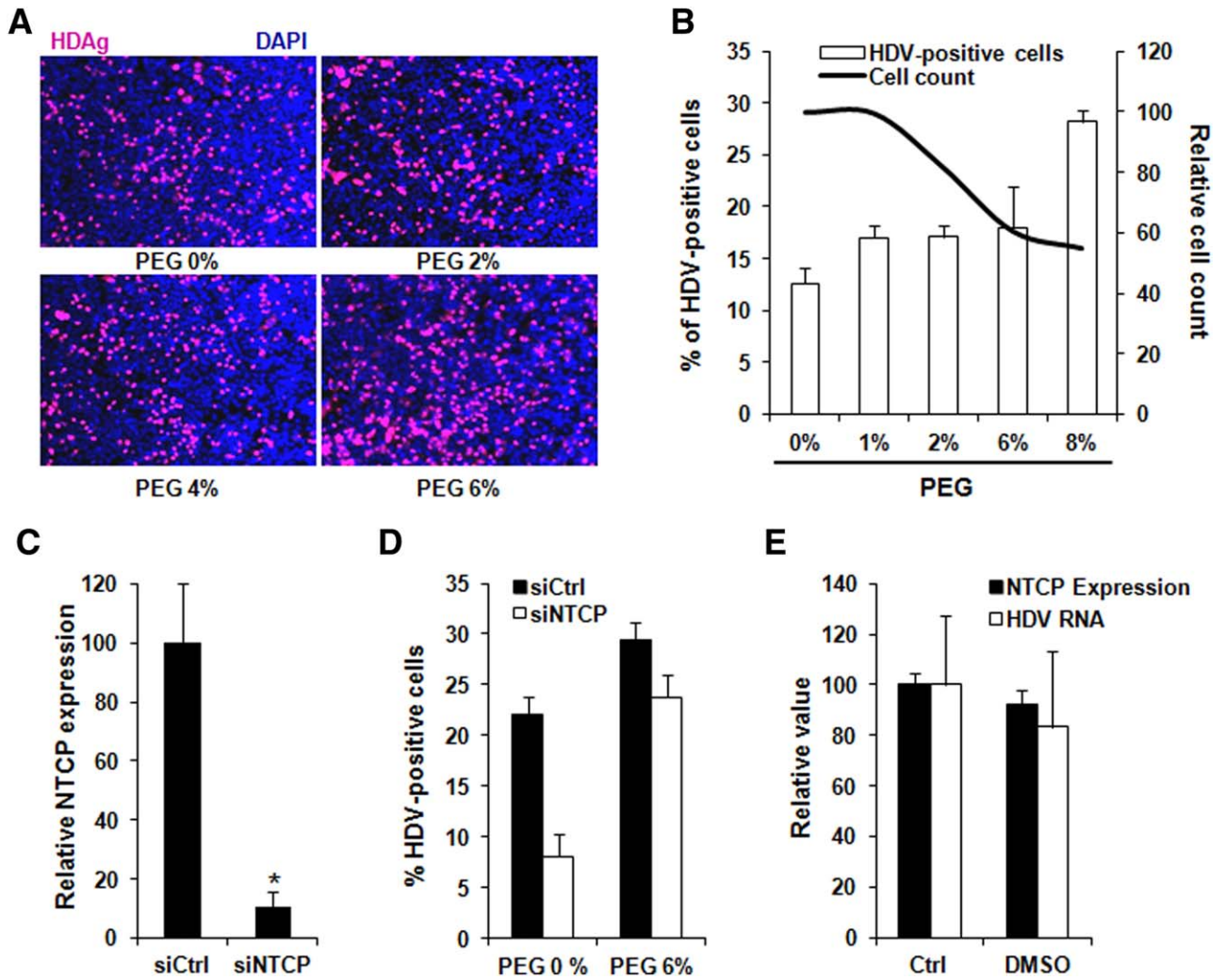


Fig. 2. HDV infection in Huh-106 cells is PEG-independent and DMSO-independent. (A) The effect of PEG on HDV infection. Huh-106 cells were infected with HDV in the presence of increasing concentrations of PEG (0%, 2%, 4%, 6%). Infection was assessed 7 dpi by IF. (B) Effect of PEG on cell viability. Huh-106 cells were infected with HDV in the presence of increasing concentrations of PEG. Infection was measured 7 dpi by quantification of HDV-positive cells. Results are expressed as mean ± standard deviation percentage of HDV-infected cells from one representative experiment performed in triplicate. The number of cells 7 dpi was evaluated by quantification of viable 4′,6-diamidino-2-phenylindole–stained nuclei. Results are expressed as means ± standard deviation percentage of cells relative to cells infected in the absence of PEG from one representative experiment performed in triplicate. (C,D) Effect of PEG treatment on silencing of NTCP expression. NTCP expression was silenced in Huh-106 cells. Two days posttransfection silencing efficacy was assessed by qRT-PCR (C), and cells were infected with HDV in the presence or absence of 6% PEG. Infection was measured 7 dpi by quantification of HDV-positive cells (D). Results are expressed as means ± standard deviation percentage of infected cells from three independent experiments performed in triplicate. (E) Effect of DMSO treatment on NTCP expression and HDV infection. Huh-106 cells were treated for 2 days with 2% DMSO. NTCP expression at the cell surface was assessed by flow cytometry as described in Fig. 1. Cells were then infected with HDV, and infection was assessed 7 dpi by quantification of HDV RNA. Results are expressed as means ± standard deviation percentage NTCP expression at the cell surface or percentage HDV infection relative to control from three independent experiments performed in triplicate. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

simple 4′,6-diamidino-2-phenylindole–HDAg costaining allows for discrimination of infected versus noninfected cells. To determine the best conditions for infection assay, HDAg detection was performed at 5, 7, and 9 dpi. While about 5% of cells were HDAg-positive at 5 dpi, 20% were positive at 7 dpi (Fig. 3A-D), and no further increase was observed at 9 dpi (Fig. 3A). Furthermore, the level of intracellular HDV RNA was measured at 5, 7, and 9 dpi. A significant increase was observed between 5 and 7 dpi, and no further increase was observed at 9 dpi (Fig. 3C).

According to these data, we developed a functional high-throughput HDV infection protocol by infecting Huh-106 cells in 96-well or 384-well plates with HDV (without PEG and DMSO preincubation) for 7 days before quantification of HDAg-positive cells using IF. To ascertain the robustness of this model to monitor inhibition of HDV infection, we silenced NTCP

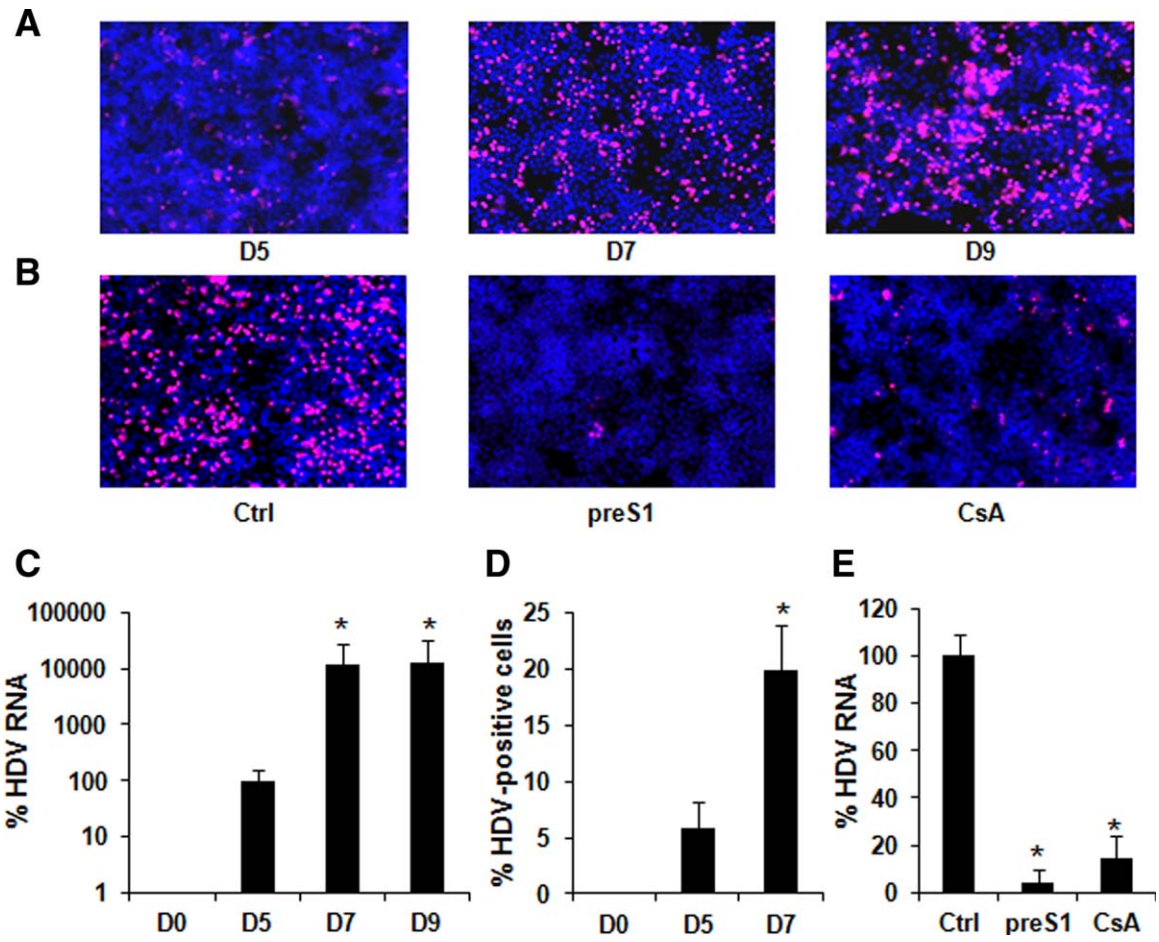
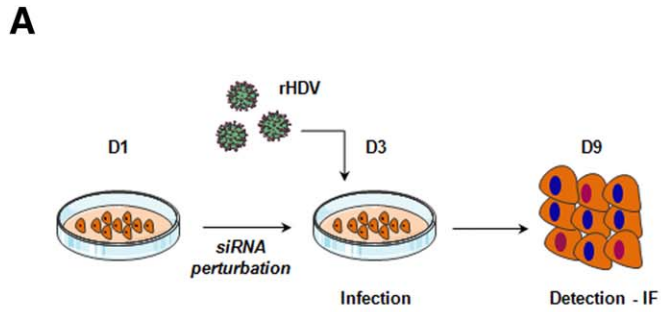


Fig. 3. A high-throughput assay for drug screening and functional genomics of HDV infection using Huh-106 cells. (A,C,D) HDV time-course of infection. Huh-106 cells were infected in 96-well (A,C) and 384-well (D) plates with HDV for 5, 7, and 9 days. (A,D) Infection was assessed by quantification of HDAg-positive cells after HDAg immunodetection. One representative experiment (A) and means ± standard deviation percentage HDV-positive cells from three independent experiments performed in triplicate (D) are shown. (C) Total RNA was extracted after 5, 7, and 9 dpi; and HDV infection was assessed by qRT-PCR quantification of HDV RNA normalized to GAPDH mRNA. Results are expressed as means ± standard deviation relative to the HDV RNA level at 5 dpi, set at 100% from three experiments performed in triplicate. (B,E) Inhibition of HDV infection using NTCP inhibitors. Cells were treated for 1 hour with HBV-derived preS1 peptide (200 nM) or with CsA (8 μM) and subsequently infected with HDV for 7 days. Infection was assessed by HDAg immunodetection (B) or qRT-PCR quantification of HDV RNA (E). Results are expressed as means ± standard deviation percentage HDV infection from three independent experiments performed in triplicate.

expression using siRNA and incubated Huh-106 cells with NTCP inhibitors. As shown by a marked decrease in the number of HDAg-positive cells (Fig. 3B) and HDV RNA levels (Fig. 3E), HDV infection was impaired by preincubation with an HBV preS1-derived peptide or CsA, which are known to block NTCP function,[8,9,18,33] and following silencing of NTCP expression (Fig. 2D). Collectively, these data demonstrate that our model is suitable for high-throughput perturbation screens.

A Targeted RNAi Entry Screen Identifies GPC5 as a Host Cell Entry Factor. We next applied this cell-based model for a targeted RNAi HDV entry screen (Fig. 4A). Given the importance of HSPGs for HDV/HBV attachment and entry, we investigated their role in HDV infection by silencing the expression of all genes belonging to the HSPG families (Fig. 4B). Huh-106 cells were transfected with pools of four siRNAs 2 days before exposure to HDV for 24 hours (Fig. 4A). Only silencing of GPC5 expression inhibited HDV infection, leading to a 45% decrease in the number of HDAg-positive cells at 7 dpi (Fig. 4B). Nonspecific toxic effects were excluded by two independent cell viability assays (Supporting Fig. S1). To validate the role of GPC5 in HDV entry, we performed additional silencing studies using individual GPC5-specific siRNAs. A 50% decrease in the number of HDAg-positive cells and a strong reduction of HDV RNA were observed after transfection of Huh-106 cells with GPC5 siRNA #3, similar to the siRNA pool (Fig. 4C,D). In contrast, only a minimal effect on HDV infection was observed after silencing of SDC2, an HSPG that was not identified in



B

Gene symbol	Gene - family	HDV-positive cells (% relative to Ctrl)	Cell count (% relative to Ctrl)
Ctrl		100.0 ± 1.1	100.0 ± 1.4
SDC1	Syndecan	91.3 ± 3.3	105.6 ± 1.9
SDC2		75.4 ± 0.9	118.0 ± 3.9
SDC3		81.2 ± 2.1	93.0 ± 11.0
SDC4		87.0 ± 0.4	118.2 ± 4.1
GPC1	Glypican	83.3 ± 1.5	111.0 ± 1.4
GPC2		80.2 ± 1.4	115.4 ± 5.4
GPC3		88.6 ± 1.6	99.7 ± 0.4
GPC4		83.4 ± 1.5	115.6 ± 8.4
GPC5		**55.7 ± 3.3**	**98.7 ± 5.9**
GPC6		89.0 ± 1.6	110.9± 4.0
HSPG2	Perlecan	117.5 ± 2.1	81.7 ± 6.9
AGRN	Agrin	95.6 ± 1.5	108.8 ± 9.5

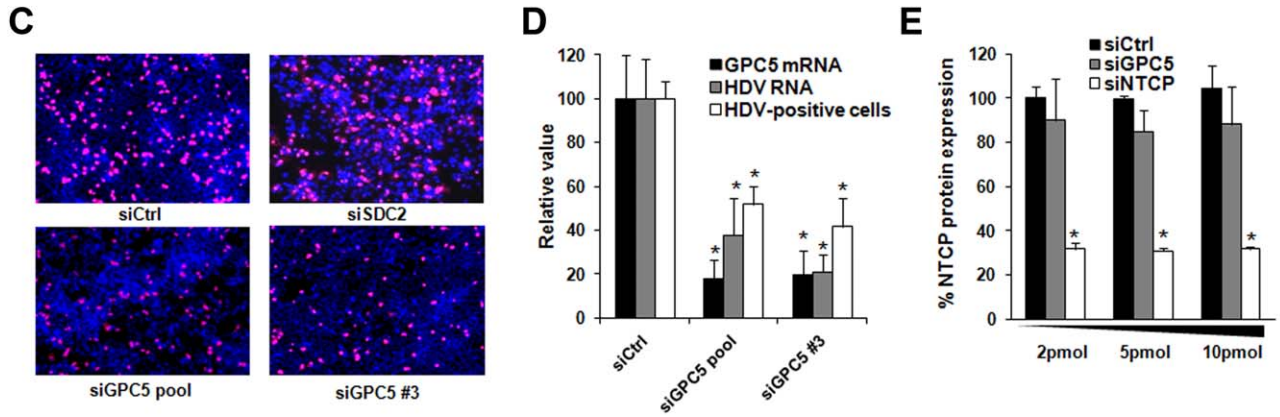


Fig. 4. A targeted functional RNAi HDV entry screen uncovers GPC5 as an HDV entry factor. (A) Flowchart of the siRNA screen. Huh-106 cells were reverse-transfected with siRNAs 48 hours prior to infection with HDV for 24 hours. HDV infection was measured 7 dpi by IF and quantification of HDV-positive cells. (B) Effect of HSPG gene silencing on HDV infection. Cells were transfected with siRNAs targeting the transcripts of genes belonging to members of different HSPG families and then infected as described in Fig. 3. Results are expressed as means ± standard deviation percentage HDV-positive cells or percentage cell count relative to control siRNA transfected cells from one experiment performed in triplicate. (C,D) Confirmation of GPC5 involvement in HDV infection using an individual siRNA. Huh-106 cells were silenced using an SDC2-specific siRNA pool, a GPC5-specific siRNA pool, or the individual GPC5-specific siRNA #3 for 48 hours and then infected with HDV. Infection was revealed 7 dpi by IF (C), quantification of HDV-positive cells, and qRT-PCR quantification of HDV RNA (D). Silencing efficacy was assessed by qRT-PCR (D). Results are expressed as means ± standard deviation percentage mRNA expression, HDV-positive cells, or HDV RNA from three independent experiments performed in triplicate. (E) Effect of GPC5 silencing on NTCP expression at the cell surface. Huh-106 cells were silenced for NTCP of GPC5 expression for 2 days using increasing quantities of siRNA. NTCP expression at the cell surface was assessed by flow cytometry. Results are expressed as means ± standard deviation percentage NTCP expression from three independent experiments performed in triplicate.

the RNAi screen. To verify that GPC5 silencing did not simply affect NTCP expression, we evaluated NTCP expression in GPC5-silenced Huh-106 cells. NTCP expression at the cell surface was not affected by GPC5 silencing (Fig. 4E). These data identify GPC5 as an HDV entry factor candidate.

GPC5 Mediates HDV Entry Into Human Hepatocytes. To validate the role of GPC5 as an HDV entry factor, we performed mechanistic studies using an antibody targeting GPC5. The GPC5-specific antibody inhibited HDV infection of Huh-106 cells in a dose-dependent manner, contrary to an antibody targeting SDC4, another HSPG expressed in the liver and involved in hepatitis C virus (HCV) attachment[34] but not identified in our RNAi screen (Fig. 5B). Finally, we validated the functional role of GPC5 as an HDV entry factor in PHHs, the natural target cells of HDV, which express high levels of GPC5 protein (Fig. 5A). PHH cultures were silenced for GPC5 expression using siRNAs (Fig. 5C), inoculated with HDV before assessing viral infection using IF. In contrast to cell lines, HDAg was detected predominantly in the cytoplasm of PHHs. Silencing of NTCP and GPC5 similarly resulted in a marked decrease of HDAg-positive cells (Fig. 5D), in agreement with a functional role of GPC5 as an entry factor for HDV infection of human hepatocytes.

GPC5 Mediates HBV, but not HCV, Entry. Because HBV and HDV are believed to use the same receptors and early steps of viral entry, we next evaluated whether GPC5 is also an entry factor for HBV. Because HepG2

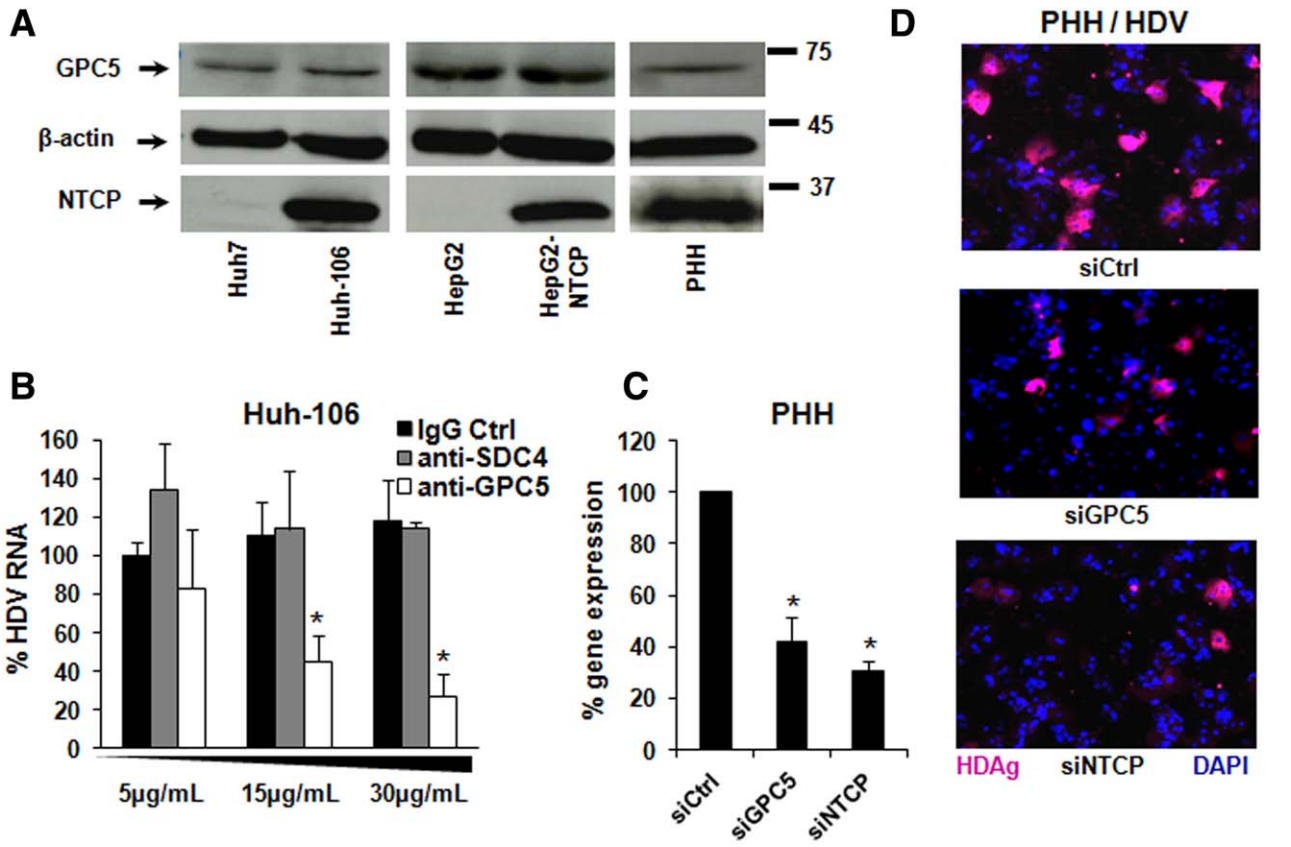


Fig. 5. GPC5 is a specific HDV entry factor in hepatoma cells and human hepatocytes. (A) GPC5 and NTCP protein expression in different cell culture models assessed by western blot. (B) Inhibition of HDV infection using anti-GPC5 antibody. Huh-106 cells were treated for 1 hour with an antibody targeting GPC5, an antibody targeting SDC4, or a control IgG at different concentrations. Cells were then infected with HDV in the presence of the antibody. Infection was assessed 7 dpi by qRT-PCR quantification of HDV RNA. Results are expressed as means ± standard deviation percentage HDV infection from three independent experiments performed in triplicate. (C,D) GPC5 silencing inhibits HDV infection in PHHs. PHHs were transfected with siRNAs targeting GPC5 or NTCP 2 days prior to infection with HDV for 7 days. Silencing efficacy was assessed by qRT-PCR of GPC5 and NTCP mRNA (C), and HDV infection was assessed by IF as described in Fig. 1 (D). One representative experiment is shown. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

cells were shown to be better adapted to HBV infection assays than Huh7 cells,[8,9] we established an HepG2-NTCP cell line for productive HBV infection, as confirmed by immunodetection of HBsAg and HBV-specific pgRNA postinoculation (Supporting Fig. S2). The specificity of RT-PCR for pgRNA, but not viral DNA, was confirmed by the lack of signal upon prior DNAse treatment (Supporting Fig. S2). Inhibition of HBV infection using a preS1-derived peptide (Supporting Fig. S2) and time-course experiments demonstrating *de novo* synthesis of HBeAg (assessed by ELISA), HBsAg (assessed by IF), covalently closed circular DNA (assessed by qPCR), and HBV RNA (assessed by qRT-PCR of HBV pgRNA or total HBV RNA using RNA-seq) confirmed that the signals were from *de novo* synthesis and not from incoming virions (Supporting Fig. S3).

To determine whether GPC5 is also required for HBV infection, GPC5 expression was silenced in HepG2-NTCP cells. Silencing of GPC5 expression decreased HBV infection, as measured by the number of HBsAg-positive cells and the level of intracellular HBV pgRNA at 10 dpi, similar to observations following NTCP silencing (Fig. 6A-D). To assess whether GPC5 is a specific entry factor for HBV and HDV or may also contribute to HCV entry, we studied its functional role for HCV entry using HCVpp. The silencing of GPC5 expression does not affect HCVpp entry (Fig. 6E,F), indicating that GPC5 specifically mediates HBV and HDV entry into hepatocytes.

GPC5 Mediates Attachment of the Virion to the Target Cell Surface. To investigate the steps of the HBV entry process involving GPC5, we tested its role in HBV binding to HepG2-NTCP cells. Similar to pretreatment of virions with heparin, which is known to inhibit HBV-HSPG binding, silencing of GPC5 expression significantly decreased HBV binding (Fig. 7A), strongly suggesting that GPC5 is a major player in HBV/HDV attachment at the cell surface of hepatocytes. To ascertain

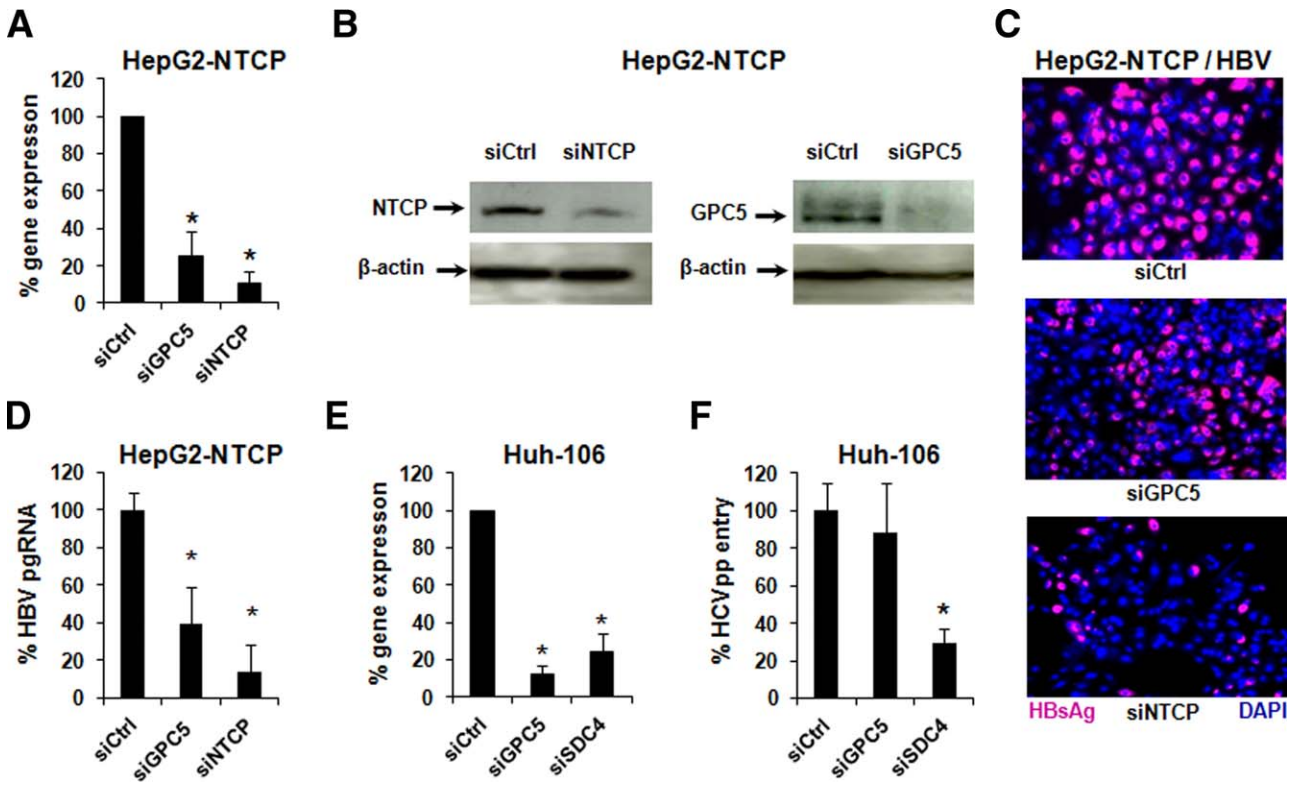


Fig. 6. GPC5 is an HBV host entry factor. (A-D) GPC5 silencing inhibits HBV infection in HepG2-NTCP cells. HepG2-NTCP cells were transfected with siRNAs targeting GPC5 and NTCP. Silencing efficacy was assessed after 2 days by qRT-PCR (A, means ± standard deviation percentage mRNA expression normalized to GAPDH mRNA expression from three independent experiments performed in duplicate) and western blot (B, one representative experiment is shown). Cells were then infected by HBV for 10 days, and infection was detected by IF (C, one representative experiment is shown) and qRT-PCR quantification of HBV pgRNA (D). Results are expressed as means ± standard deviation percentage HBV pgRNA levels from three independent experiments performed in triplicate. (E,F) GPC5 silencing does not affect HCVpp entry. Huh-106 cells were transfected with siRNAs targeting GPC5 and SDC4 48 hours prior to infection with HCVpp genotype 1b for 3 days. Silencing efficacy was assessed by qRT-PCR (E). HCVpp entry was measured by quantification of luciferase activity (F). Results are expressed as means ± standard deviation percentage HCVpp entry relative to control siRNA transfected cells from three independent experiments performed in triplicate. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

the ability of GPC5 to interact with the HBV envelope, we performed a competition experiment in which HBV was pretreated with soluble recombinant GPC5 protein (rGPC5). Recombinant GPC5 neutralized HBV at concentrations similar to heparin, unlike soluble recombinant SDC4 (rSDC4) (Fig. 7B). Moreover, pretreatment of Huh-106 and HepG2-NTCP cells with an anti-GPC5 antibody inhibited HDV and HBV binding, respectively (Fig. 7C). However, HBV binding was no longer inhibited when the anti-GPC5 antibody or recombinant protein was added 12 hours after virus inoculation (Fig. 7D; Supporting Fig. S4), confirming that GPC5 plays a role in the early steps of virus entry. Furthermore, to rule out that GPC5 inhibits HBV infection by interfering with other steps of the HBV life cycle, we studied the effect of GPC5 silencing on HBV replication in stably HBV-replicating HepAD38 cells.[23] Neither HBsAg nor HBV pgRNA was affected by silencing of GPC5 expression, suggesting that GPC5 has no functional role in HBV replication (Fig. 8A,B). To confirm that GPC5 has no effect on HBV replication when HBV DNA and GPC5 siRNA are present in the same cells, Huh-106 cells were cotransfected with siRNA and a plasmid encoding the HBV genome prior to measurement of soluble HBsAg and HBeAg in cell culture supernatants and quantification of viral capsids (by HBV core antigen ELISA) and viral DNA (by qPCR) in cytoplasmic lysates. Using this approach, HBV DNA and protein levels were not affected by GPC5 silencing (Fig. 8C,D,E). Collectively, these data demonstrate that GPC5 contributes to viral entry by mediating the initial attachment of HBV to its target cell.

Discussion

Here, we report a novel cell-based infection model system that allows the screening of host factors involved in HDV infection in functional assays (Fig. 4). Using this model and a loss-of-function approach, we identified GPC5 as an HDV/HBV entry factor. The

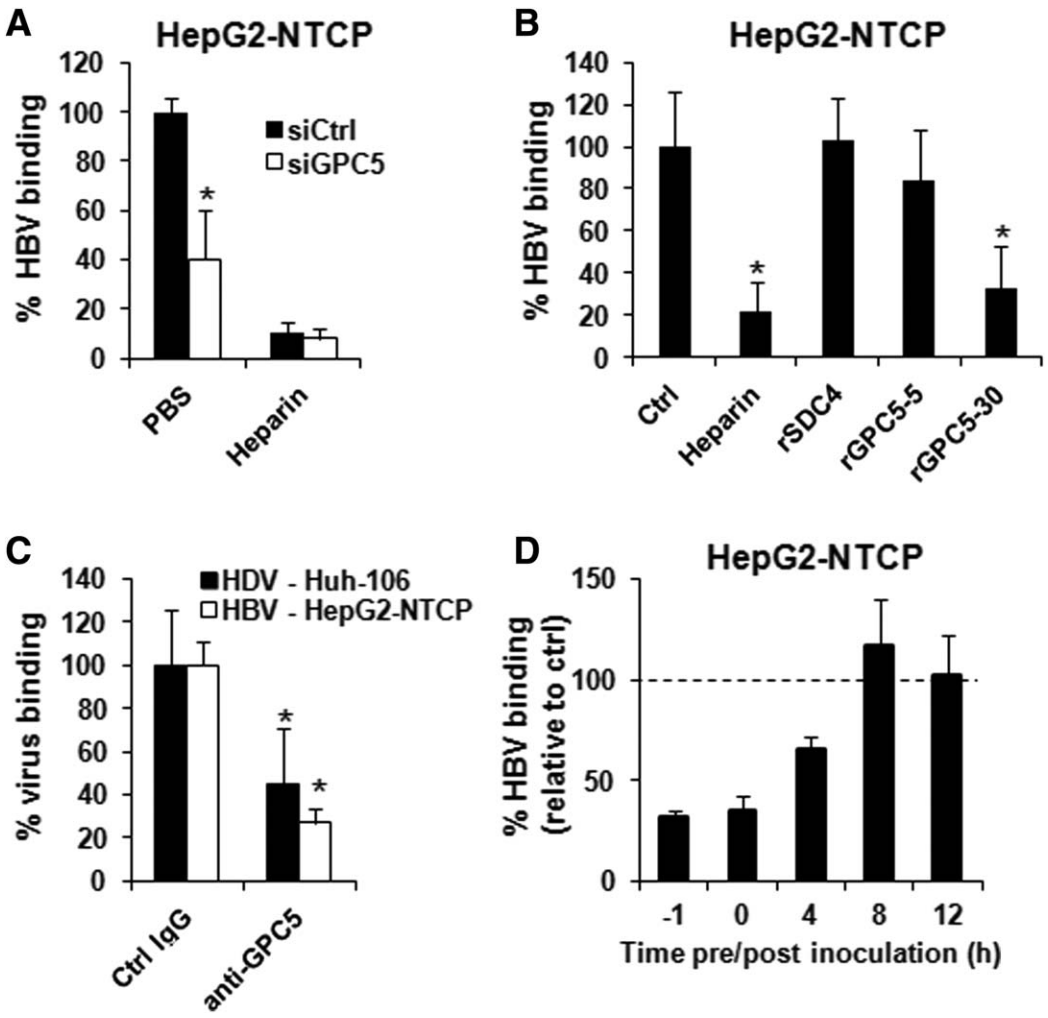


Fig. 7. GPC5 mediates binding of HBV and HDV virions to the liver cell surface. (A) GPC5 silencing inhibits HBV binding. HepG2-NTCP cells silenced for GPC5 expression were treated for 24 hours at 16°C with HBV particles pretreated or not (PBS) with heparin (30 μg/mL). HBV binding was measured by qPCR quantification of total HBV DNA bound to cells after 24 hours. Results are expressed as means ± standard deviation percentage HBV binding relative to control siRNA transfected cells treated with PBS from three independent experiments performed in duplicate. (B) Neutralization of HBV infectious particles using soluble recombinant GPC5 protein (rGPC5). HBV particles were pretreated for 30 minutes at 37°C with heparin (30 μg/mL), with soluble recombinant SDC4 (rSDC4, 30 μg/mL), or with rGPC5 at the indicated concentrations. HBV binding was measured by qPCR quantification of total HBV DNA bound to cells after 24 hours. Results are expressed as means ± standard deviation percentage HBV binding relative to control cells from three independent experiments performed at least in duplicate. (C) A specific anti-GPC5 antibody inhibits HDV and HBV binding at the cell surface. Huh-106 and HepG2-NTCP cells were incubated for 1 hour at 37°C with an anti-GPC5 antibody or a rabbit control IgG and then incubated for 24 hours at 16°C with HDV and HBV infectious particles, respectively. HBV and HDV binding was measured by qPCR quantification of total HBV DNA and HDV RNA bound to cells after 24 hours. Results are expressed as means ± standard deviation percentage HBV or HDV binding relative to control cells treated with a control IgG from three independent experiments performed at least in duplicate. (D) Time course of antibody-mediated inhibition of HBV binding. HepG2-NTCP cells were incubated with an anti-GPC5 antibody at various time points before and after incubation with HBV particles as indicated. HBV binding was measured by qPCR quantification of total HBV DNA bound to cells after 24 hours. Results are expressed as means ± standard deviation percentage HBV binding relative to cells incubated with control antibody from two independent experiments performed in triplicate.

functional role of GPC5 as an entry factor for these two viruses was confirmed by several lines of evidence and complementary approaches: (1) silencing of GPC5 expression decreased HDV and HBV infection in NTCP-expressing hepatoma cells and PHHs (Figs. 4–6), (2) an anti-GPC5 antibody impaired viral infection (Fig. 5), and (3) HBV binding to the cell surface decreased after silencing GPC5 expression or pretreatment of HBV with rGPC5 (Fig. 7), confirming a direct interaction between the virus and GPC5. Notably, the HBV–GPC5 interaction was independent of NTCP expression (Figs. 4 and 5).

PEG has been shown to favor HBV infection in PHH cultures,[35] and it is used, as well as DMSO, in most of the *in vitro* HBV and HDV infection systems based on NTCP-expressing hepatoma cells.[8,9,18] In our

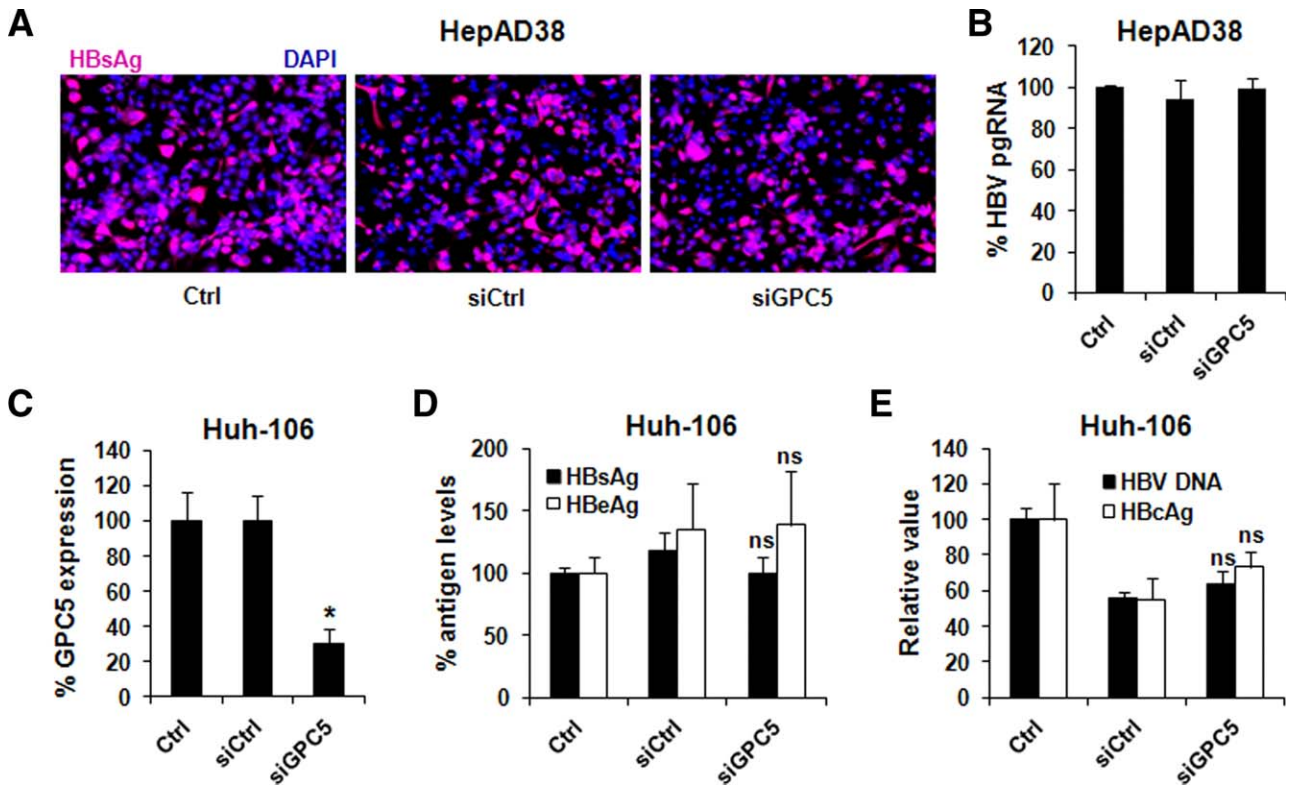


Fig. 8. GPC5 is not involved in HBV replication and production. (A,B) Absent effect of GPC5 silencing on HBV production in HepAD38 cells. HepAD38 cells stably replicating HBV were transfected with siRNAs targeting GPC5. Three days after transfection, the HBsAg level was evaluated by IF (A, one representative experiment is shown) and HBV pgRNA level was assessed by qRT-PCR quantification (B). Results are expressed as means ± standard deviation percentage HBV pgRNA levels relative to control nontransfected cells from three independent experiments performed in triplicate. (C-E) Absent effect of GPC5 silencing on HBV replication in Huh-106 cells. Huh-106 cells were reverse-cotransfected with a plasmid encoding the HBV genome (adwR9) or control plasmid and siGPC5 or control siRNA. Silencing efficacy was assessed by qRT-PCR 3 days after transfection (C). Three days after transfection, HBV replication was assessed by quantification of HBeAg and HBsAg in the supernatants (D) and total HBV DNA and HBV core antigen in cell lysates (E). Results are expressed as means ± standard deviation percentage GPC5 expression, HBV antigen, and DNA levels normalized to the HBV plasmid-only control (control) set as 100% from three independent experiments performed in triplicate. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole; ns, nonsignificant difference between nontargeting control siRNA and siGPC5.

study, we observed that the use of PEG and DMSO was not necessary for conducting high-throughput *in vitro* HDV infection assays that are adapted to functional siRNA and small molecule screening. We thus made the choice of using these PEG/DMSO-free conditions for practical reasons in conducting the assays but also for clarity in the interpretation of the data. Although DMSO is known to induce polarization and differentiation of hepatocytes in culture, it may also affect transcriptomic patterns of cells[36] and modulate the expression of factors involved in viral entry independently of differentiation.

Using this model system and an RNAi approach to uncover which HSPG(s) mediate(s) HBV/HDV entry, we identified GPC5 as a host factor involved in the initiation of HDV and HBV infection. GPC5 is a member of the glypican family, a group of six HSPGs (GPC1-6) that are attached to the cell membrane by a glycosylphosphatidylinositol anchor.[37] Glypicans are coreceptors for numerous heparin-binding factors, and they regulate the signaling activity of many growth factors, including Wnts and hedgehogs.[37] GPC5 is highly expressed during development in a tissue-specific manner, suggesting a major role in morphogenesis.[38] In adult tissues, GPC5 is mainly expressed in the brain, kidney, and liver.[39] Modulation of GPC5 expression is observed in various cancers and other disorders, including nephrotic syndrome,[39] alveolar and embryonal rhabdomyosarcoma,[40] and lung cancer in nonsmoker patients.[41] However, a comprehensive understanding of the GPC5 functions has yet to be established. Here, we report that GPC5 acts as an entry factor for both HBV and HDV through its interactions with the HBV envelope proteins during viral attachment. Given its tissue distribution and high expression level in the liver, GPC5 may contribute to the hepatotropism of HBV and HDV. Unlike other glypican members, GPC5 carries chondroitin sulfate chains in addition to heparan sulfate chains.[37] Chondroitin sulfate chains can function as receptors for porcine circovirus 2 and herpes simplex virus,[42,43] suggesting a role for these polysaccharides in

microbial adherence. The presence of chondroitin sulfate, in addition to heparan sulfate, could explain the specificity of GPC5 as an HBV/HDV attachment factor compared to other glypican members. In line with a recent study,[44] we observed that silencing of GPC5 expression had no effect on HCV entry. Similar to HBV, HCV attachment to hepatocytes is mediated by HSPGs[45]; and two recent studies identified SDC1 and SDC4 as mediators of HCV attachment.[34,44] Our data demonstrate that neither of these two HSPGs is involved in HBV entry, highlighting high specificity of virus–HSPG interactions at the surface of hepatocytes. Our binding data indicate that GPC5 is involved in the initial attachment of the virus at the hepatocyte surface, likely at a step prior to HBV preS1 binding to NTCP, which would subsequently trigger viral entry. The interaction between the HBV envelope and GPC5 may involve both glycosaminoglycans and the GPC5 core protein.

Because glypicans have been shown to play a role in the control of cell division and growth regulation, virus–GPC5 interactions may also play a role for pathogenesis of virus-induced liver disease and cancer. Indeed, HBV[46] and HCV[47] can transmit signals during hepatocyte binding and cell entry. In turn, glypicans are involved in the modulation of several signal transduction pathways. In particular, GPC3, a closely related member in the glypican family, may serve as a biomarker and target of HCC therapy.[48] GPC3 modulates fibroblast growth factor 2 and hedgehog signaling pathways in HCC (reviewed in Ho and Kim[48]). Similarly, GPC5 modulates fibroblast growth factor 2 signaling and stimulates hedgehog signaling in rhabdomyosarcoma cells.[49,50] However, its potential role in liver disease has not yet been studied. Collectively, the interaction between GPC5 and HBV envelope proteins may modify glypican-specific signaling pathways with potential implications for liver disease progression and hepatocarcinogenesis.

Furthermore, GPC5 may represent a previously undiscovered target for urgently needed antiviral therapies. Targeting viral entry using NTCP inhibitors is a promising approach for HBV treatment.[16] Myrcludex B, an HBV preS1 NTCP-targeting peptide, strongly inhibits HBV infection *in vivo*[19] and is currently being evaluated in a phase 2 clinical trial.[17] Thus, inhibiting HBV–GPC5 interactions using small molecules or antibodies (Fig. 5) opens new perspectives for control or cure of HBV and HDV infections.

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Author names in bold designate shared co-first authorship.

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