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► **To cite this version:**

Christophe Gallard, Nadjat Lebsir, Hira Khursheed, Emma Reungoat, Marie-Laure Plissonnier, et al.. Heparanase-1 is upregulated by hepatitis C virus and favors its replication. *Journal of Hepatology*, 2022, 10.1016/j.jhep.2022.01.008 . hal-03608032

HAL Id: hal-03608032

<https://hal.science/hal-03608032>

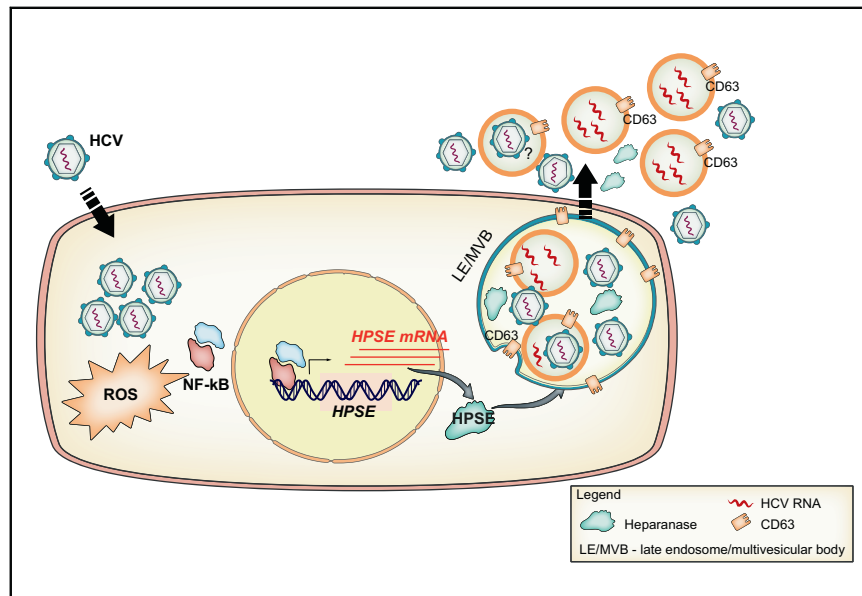
Submitted on 14 Mar 2022

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Heparanase-1 is upregulated by hepatitis C virus and favors its replication

Graphical abstract



Highlights

- Heparanase-1 (HPSE) is upregulated by HCV in an NF-κB-dependent manner.
- HPSE favors HCV replication cycle at the virus release step.
- HCV release is dependent on the tetraspanin CD63.
- High HPSE levels may favor pathologic liver alterations.

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Lay summary

Chronic hepatitis C virus (HCV) infection can lead to hepatocellular carcinoma development in a process that involves derangement of the extracellular matrix (ECM). Herein, we show that heparanase-1, a protein involved in ECM degradation and remodeling, favors HCV infection and is upregulated by HCV infection; this upregulation may result in pathogenic alterations of the ECM.

Heparanase-1 is upregulated by hepatitis C virus and favors its replication

Christophe Gallard^{1,†}, Nadjet Lebsir^{1,†}, Hira Khursheed¹, Emma Reungoat¹, Marie-Laure Plissonnier¹, Jennifer Bré¹, Maud Michelet¹, Yasmina Chouik^{1,2}, Fabien Zoulim^{1,2}, Eve-Isabelle Pécheur^{1,*}, Birke Bartosch^{1,§}, Boyan Grigorov^{1,*}

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Background & Aims: Over time, chronic HCV infection can lead to hepatocellular carcinoma (HCC), a process that involves changes to the liver extracellular matrix (ECM). However, the exact mechanisms by which HCV induces HCC remain unclear. Therefore, we sought to investigate the impact of HCV on the liver ECM, with a focus on heparanase-1 (HPSE).

Methods: HPSE expression was assessed by quantitative reverse-transcription PCR, immunoblotting and immunofluorescence in liver biopsies infected or not with HCV, and in 10-day-infected hepatoma Huh7.5 cells. Cell lines deficient for or overexpressing HPSE were established to study its role during infection.

Results: HCV propagation led to significant HPSE induction, *in vivo* and *in vitro*. HPSE enhanced infection when exogenously expressed or supplemented as a recombinant protein. Conversely, when HPSE expression was downregulated or its activity blocked, HCV infection dropped, suggesting a role of HPSE in the HCV life cycle. We further studied the underlying mechanisms of such observations and found that HPSE favored HCV release by enhancing CD63 synthesis and exosome secretion, but not by stimulating HCV entry or genome replication. We also showed that virus-induced oxidative stress was involved in HPSE induction, most likely through NF- κ B activation.

Conclusions: We report for the first time that HCV infection is favored by HPSE, and upregulates HPSE expression and secretion, which may result in pathogenic alterations of the ECM.

Lay summary: Chronic hepatitis C virus (HCV) infection can lead to hepatocellular carcinoma development in a process that involves derangement of the extracellular matrix (ECM). Herein, we show that heparanase-1, a protein involved in ECM degradation and remodeling, favors HCV infection and is upregulated by HCV infection; this upregulation may result in pathogenic alterations of the ECM.

Keywords: HCV; Heparanase-1; CD63.

Received 2 June 2021; received in revised form 3 January 2022; accepted 13 January 2022; available online xxx

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<https://doi.org/10.1016/j.jhep.2022.01.008>

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is ranked as the fourth-leading cause of death due to cancer.¹ According to the WHO, HCV is a leading etiology for HCC with an estimated 71 million chronic carriers globally. Chronic HCV infection evolves through stages of fibrosis, cirrhosis and may result in liver decompensation or HCC in severe cases, decades after the initial infection. How exactly HCV induces liver cancer is not yet fully understood since it is a non-integrative virus, but epidemiological observations suggest that HCV could fuel liver carcinogenesis by creating an oxidative, inflammatory microenvironment characterized by concomitant wound repair processes.² In the long term, these processes lead to genomic instability and deform the liver architecture, thus favoring HCC. Importantly, oxidative stress, either directly induced by the virus or due to inflammatory immune responses against the virus, leads to a remodelling of the extracellular matrix (ECM).³

To better understand how chronic hepatitis C leads to liver disease, we sought to investigate the impact of viral infection on the liver ECM by focusing on heparanase-1 (HPSE). Abnormal ECM dynamics lead to pathological processes such as tissue fibrosis and cancer.^{4–6} Major components of the ECM are heparan sulfate proteoglycans (HSPGs). They not only provide a storage depot for heparan sulfate (HS)-binding molecules such as growth factors, cytokines, chemokines and enzymes, but also regulate their accessibility, function and mode of action.⁷ HPSE is the sole endo- β -D-glucuronidase that cleaves HS side chains, thereby participating in ECM degradation and remodelling. It is synthesized as a 65 kDa-proenzyme in the Golgi and further processed by cathepsin L in late endosomes into 2 subunits of 50 kDa and 8 kDa that form a proteolytically active enzyme. HPSE expression is rather restricted to placenta, keratinocytes, platelets, activated immune cells, with little expression in connective tissues and epithelia, whereas an enzymatically inactive form of HPSE (HPSE2), is widely spread in normal tissues.^{8–11} High HPSE levels have been shown to correlate with cell migration and invasion, inflammation, and angiogenesis,¹² and have been observed in various human malignancies such as breast, colon, lung, prostate and liver cancers.^{9,10}



Apart from its pro-carcinogenic role, accumulating evidence revealed a role of HPSE in regulating the lifecycle of several human viruses including herpes simplex virus 1 (HSV-1), dengue, human papilloma (HPV), respiratory syncytial (RSV), and the porcine respiratory and reproductive syncytial virus (PRRSV).^{13–17} However, very little is known about HPSE and HCV infection. Indeed, one report suggests that HCV may enhance HPSE expression in HCV-related HCC,¹⁸ but nothing is known about the impact of HPSE on HCV replication and pathogenesis, nor the underlying mechanisms. Therefore, we investigated whether there was any relationship between HPSE and HCV infection. We found that HCV induced HPSE in an NF- κ B-dependent manner *in vitro* and in patient biopsies, suggesting a possible role of HPSE in the HCV lifecycle. Indeed, genetic modulation of HPSE expression altered HCV infection and virus release. Lastly, we identified the tetraspanin CD63, a molecule involved in exosome biogenesis, as a key player in this process.

Materials and methods

RT-qPCR

To detect the role of HPSE on HCV replication, Huh7.5 cells were seeded in 6- or 12-well plates. Virus replication or HPSE expression was quantified by reverse-transcription quantitative PCR (RT-qPCR). For RNA extraction, cells were lysed in ExtractAll[®] solution according to the manufacturer's protocol. For the next step, 1.5 μ g of RNA was treated with DNase (Roche) and reverse transcribed by 5X RT master mix (Abm ref#G592) following manufacturer's instructions. Samples were diluted 1:5 and mixed with SYBR Green (Roche) and primers (Table S1) to amplify cDNA on QuantStudio7 (Applied Biosystems). Beta-glucuronidase (GUS) was used as a housekeeping gene.⁶⁵

Biopsies

RNA was directly extracted from the biobank. Samples came from 60 patients uninfected by HCV and 93 patients infected by HCV as described in.⁶⁶ The study protocol was approved by the French IRB "Comité de Protection des Personnes (CPP) Sud-Est IV" (#11/040). Clinical, biological, and virological parameters of virus-free and HCV-positive patients are indicated. Written informed consent was obtained from each patient included in this study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the institution's human research committee. Uninfected patients had autoimmune disease, alcohol-related liver disease or non-alcoholic fatty liver disease, which included metabolic and genetic diseases. Some samples were also undetermined. The details of the samples used are grouped in Table S2. Concerning infected patients, we assume that fibrosis is due to the infection itself.

For further details regarding the materials and methods used, please refer to the [CTAT table](#) and [supplementary information](#).

Results

HCV-dependent heparanase induction in patients

We recently reported that HCV infection resulted in the down-regulation of the HSPG syndecan-1 in hepatocytes,¹⁹ implying that viral replication might lead to ECM alterations. Therefore, we asked whether persistent HCV infection had an impact on endogenous HPSE expression. We examined HPSE expression *in vivo* on liver biopsies from patients chronically infected with HCV or not, at different stages of liver disease according to

METAVIR scoring: F0 (no fibrosis), F1 (portal fibrosis without septa), F2 (portal fibrosis with few septa), F3 (septal fibrosis) and F4 (cirrhosis).²⁰ Biopsies from uninfected patients were mainly collected from patients with autoimmune liver disease, alcohol-related liver disease, or non-alcoholic fatty liver disease. Principal component analysis was performed on virological parameters (viremia, genotypes) as well as on different parameters of liver injury such as fibrosis score. HPSE expression was positively correlated with viremia ($r = 0.25$) and negatively correlated with fibrosis score ($r = -0.22$) in a significant manner ($p = 0.05$). When all disease stages were included, and HCV-positive biopsies ($n = 87$) compared to uninfected ones ($n = 58$), HPSE mRNA expression was found to be significantly upregulated in infected patients (Fig. 1A). Moreover, this expression was positively correlated with HCV infection rate ($r = 0.328$) as measured by intrahepatic HCV RNA (Fig. S1A-B). After further stratification of the biopsies as "mild/moderate fibrosis" (F1/F2) and "severe fibrosis/cirrhosis" (F3/F4), HPSE expression was found to be higher in F1/F2 biopsies compared to F3/F4 ones (Fig. 1B), which is in line with HCV infection within the 2 groups (Fig. 1C). In uninfected patients, HPSE mRNA expression did not seem to be related to disease etiology (Fig. S1C), and no significant increase in expression was found in F1/F2 or F3/F4 compared to F0 (Fig. 1D). Thus, our data demonstrate for the first time that HPSE can be induced by HCV *in vivo*, with overexpression more pronounced at early stages of liver fibrosis when HCV titers are higher.

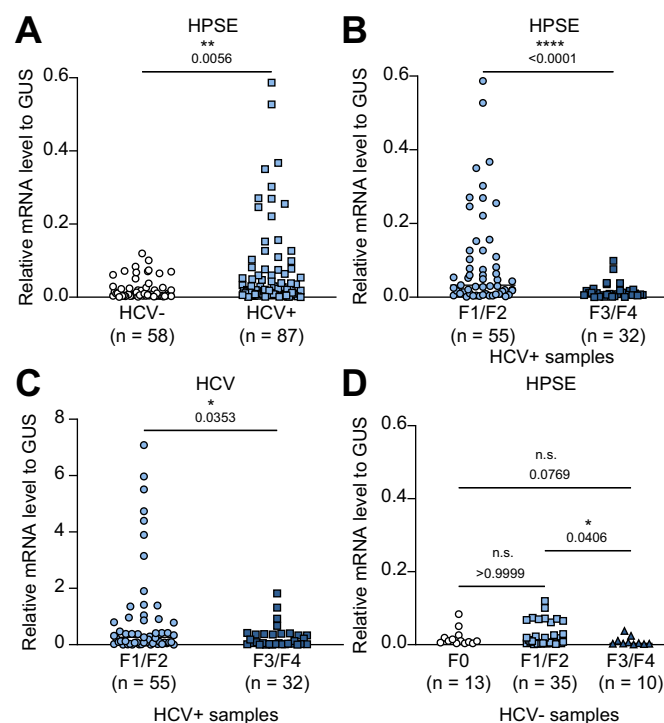


Fig. 1. HPSE expression in liver biopsies. (A) mRNA analysis of liver biopsies from HCV-infected or uninfected patients at various stages of chronic liver disease; n, number of patients. HPSE is significantly induced in HCV-infected patients. (B) HPSE is significantly induced in HCV-infected patients with mild/moderate fibrosis compared to severe fibrosis/cirrhosis. (C) HCV infection is more pronounced in patients with moderate/mild fibrosis compared to patients with severe fibrosis/cirrhosis. (D) HPSE expression in uninfected disease-free patients vs. mild/moderate fibrosis and severe fibrosis/cirrhosis. Statistical significance was calculated using Mann-Whitney *U* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

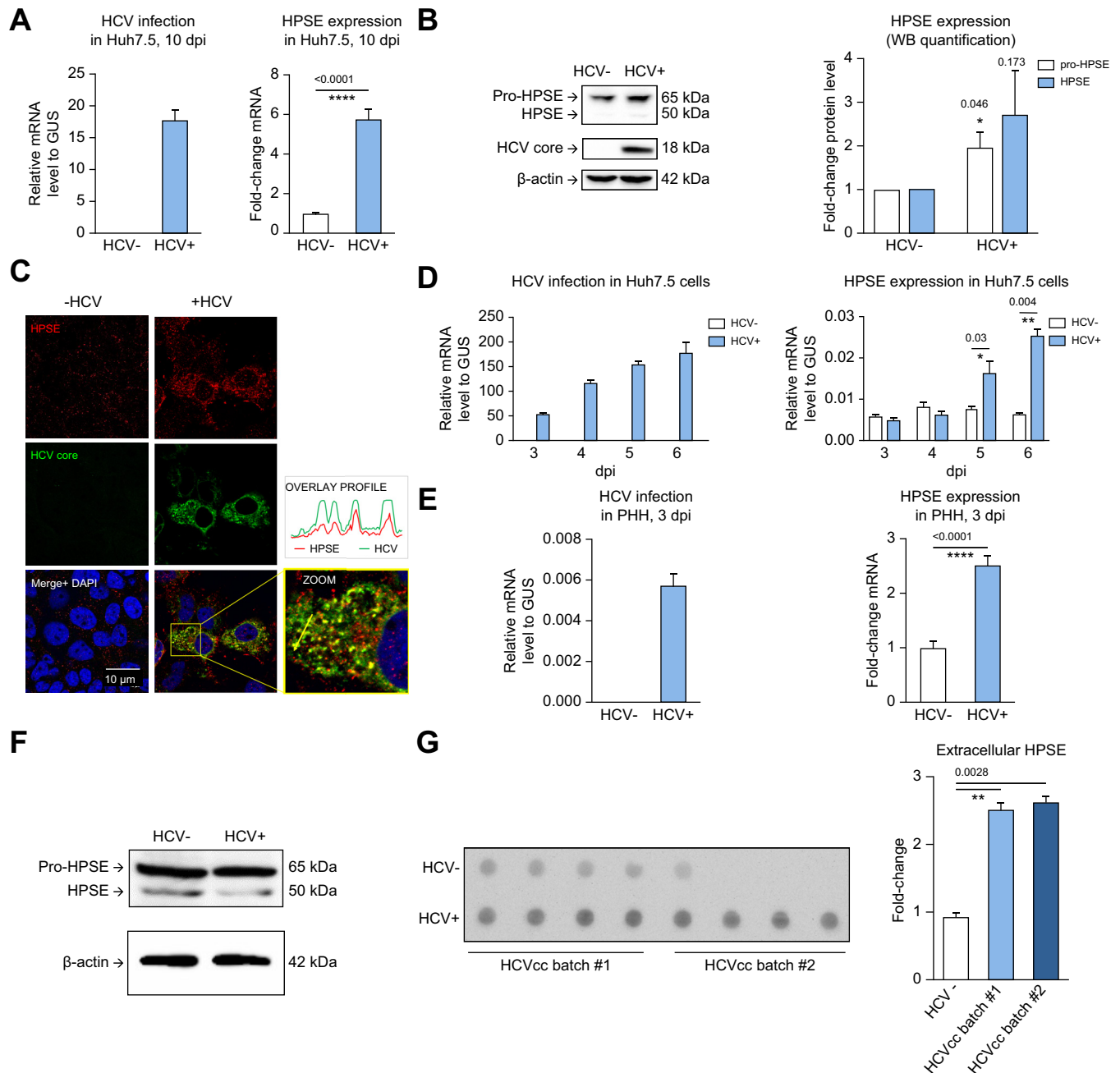


Fig. 2. Overexpression of heparanase upon HCV infection in different cells. (A) Huh7.5 cells were infected with HCVcc at MOI=0.1. At 5 dpi, cells were passaged and at 10 dpi recovered for RT-qPCR analysis of mRNAs. GUS was used as a housekeeping gene. Mean \pm SD of 4 independent experiments in triplicates. (B) Western blot analysis of Huh7.5 cells infected or not by HCV, for HPSE and HCV core protein expression at 10 dpi. HPSE antibody recognizes both forms of HPSE, i.e. the precursor pro-HPSE (~65 kDa) and the active HPSE (~50 kDa). Right panel: quantification of the bands for HPSE normalized on actin level (ImageJ analysis). Mean \pm SD of 3 blots. (C) Huh7.5 cells were infected or not with HCVcc and 10 days afterwards co-stained for HPSE (red) and HCV core (green). Zoomed image shows colocalization profile between HCV core and HPSE. Representative images are shown. (D) Kinetics of HCV infection in Huh7.5 cells up to 6 dpi. Cells were infected with HCVcc at MOI=0.1. At indicated dpi, cells were collected, and mRNA quantified. Infection augments with time (left chart). On the right chart, HPSE expression is significantly induced at 5 dpi. Mean \pm SD of 2 independent experiments in triplicates. (E) Expression of HPSE in PHHs. PHHs were infected with HCVcc at an MOI of 1, and were lysed at 3 dpi for RT-qPCR analysis of viral RNA (left panel) or HPSE mRNA (right panel). Mean \pm SD from 3 donors in triplicates. (F) Lysates of infected or uninfected PHHs were analyzed by Western blot for HPSE and HCV expression. (G) Extracellular HPSE measurement. Huh7.5 cells were seeded in 12-well plates and infected or not by HCVcc at MOI=0.1 for 6 days. Cell supernatants were collected, centrifuged to discard cell debris, and 200 μ l were used for dot blot analysis using a HPSE antibody. Pixel densitometry of the dots was performed using ImageJ, and results presented as fold-change from the uninfected cells. Statistical significance was calculated using Mann-Whitney *U* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001. dpi, days post infection; HCVcc, HCV cell culture; MOI, multiplicity of infection; PHHs, primary human hepatocytes; RT-qPCR, reverse-transcription quantitative PCR.

Heparanase expression is induced in HCV-infected hepatocytes

Next, we assessed the effect of HCV infection on HPSE using Huh7.5 cells in which the HCV cell culture (HCVcc) JFH1 strain had been passaged for 10 days. We detected a ~6-fold increase in HPSE mRNA expression, concomitant to an increase in protein level (Fig. 2A,B). Immunofluorescence analyses confirmed HPSE induction upon infection and furthermore revealed a punctate distribution of HPSE and its colocalization with HCV core (Fig. 2C, Fig. S2A), suggesting a link between both molecules. We also followed HCV infection for 6 days post infection (dpi) in Huh7.5 cells which were not passaged (Fig. 2D) and for up to 17 dpi in Huh7.5 cells incubated in DMSO-containing medium (Fig. S2B). The latter leads to hepatocyte differentiation, as evidenced by expression of markers such as cytokeratin-18, albumin, or alpha-1-antitrypsin (Fig. S2C), which obviated the need for cell passaging since cells become growth-arrested: they can be kept in cell culture and infected for more than 2 weeks.²¹ These kinetics revealed that HPSE mRNA expression increased over time, together with HCV infection, reaching its maximum at 4-5 dpi when the replication rate was high, suggesting that HPSE overexpression occurs once HCV infection becomes persistent. To recreate a more physiological setting, we also infected primary human hepatocytes (PHHs) with HCV. Since these cells exhibit functional innate immunity, they rapidly clear HCV infection. Therefore, we evaluated HCV RNA expression 3 dpi, which is within the infection peak as demonstrated by us (Fig. S2D) and

others.²² A significant increase (~2.5-fold) of HPSE mRNA expression was observed in HCV-infected PHHs from 3 different donors (Fig. 2E), compared to uninfected cells. However, no change in HPSE expression could be seen at the protein level, compared to Huh7.5 (compare Fig. 2 panels B&F), and HCV core was not detected. This could come from the low infectivity rate of PHHs *in vitro* – 2,000 to 20,000-fold less than that of Huh7.5 cells based on ΔCt values (compare Fig. 2 panels A&E left), most likely due to the early activation of interferon-related genes in primary cells.^{23,24} Since HPSE plays its physiological or pathological roles when released in the ECM, we further examined its level in cell supernatants by dot blot. Our results showed a >2.5-fold increase in extracellular HPSE level when cells were infected (Fig. 2G). Collectively, our results show that HCV infection enhanced HPSE expression and release in cultured hepatocytes.

HPSE expression returns to baseline upon HCV treatment

Whether HPSE overexpression was indeed linked to HCV infection was then studied in HCV-cured cells. Huh7.5 cells were infected with HCV under conditions that allowed for a massive spread of infection, as we documented.²⁵ Five dpi and prior to cell passaging, when HCV infection was established (Fig. 3A), HPSE expression exhibited a 2-fold increase in HCV-infected cells compared to uninfected ones (Fig. 3B), as previously observed (Fig. 2). Cells were then passaged and cultured for 6 additional days in the absence or presence of the NS5B polymerase inhibitor sofosbuvir to clear HCV infection. Eight dpi, sofosbuvir

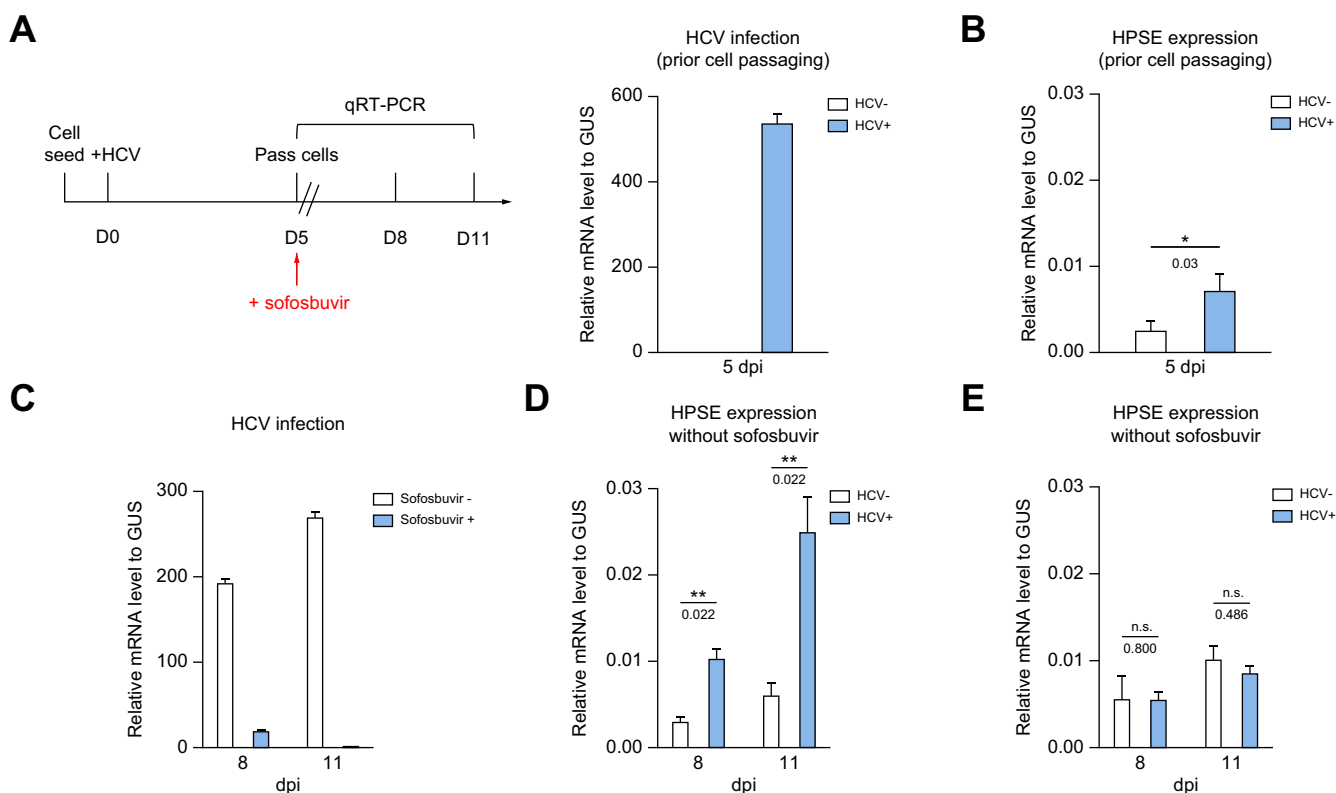


Fig. 3. Reversibility of the HPSE induction upon HCV elimination. (A) HCV RNA quantification at day 5 (prior cell passaging and sofosbuvir treatment). (B) HPSE mRNA quantification at day 5 (prior cell passaging and sofosbuvir treatment). (C) HCV RNA quantification at days 8 and 11 in the absence or presence of 10 μ M sofosbuvir. (D) HPSE mRNA quantification at days 8 and 11 in the absence of treatment in HCV-infected or uninfected cells. (E) HPSE mRNA quantification at days 8 and 11 in the presence of sofosbuvir in HCV-infected or uninfected cells. All experiments are mean \pm SD of 2 independent experiments in triplicates. Statistical significance was calculated using Mann-Whitney *U* test. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001. dpi, days post infection.

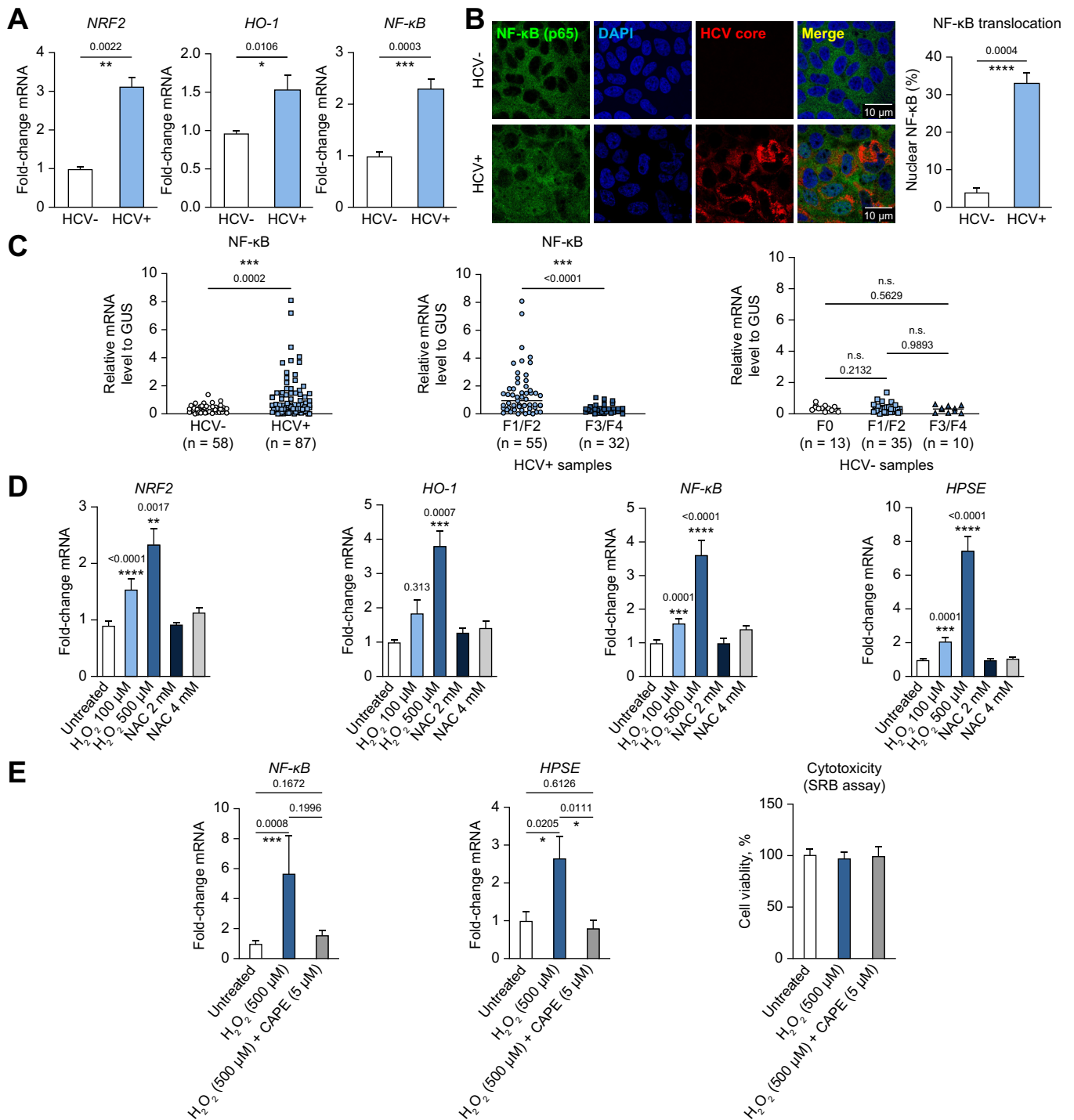


Fig. 4. HPSE is induced by oxidative stress in an NF-κB-dependent manner. (A) Huh7.5 cells were infected with HCVcc at MOI=0.1 for 6 days. mRNA analysis of *NRF2* (left chart), *HO-1* (middle chart), and *NF-κB* (right chart) expression. Mean±SD of 3 independent experiments in triplicates. (B) Huh7.5 cells were seeded on coverslips and infected or not with HCVcc at MOI=0.1. Cells were fixed at 6 dpi and stained for NF-κB p65 (green), HCV core protein (red) and nuclei (blue). Representative images are shown. Nuclear translocation of NF-κB was quantified according to the formula [(number of cells with nuclear NF-κB/all cells)*100]. Results are presented as mean ± SD of translocation counted in 6 microscope images. (C) NF-κB expression was assessed *in vivo* (same samples as in Fig. 1, n, number of patients). Left chart: HCV-infected or uninfected biopsies, all stages included; middle chart: patients with mild/moderate fibrosis (F1+F2) or severe fibrosis/cirrhosis (F3+F4); right chart: uninfected. (D) Huh7.5 cells were seeded and treated (or not) with H₂O₂ or NAC for 4 days. Cells were then collected for evaluation of *NRF2*, *HO-1*, *NF-κB* and *HPSE* mRNA expression. Mean±SD of 3 independent experiments in triplicates. (E) Huh7.5 cells were seeded, then pre-treated for 24 h with CAPE, followed by a 48 h stimulation with H₂O₂. *NF-κB* and *HPSE* mRNA were then analyzed by RT-qPCR. Cell cytotoxicity of the treatments was evaluated by the SRB assay. Mean±SD of 3 independent experiments in triplicates. Statistical significance was calculated using Mann-Whitney *U* test. **p* <0.05, ***p* <0.01, ****p* <0.001 and *****p* <0.0001. Dpi, days post infection; HCVcc, HCV cell culture; MOI, multiplicity of infection; NAC, N-acetyl cysteine; RT-qPCR, reverse-transcription quantitative PCR.

mediated inhibition of HCV infection was 90% and nearly complete at 11 dpi (Fig. 3C). Concomitantly baseline levels of HPSE (Fig. 3E) were observed, whereas in the absence of sofosbuvir, HPSE expression increased (Fig. 3D, ~3-fold at 8 dpi, ~4-fold at 11 dpi), as reported above. HCV infection *per se* could therefore induce the upregulation of HPSE expression, and this effect was reversible upon viral clearance.

HCV-related oxidative stress induces HPSE expression in an NF- κ B-dependent manner

We have already documented that during its replication in Huh7.5 cells, HCV creates an oxidative stress due to the accumulation of peroxide ions.²⁶ We therefore analyzed the expression of oxidative stress-related genes like the nuclear factor erythroid-2 related factor 2 (NRF2) and its downstream target – hemeoxygenase-1 (HO-1). NRF2 is a major transcription factor which is involved in cellular defense against oxidative stress and is upregulated in such conditions.^{27,28} The quantification of NRF2 and HO-1 mRNA levels in HCVcc-infected Huh7.5 cells showed a significant increase compared to controls (Fig. 4A). Oxidative stress has been shown to stimulate the expression and activation of the transcription factor NF- κ B.^{29,30} Since HPSE is an NF- κ B target gene,³¹ upregulated through NF- κ B during HSV-1 and PRRSV infections,^{13,14} we checked if this occurred with HCV. As reported by others,³² we found that NF- κ B was induced in HCV-infected Huh7.5 cells (Fig. 4A), and observed the nuclear translocation of NF- κ B p65 (Fig. 4B), as an indication of its activation. In human liver biopsies, NF- κ B was induced in HCV-infected samples (Fig. 4C) and correlated positively to HCV infection rate as measured by intrahepatic HCV RNA ($r = 0.5$) (Fig. S3A-B). Interestingly, this induction occurred at the early stages of liver disease (F1/F2), corresponding to high levels of HCV infection and HPSE expression (Fig. 1). Indeed, a significant positive correlation ($r = 0.6$) between NF- κ B and HPSE mRNA expression was found (Fig. S3C), suggesting a functional link between HCV infection, NF- κ B and HPSE expression. To identify whether NF- κ B upregulation is due to oxidative stress, Huh7.5 cells were treated with H₂O₂. The results showed a dose-dependent increase in NRF2 and HO-1 expression, correlating with NF- κ B and HPSE expression (Fig. 4D). As a control, cells treated with the antioxidant N-acetyl cysteine did not display any change in any of the studied genes. Alternatively, we activated NF- κ B by a different mechanism than oxidative stress: cells were treated with PMA (phorbol-12-myristate-13-acetate ester), an activator of NF- κ B transcription.³³ Our results reveal that HPSE is upregulated by PMA treatment (Fig. S3D) and are in favor of a mechanism whereby HPSE upregulation is induced by NF- κ B. Lastly, we explored the effect of the NF- κ B inhibitor CAPE³⁴ on HPSE expression. When cells were pretreated with CAPE prior to H₂O₂ stimulation, at concentrations where no cytotoxicity was observed, there was no increase in NF- κ B and HPSE mRNA expression compared to the condition where H₂O₂ alone was used (Fig. 4E, cf Fig. 4D). These results validate the link between oxidative stress and NF- κ B upregulation and suggest that the oxidative stress-induced upregulation of HPSE depends on NF- κ B.

Heparanase favors HCV infection

A link between HPSE upregulation and infection by viruses such as HSV-1, PRRSV, HPV and RSV was recently reported, where HPSE contributed to enhanced virus release.^{13,14,16,17} We

investigated this further by establishing Huh7.5 cells that constitutively expressed the canonical form of HPSE using lentiviral vectors. HPSE overexpression was evidenced at the protein and mRNA levels (Fig. 5A-B). In these cells (Huh7.5 HPSE+), a ~3-fold increase in HCV infection was observed, compared to normal Huh7.5 cells (Fig. 5C), suggesting that HPSE promotes the HCV infectious cycle. A dose-dependent increase in HCV infection and release was also observed when recombinant HPSE (rHPSE) was added to the cell medium (Fig. 5D-E). Conversely, when HPSE expression was downregulated using a short-hairpin (sh) RNA approach (Fig. 5F-G), HCV infection was reduced by 70% compared to control cells (Fig. 5H) and was partially restored when rHPSE was added (Fig. 5I-J). This was also confirmed by a CRISPR/Cas9 knockout approach (Fig. S4). Lastly, we used a pharmacological approach to block HPSE activity. HCV-infected Huh7.5 cells were treated with the specific HPSE inhibitor PI-88, and HCV infection was assessed 3 dpi. We observed a dose-dependent decrease in HCV RNA at PI-88 concentrations displaying no significant cell cytotoxicity (Fig. 5K). Taken together, these results suggest that HPSE plays a role in the HCV life cycle.

Heparanase is not involved in HCV entry or virus genome replication

To elucidate the mechanism(s) by which HPSE favors HCV infection, viral entry was first investigated. For that purpose, we used pseudo-particles displaying HCV E1/E2 (HCVpp) and vesicular stomatitis virus G (VSVGpp), bearing a GFP marker gene, as a control.²⁵ Neither VSVGpp nor HCVpp entry was blocked by the HPSE inhibitor PI-88, indicating that HPSE is dispensable for HCV entry (Fig. 6A). Conversely, control reactions with the anti-CD81 antibody JS81 showed an efficient dose-dependent inhibition of HCVpp entry. Treating cells with rHPSE prior to transduction had no effect on HCVpp entry (Fig. S5). Additionally, a time of addition experiment was set up in the context of HCVcc: PI-88 was added at -1, 0, +2, +3, and +5 hours post infection, and kept until 1 dpi when HCV RNA was quantified by RT-qPCR (Fig. 6B). Adding the drug even 5 hours post infection led to inhibition of HCV infection at all time points, as was the case with sofosbuvir, indicating that the PI-88 was active at a post entry step. In contrast, JS81 inhibited HCV infection only when added at the earliest time points (-1 h and 0 h), in agreement with the involvement of CD81 in viral entry. Next, we set up an HCV replicon system³⁵ to specifically assess HCV replication. This system is based on measuring luciferase activity, since the *Renilla* luciferase reporter gene was encoded within the HCV RNA. HCV replication in Huh7.5 cells was comparable in Huh7.5 HPSE+ and Huh7.5 shHPSE cells (Fig. 6C), as well as in cells treated with PI-88 (Fig. 6D), arguing against any involvement of HPSE in HCV genome replication.

HPSE induction modifies the expression of the tetraspanin CD63 and enhances HCV release

Finally, we investigated the impact of HPSE on HCV release as described for HSV-1.¹⁴ Specifically, we focused on the tetraspanin CD63, considered as an endosomal and exosomal marker, and involved in vesicle secretion.^{36,37} Indeed, the processing of the inactive pro-HPSE to its active form by the protease cathepsin L requires acidic conditions, met in the late endosomal/lysosomal compartments.³⁸ Moreover, HPSE was reported to enhance exosome secretion when overexpressed in cancer cells,³⁹ and the release of the exosomal cargo CD63.⁴⁰

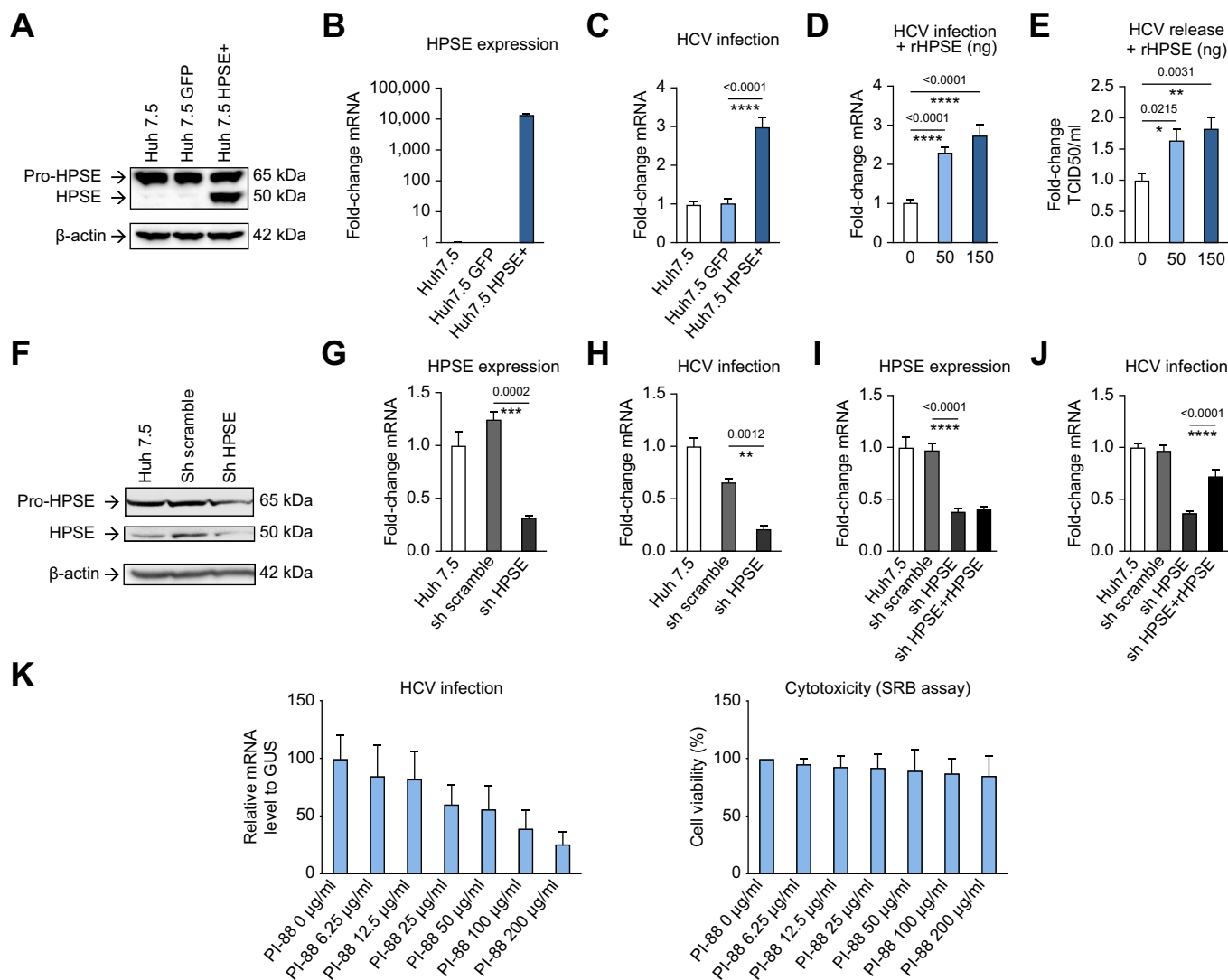


Fig. 5. Heparanase favors HCV infection. (A) Huh7.5 cell line constitutively expressing exogenous HPSE was developed and evaluated for HPSE expression by Western blot. GFP-expressing cell line was developed using the same plasmid and used as a control. (B) Cells were then infected with HCVcc at MOI=0.1 and 3 days afterwards, collected and analyzed by RT-qPCR for mRNA expression of HPSE. Results are presented as fold-change expression from control Huh7.5 cells. (C) HCV infection was evaluated by quantification of HCV RNA. Mean \pm SD of 3 independent experiments in triplicates. (D) Huh7.5 cells were infected with HCVcc at MOI=0.2. One dpi, the cells were treated with the indicated concentrations of rHPSE for 24 h. Two dpi, cells were recovered and assessed for intracellular HCV RNA. Mean \pm SD of 4 independent experiments in triplicates. (E) Supernatants from (D) were subjected to the TCID50 assay to measure HCV virus release, expressed as fold-change compared to the titer of rHPSE-untreated condition. Mean \pm SD of 4 independent experiments in triplicates. (F) Huh7.5 cells deficient in HPSE were established by shRNA approach and evaluated for HPSE protein expression. A control cell line was developed by transduction of the cells with the same vector containing a scrambled sh sequence. (G) Cells were infected with HCVcc at MOI=0.1 and 3 dpi, HPSE mRNA analysis was performed. Mean \pm SD of 3 independent experiments in triplicates. (H) HCV infection was evaluated by quantification of HCV RNA. Mean \pm SD of 3 independent experiments in triplicates. (I) Huh7.5, sh-scrambled, and shHPSE cells were infected as in (G). Six hours post infection, cells were washed and 150 ng of rHPSE were added to the shHPSE condition. After 48 h, cells were recovered and HPSE mRNA assessed. Mean \pm SD of 3 independent experiments in triplicates. (J) HCV infection was evaluated by quantification of HCV RNA. Mean \pm SD of 3 independent experiments in triplicates. (K) HCV infection was evaluated in the presence of PI-88. Three dpi, HCV RNA was quantified by RT-qPCR (left panel). Cytotoxicity was measured by the SRB assay (right panel). Mean \pm SD of 3 independent experiments in triplicates. Statistical significance was calculated using Mann-Whitney *U* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001. dpi, days post infection; HCVcc, HCV cell culture; MOI, multiplicity of infection; PHHs, primary human hepatocytes; rHPSE, recombinant HPSE; RT-qPCR, reverse-transcription quantitative PCR; sh, short-hairpin.

Although the precise mechanism of HCV assembly and release remains unclear, there is evidence that these processes are linked to the exosomal pathway,^{41–43} involving CD63 in particular, identified to be associated to the viral particle.⁴⁴ Therefore, we investigated CD63 expression during HCV infection. A significant increase in CD63 mRNA expression was observed in infected liver biopsies and in Huh7.5 cells, but no association with fibrosis stages was found (Fig. 7A-B).

Furthermore, CD63 protein expression was higher in HCV-infected Huh7.5 cells compared to uninfected cells, and it localized in large aggregates (Fig. 7C). CD63 overexpression appeared to be due to HPSE induction during infection rather than to viral infection *per se*, since uninfected Huh7.5 cells expressing exogenous HPSE displayed CD63 profiles comparable to infected cells (Fig. 7C). Earlier reports indicated that cells overexpressing HPSE showed a high number of cytosolic

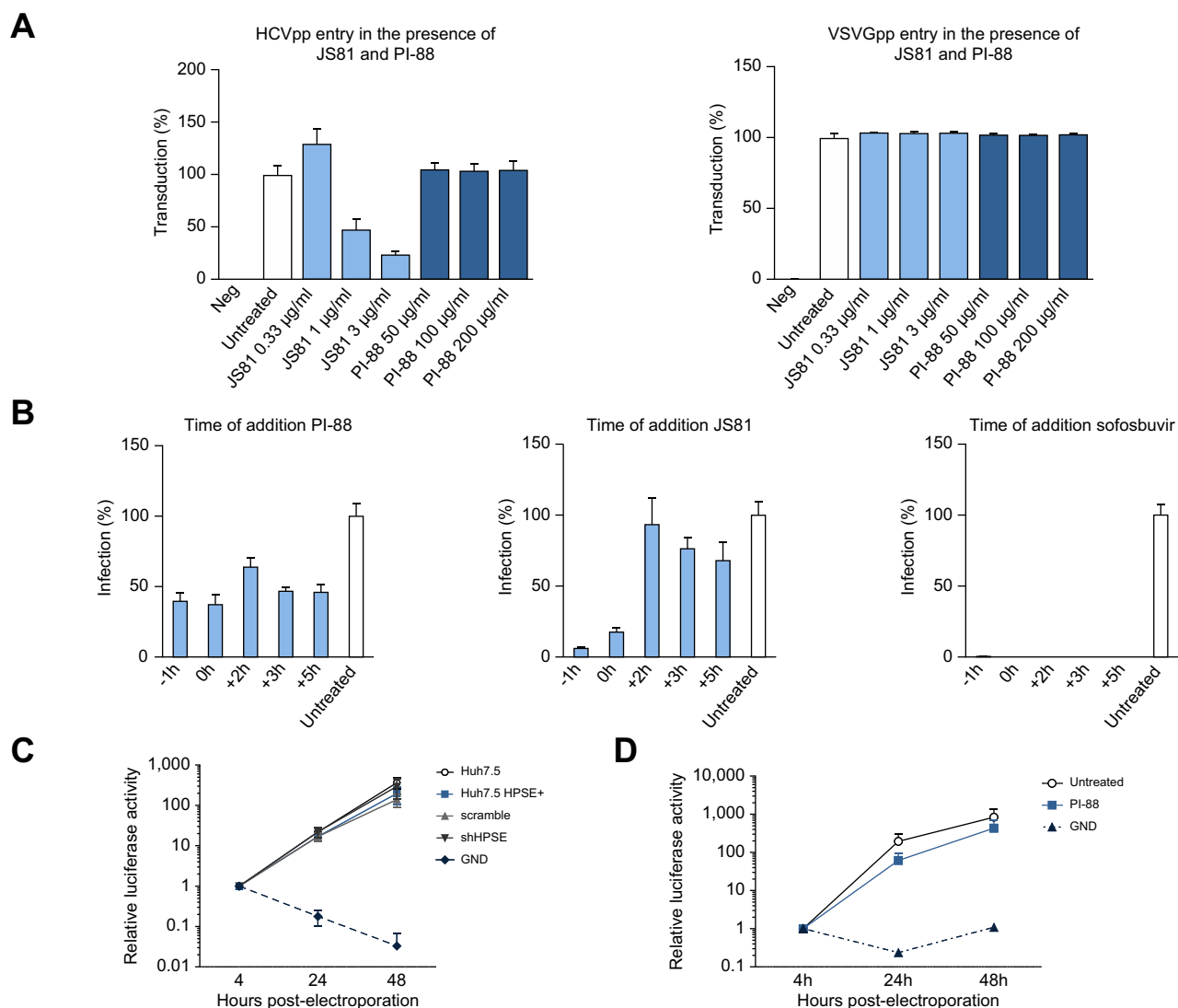


Fig. 6. Heparanase is not involved in HCV entry or virus genome replication. (A) Huh7.5 cells were seeded, and HCVpp (left panel) or VSVGpp (right panel) added in the absence or presence of the HCV entry inhibitor JS81, or PI-88. After 72 hours, cells were detached by trypsin and analyzed by flow cytometry for GFP expression. Results are presented as % from the untreated controls for each type of pseudo-particles which were set to 100% transduction. Mean±SD of 2 independent experiments in triplicates. (B) Time of addition experiment: PI-88 (200 µg/ml), JS81 (3 µg/ml) or sofosbuvir (10 µM) were added at the indicated time points. HCVcc at MOI=1 was added at t = 0. After 2 h, cells were washed twice with PBS, and medium replaced. 24 hours post infection, cells were recovered for HCV RNA quantification. Mean±SD of 2 independent experiments in triplicates. Note that HCV infection is only partially blocked by PI-88, as already shown in Fig. 5K. (C) *In vitro*-transcribed RNA of Δ E1E2-JFH-1 construct was electroporated in Huh7.5 cells, Huh7.5 HPSE+ cells or Huh7.5-shHPSE and Huh7.5-sh scrambled. The non-replicative GND replicon (with point mutation in the GDD motif of NS5B, abolishing its activity) was electroporated in Huh7.5 cells as a negative control. Cells were lysed at indicated times post-electroporation and luciferase activity was measured. Data represent means from 3 independent experiments performed in triplicates. (D) *In vitro*-transcribed RNA of Δ E1E2-JFH-1 construct and the non-replicative GND replicon were electroporated in Huh7.5 cells in the absence or presence of PI-88 (200 µg/ml). Cells were lysed at indicated times post-electroporation and luciferase activity was measured. Data represent means from 3 independent experiments performed in triplicates. HCVcc, HCV cell culture; HCVpp, HCV pseudo-particles; MOI, multiplicity of infection; sh, short-hairpin; VSVGpp, vesicular stomatitis virus G pseudo-particles.

vesicles and an abundant release of exosome-like vesicles.^{39,40} This pointed to a potential link between HPSE, HCV and CD63, further investigated by silencing CD63 in Huh7.5 cells by RNA interference. CD63 was efficiently knocked down by 2 shCD63 constructs (Fig. 7D, left panel). CD63-deficient cells displayed drastic reductions in HCV infection compared to control cells (sh-scrambled), with HCV RNA and virus release as readouts (Fig. 7D, graphs). These data delineate for the first time a functional link between HPSE, CD63 and HCV. We next investigated whether this enhanced HCV release was linked to

exosomes. Three Huh7.5 cell lines (naïve, CD63-deficient, and overexpressing exogenous HPSE) were infected or not, and analyses were performed on the exosome-containing fraction and cell lysates (*cf. supplementary materials and methods*). In line with results above, CD63 expression was induced in the presence of HCV and in cells overexpressing HPSE and down-regulated in shCD63 cells (Fig. 7E “Intracellular”). Similarly, in the pellet containing extracellular exosomes, the CD63 signal was stronger in HCV-infected cells or when HPSE was overexpressed (Fig. 7E “Extracellular”). The infectivity assay of the

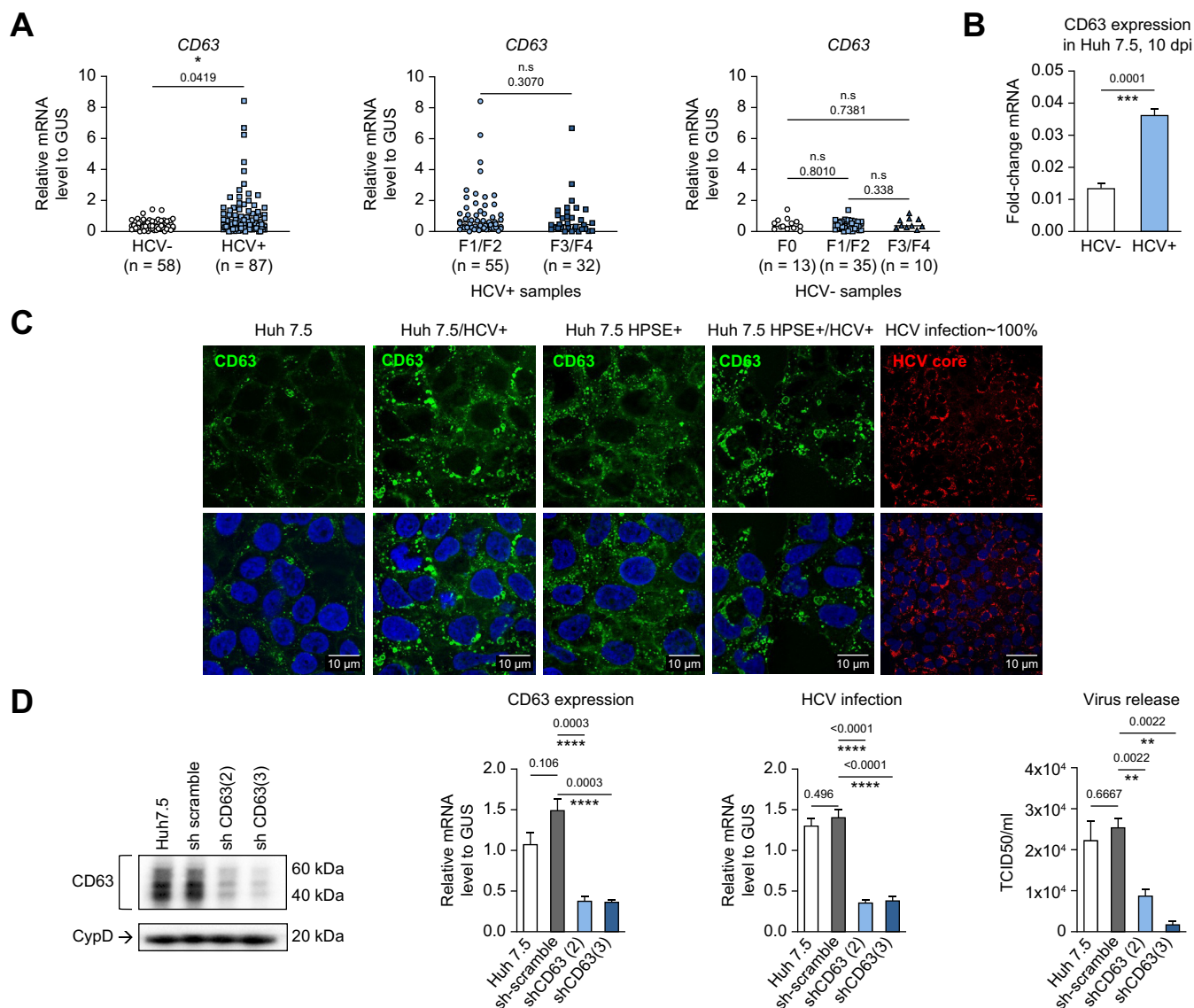


Fig. 7. HPSE is involved in exosome production which favors HCV release. (A) CD63 expression was assessed *in vivo* (same samples as in Figs 1 & 4, n, number of biopsies). Left chart: HCV-infected or uninfected biopsies, all stages included; middle chart: patients with mild/moderate fibrosis (F1+F2) or severe fibrosis/cirrhosis (F3+F4); right chart: uninfected; (B) CD63 expression in Huh7.5 cells. Cells were infected or not with HCVcc at MOI=0.1 and maintained in cell culture for 10 days, when CD63 and HCV RNA quantification was performed. Mean \pm SD of 4 independent experiments in triplicates. (C) Huh7.5 or Huh7.5 HPSE+ cells were infected or not with HCVcc at MOI=0.1 and 10 dpi, fixed and co-stained for CD63 and DAPI. Representative images are shown in couples with or without DAPI (blue). The first 4 couples are cells stained for CD63 (green). The fifth couple represents cells stained for HCV core protein (red) to visualize HCV spread. (D) CD63 downregulation by shRNA. Two different shRNA sequences against CD63 or a scrambled sequence were used to transduce Huh7.5 cells. CD63 protein expression was checked by immunoblot (left panel). Cells were then infected with HCVcc and collected at 3 dpi for RNA analysis of CD63 (left chart), and intracellular HCV RNA (middle chart). Cell supernatants were quantified by the TCID50 method to assess virus release and presented as fold-change from the titer of the Huh7.5 condition (right chart). Mean \pm SD of 3 independent experiments in triplicates. (E) Huh7.5 cells, Huh7.5 shCD63 (sh3) cells, and Huh7.5 HPSE+ cells were infected or not with HCVcc and 3 dpi supernatants and cells were collected and subjected to purification and lysis respectively (cf. supplementary materials and methods). Fifty μ g of proteins (left panel “intracellular”), and equal volumes of exosome-containing pellets (right panel “extracellular”) were loaded on a gel and immunoblotted under non-reducing conditions. (F) Cell supernatants were quantified by the TCID50 method to assess virus release and presented as fold-change from the titer of the Huh7.5 condition. (G) Exosome purification on iodixanol gradients. Supernatants from HCV-infected Huh7.5 cells were processed as described in the supplementary materials and methods. Exosomes were isolated by ultracentrifugation and subjected to iodixanol gradient ultracentrifugation. Fractions were taken from the top and equal volumes of each were used either for Western blot detection (by anti-CD9, anti-CD63, anti-HCV core or anti-HPSE antibodies), or RNA quantification. Statistical significance was calculated using the Mann-Whitney *U* test. **p* <0.05, ***p* <0.01, ****p* <0.001 and *****p* <0.0001. HCVcc, HCV cell culture; MOI, multiplicity of infection; sh, short-hairpin.

supernatants showed a drop in HCV release in shCD63 cells, and an increase in HPSE-positive cells compared to naïve Huh7.5 cells (Fig. 7F).

In order to determine whether HCV virions are secreted along CD63-positive exosomes, we investigated a potential co-

fractionation of the exosomal markers CD63 and CD9 with HCV virions. The exosome-containing pellet fraction from supernatants of HCV-infected cells was layered on iodixanol density gradients. Their analysis revealed that CD63 and CD9 were both present in fractions from 1.09 to 1.15 g/ml density, with a peak at

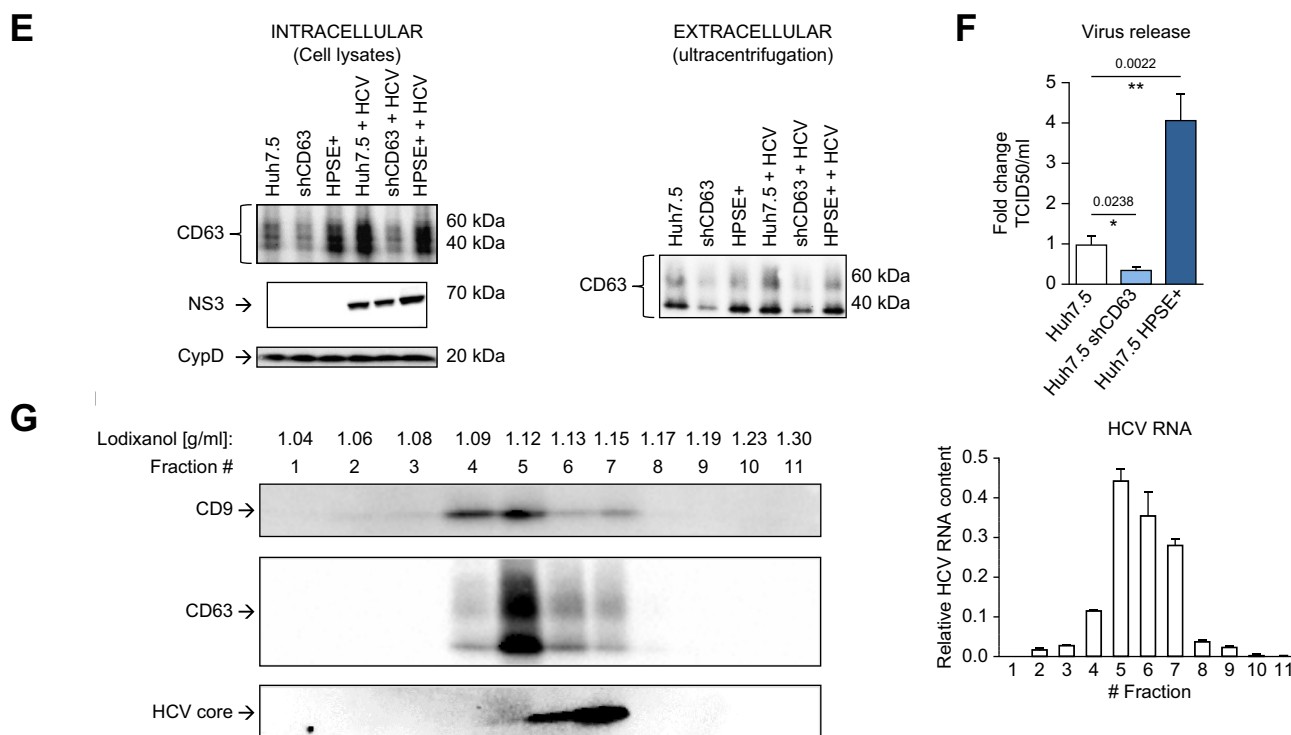


Fig. 7. (continued).

1.12 g/ml (Fig. 7G). Most classical (MVB-related) exosomes display buoyant densities in iodixanol from 1.08 to 1.15 g/ml,⁴⁵ indicating that CD63 is mainly of exosomal origin. HCV RNA co-fractionated with CD9 and CD63, peaking in the 1.12 g/ml fraction, consistent with recent data reporting that HCV RNA traffics in exosomes.⁴⁶ However, virions sedimented with a shifted profile compared to that of exosomes, with HCV core peaking in the 1.15 g/ml fraction, in line with previous data.^{47,48} Therefore, our data would indicate that HCV virions are not contained in exosomes, although at this stage, we cannot exclude that some virions might traffic with exosomes. The dependence of HCV infection on CD63 expression nevertheless suggests that virions may share common secretion mechanisms with exosomes. Altogether, these data imply that HPSE might favor HCV release by enhancing CD63-positive exosome production and secretion.

Discussion

Our studies show that HPSE may play important roles in the life cycle as well as the pathophysiology of HCV infection. We show that HPSE expression is significantly upregulated in HCV-positive patients (Fig. 1A) and particularly at mild/moderate (F1/F2) compared to severe (F3/F4) fibrosis stages (Fig. 1B) which is consistent with HCV infection (Fig. 1C). In uninfected liver samples (Fig. 1D), we found that HPSE was slightly but significantly upregulated in patients with mild/moderate fibrosis compared to severe fibrosis/cirrhosis (Fig. 1B) implying that liver inflammation and/or scarring *per se* could induce HPSE, as previously described⁴⁹ but also reported for other pathologies such as chronic pancreatitis and Crohn's disease.⁵⁰ Indeed, studies in rat liver post partial hepatectomy and upon pharmacologically induced liver fibrosis showed elevated HPSE expression during

the tissue regeneration process and early fibrosis, but a decrease to baseline as the severity of fibrosis increased.^{51,52} Our results extend the notion that HPSE expression is induced in early fibrosis to human liver biopsies and are in agreement with other studies showing that HPSE expression levels between cirrhotic tissues and normal liver tissues are not significantly different.⁵³ Low HCV replication in end-stage liver disease has been reported,⁵⁴ which further strengthens our data that HPSE expression is linked to HCV infection rate. Moreover, high HPSE level was also shown to have a cytoprotective role by upregulating pro-survival genes in the heart.⁵⁵ We can speculate that at the early stages of liver disease where HCV infection is highly active, high HPSE levels may favor ECM alterations and inflammation but also cell survival, thus favoring cancer development. HPSE plays a role in angiogenesis and its levels rise in some HCC but remain low in others, depending on the tumor differentiation grade and heterogeneity.^{56,57} Moreover, Huh7.5 which are hepatoma cells, present very low levels of HPSE expression compared to PHHs and other cells (data not shown). It is noteworthy that, in renal fibrosis, HPSE can regulate TGF- β expression and activity,⁵⁸ a cytokine thought to be responsible for viral persistence and liver fibrogenesis. Therefore, specific anti-HPSE inhibitors may be developed to target not only HPSE activity (anti-oncogenic effect) but indirectly TGF- β (anti-fibrotic effect) for which no suitable inhibitors could be found.⁵⁹

HPSE is a target gene of NF- κ B and is upregulated by other viruses (e.g. HSV-1 and PPRSV) through that pathway.^{13,14,60} Analyses of liver biopsies revealed that HCV infection resulted in NF- κ B upregulation especially at the early stages of fibrosis. The common view that NF- κ B has an anti-apoptotic effect would suggest that its expression/activity will be enhanced in the context of F3/F4 where it would serve to promote carcinogenesis.

However, data in NF- κ B-deficient mice rather points to NF- κ B as a tumor suppressor gene.⁶¹ The precise role of NF- κ B in liver regeneration and HCC remains controversial⁶² but our results suggest that downstream targets of NF- κ B such as HPSE might favor tumor development. In our *in vitro* system NF- κ B also appeared to be upregulated, most likely due to HCV-induced oxidative stress, as evidenced by the overexpression of NRF-2 and HO-1. In support of this hypothesis, when naïve cells were treated with H₂O₂, there was a dose-dependent increase in NF- κ B mRNA levels, concomitant to HPSE induction. Accordingly, H₂O₂-related oxidative stress can induce HPSE production⁶³ and in our study we further show that this induction is NF- κ B dependent. However, we cannot exclude the possibility that additional pathways are implicated in HPSE upregulation in HCV-infected cells. Reactive oxygen species accumulation may lead to cell damage and death, but can also induce NF- κ B, which targets genes that typically promote cell survival.³⁰

Recent evidence showed that HPSE plays an important role during the replication of different viruses but nothing was known about its role in the HCV life cycle.⁶⁴ Through genetic or pharmacological modulation of HPSE expression or activity, we give the first demonstration that HPSE plays a role in HCV infection. Assuming that HPSE may be involved in the later steps of the viral cycle, we focused on virus release, since this was reported to be the case for other viruses like HSV-1, HPV, PRRSV.^{13,14,16} In fact, the exact mechanism of HCV assembly and release is still not completely elucidated but evidence indicates that these processes are linked to the exosomal pathway.^{41–43,46} Besides, HPSE has been shown to enhance exosomal secretion when overexpressed,³⁹ and to activate the syndecan-syntenin-ALIX exosomal pathway by stimulating the exosomal secretion of syntenin-1, syndecan-1 and other exosomal cargos, such as CD63.⁴⁰ The latter is considered as a late endosomal/multi-vesicular body marker, involved in exosome vesicle formation and secretion.^{36,37} For that reason, we investigated the expression of CD63 in HCV-infected Huh7.5 and liver biopsies. Our results showed a marked increase in CD63 expression during HCV infection *in vivo* and *in vitro* and interestingly, HCV infection and release were dramatically reduced in CD63-silenced cells (Fig. 7A-D), showing for the first time a link between the tetraspanin CD63 and HCV. Such a link is also supported by the fact that CD63 is associated with HCV virions.⁴⁴ Further analysis revealed that high HPSE expression is linked to enhanced release of CD63-containing exosomes and infectious HCV particles. Moreover, we show that HCV RNA, but not necessarily HCV virions, traffic via these exosomes, suggesting that HCV shares a common secretion mechanism with exosomes.

In conclusion, our results demonstrate that HPSE is upregulated in chronic HCV infection both *in vitro* and *in vivo*, most likely through the induction of oxidative stress generated during infection. HPSE plays a role in the HCV life cycle by favoring virus release, *via* mechanisms that may be common to other viruses. Eventually, high liver HPSE expression may lead to pathogenic alterations of the ECM, such as decreased HSPG levels and enhanced exosome release, whose impact on HCC development warrants further investigation.

Abbreviations

Dpi, days post infection; ECM, extracellular matrix; GUS, Beta-D-glucuronidase; HCC, hepatocellular carcinoma; HCVcc, HCV cell culture; HVCpp, HCV pseudo-particles; HO-1, hemoxygenase-1;

HPSE, heparanase-1; HPV, human papilloma virus; HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; HSV-1, herpes simplex virus 1; NRF2, nuclear factor erythroid-2 related factor 2; PHHs, primary human hepatocytes; PRRSV, porcine respiratory and reproductive syncytial virus; rHPSE, recombinant HPSE; RSV, respiratory syncytial virus; RT-qPCR, reverse-transcription quantitative PCR; sh, short-hairpin; VSVGpp, vesicular stomatitis virus G pseudo-particles.

Financial support

BG: Fondation ARC (n°PJA 20161204585). BB: ANRS (grant 19238), the DevWeCan French Laboratories of Excellence Network (ANR 10 LABEX 61).

Conflict of interest

The authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

CG and NL: methodology, investigation, formal analysis, visualization. JB, HK, ER, MM: investigation, formal analysis. MLP, YC: biopsies analyses. FZ: resources, access to liver samples of patients with chronic liver diseases, data interpretation, draft reviewing. EIP: conceptualization, resources, draft reviewing. BB: conceptualization, resources, draft reviewing, funding acquisition. BG: project funding acquisition, conceptualization, project administration, methodology, investigation, supervision, visualization, original draft writing.

Data availability statement

At least 1 author has access to all the data and can vouch for the integrity of the data analyses.

Acknowledgements

We greatly thank Medigen Biotechnology Corp., Taiwan for providing us with the HPSE inhibitor PI-88, Dr. Yves Rouillé for providing us with HCV replicon plasmids, the Hepatology Service of Hospices Civils de Lyon for liver biopsies, Prof. Michel Rivoire (Centre Léon Bérard) for liver resections, and Jennifer Molle for PHH logistics. This project was funded by Fondation ARC. BB was supported by ANRS and ANR.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2022.01.008>.

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Author names in bold designate shared co-first authorship

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