Research Article
Viral Hepatitis

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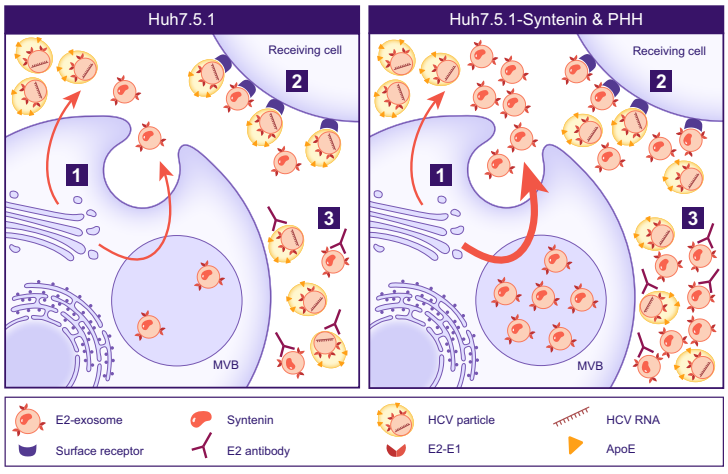
Syntenin regulates hepatitis C virus sensitivity to neutralizing antibody by promoting E2 secretion through exosomes

Graphical abstract



Highlights

- Syntenin is a key determinant of the efficiency of E2-coated exosome production.
- E2-coated exosome biogenesis is independent of HCV infectious lipo-viral-particle production.
- Robust E2-coated exosome production assists HCV infection in the presence of neutralizing antibody.

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Lay summary

This study identifies a key role for syntenin in the regulation of E2 secretion via exosomes. Efficient production of E2-coated exosomes was shown to make hepatitis C virus less sensitive to antibody neutralization. These results may have implications for the development of an hepatitis C virus vaccine.

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Syntenin regulates hepatitis C virus sensitivity to neutralizing antibody by promoting E2 secretion through exosomes

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Background & Aims: Assembly of infectious hepatitis C virus (HCV) particles is known to involve host lipoproteins, giving rise to unique lipo-viro-particles (LVPs), but proteome studies now suggest that additional cellular proteins are associated with HCV virions or other particles containing the viral envelope glycoprotein E2. Many of these host cell proteins are common markers of exosomes, most notably the intracellular adaptor protein syntenin, which is required for exosome biogenesis. We aimed to elucidate the role of syntenin/E2 in HCV infection.
Methods: Using cell culture-derived HCV, we studied the biogenesis and function of E2-coated exosomes in both hepatoma cells and primary human hepatocytes (PHHs).
Results: Knockout of syntenin had a negligible impact on HCV replication and virus production, whereas ectopic expression of syntenin at physiological levels reduced intracellular E2 abundance, while concomitantly increasing the secretion of E2-coated exosomes. Importantly, cells expressing syntenin and HCV structural proteins efficiently released exosomes containing E2 but lacking the core protein. Furthermore, infectivity of HCV released from syntenin-expressing hepatoma cells and PHHs was more resistant to neutralization by E2-specific antibodies and chronic-phase patient serum. We also found that high E2/syntenin levels in sera correlate with lower serum neutralization capability.
Conclusions: E2- and syntenin-containing exosomes are a major type of particle released from cells expressing high levels of syntenin. Efficient production of E2-coated exosomes renders HCV infectivity less susceptible to antibody neutralization in hepatoma cells and PHHs.

Lay summary: This study identifies a key role for syntenin in the regulation of E2 secretion via exosomes. Efficient production of E2-coated exosomes was shown to make hepatitis C virus less sensitive to antibody neutralization. These results may have implications for the development of a hepatitis C virus vaccine.

Introduction

Around 71 million people worldwide are infected with the hepatitis C virus (HCV).[1] Up to 80% of infected individuals are unable to clear the virus. Persistently infected individuals have a high risk of developing liver cirrhosis and hepatocellular carcinoma. Direct-acting antivirals have profoundly increased treatment efficiency, but a prophylactic vaccine required for the global control of new HCV infections is not available.

A hallmark of infectious HCV particles is their intimate association with components of very low density lipoproteins and low density lipoproteins, giving rise to hybrid virions, designated lipo-viro-particles (LVPs), which are characterized by heterogeneous buoyant densities.[2–5] Core protein, the RNA genome and the heterodimeric E1/E2 envelope glycoprotein complex are major components of infectious LVPs.[6–8] In addition, the non-exchangeable apolipotein (apo) B and several exchangeable apolipoproteins (apoE, AI, CI and CIII) were found to be incorporated into LVPs.[8–15] Apart from infectious LVPs, non-infectious lipoprotein-like particles have been reported.[16,17] These particles contain E1/E2 complexes and apoE and apoB but lack the core protein. In addition, the proteome of E2-affinity purified HCV particles identified multiple cellular proteins specifically associated with E2-containing particles.[18] Interestingly, many of these proteins are common markers of exosomes. In particular, syntenin (also known as syndecan binding protein; SDCBP), the major intracellular adaptor protein for exosome biogenesis and cargo loading[19–21] was co-purified with E2-containing particles. Gain and loss of function studies established that syntenin works as a key regulator, together with syndecan and Alix, to control the formation of intraluminal vesicles (ILV) and the release of heat shock protein 70 (HSP70)

Keywords: HCV; Syntenin; Exosomes; Neutralizing antibodies; Lipo-viro-particles; Virions.
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as well as tetraspanin-containing exosomes from multivesicular bodies (MVBs). These results suggest that in addition to being part of infectious HCV, LVPs and lipoprotein-like subviral particles, E2 might be a component of exosomes. Although E2 and exosome-like vesicle association has been implied through electron microscopy (EM) analysis,[7,22,23] the major determinant of E2-exosome production and its function were not known.

Given the pivotal role played by syntenin in exosome biogenesis and its presence in E2-containing particles, we studied the role of syntenin in the HCV life cycle. We report that syntenin expression promotes the release of E2-containing exosomes and renders HCV virions less susceptible to neutralization by E2-specific antibodies and chronic-phase patient sera.

Material and methods

Cell lines and primary human hepatocytes

The Huh7.5.1 cell line and its derivatives were cultured in Dulbecco's modified minimal essential medium (DMEM; Invitrogen) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml and 10% fetal calf serum (FCS) (complete DMEM). The exosome depleted FCS was used for exosome preparations. 293 T cells were cultured in the same medium. To prepare primary human hepatocytes (PHHs), liver cell suspensions were obtained by digesting liver tissues using collagenase 2-step perfusion.[24] Cells were pelleted by successive low-speed centrifugation at 30 × g, 40 × g and 50 × g each for 10 min at room temperature. PHH pellets were resuspended and seeded into plates coated with collagen-I using DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine and 100 IU/L insulin. Cells were incubated at 37 °C in a 5% CO_2 atmosphere and manually shaken every 10 min. After 45 min the medium was changed to remove non-adhered cells. All experiments were carried out in accordance with the approved guidelines and regulations.

Plasmids and chronic-phase patient sera

Plasmids encoding the full-length HCV chimera Jc1-E2^{Flag}, deletion mutants (Jc1-Δcore, Jc1-ΔE1E2 and Jc1-Δp7), JcR2a constructs (a Jc1-derived construct encoding a *Renilla* luciferase reporter gene) and chimeric constructs (H77, Con1 and S52) have previously been described.[15,25–29] Serum samples with chronic genotype 1b HCV infection were obtained from the First Hospital of Jilin University. All individuals signed an informed-consent form. The study protocol was approved by the Ethics Committee of the First Hospital of Jilin University.

HCV neutralization

HCV neutralization was performed as previously described.[30] Huh7.5.1 cells were seeded into 96-well plates, with 6×10^3 Huh7.5.1 cells/well. The next day, different dilutions of antibody or serum were mixed with cell culture-derived HCV (HCVcc). The mixture was incubated for 1 h at 37 °C and then was used to infect cells. Viral inoculate was removed from cells 4 h post virus infection and cultured for 72 h. Immunostaining for the HCV NS5A was performed to quantify foci number in each well.

Details of the other methods used, including knockout cell line generation, HCVcc production, affinity purification, immunoprecipitation, attachment assay, HCV infectivity quantification, western blot, quantitative PCR, exosome preparation, attachment assay, immunofluorescence, immuno-EM and density gradient analysis, are provided in the supplementary information.

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

Production of infectious HCV particles is not affected by syntenin expression

Proteome analyses of E2-affinity purified HCV virions confirmed the tight association between infectious particles and host apolipoproteins. Consistent with an earlier report,[18] these analyses also identified various host proteins that are components of exosomes, most notably syntenin (Table S3). This result suggested that exosome proteins might be components of HCV virions or other E2-containing structures and that they might play a role in the HCV life cycle.

To address this possibility, we focused on syntenin, an exosome marker with a key role in exosome biogenesis, as it was specifically recovered both in our proteome and in the analysis performed by Lussignol and coworkers.[18] Firstly, we generated syntenin-knockout pools of Huh7.5.1 cells that are highly permissive for HCV, using lentiviral transduction of 3 different single guide RNAs (sgRNAs). Knockout was most efficient with sgRNA-3 and this cell pool was used for subsequent analyses (Fig. 1A). The impact of syntenin knockout on the HCV life cycle was determined by assessing the infectivity of HCV in this cell pool and by quantifying viral RNA replication by measuring the production of HCV proteins and infectious virions at different time points after infection. Compared to control cells, Huh7.5.1 cells lacking syntenin were equally able to support HCV genome replication and the production of infectious progeny (Fig. S1C; Fig. 1B and 1C), suggesting that syntenin is not required for robust HCV replication and assembly.

Next, we compared syntenin expression level in Huh7.5.1 cells with that detected in PHHs. We found that the expression level of syntenin in PHHs is up to 6-fold higher than that in Huh7.5.1 cells (Fig. 1D; Fig. S2A and 2B). Therefore, we generated Huh7.5.1 cells overexpressing syntenin (Fig. 1D; Fig. S2C). Compared to control cells, ectopic expression of syntenin did not alter the permissiveness of Huh7.5.1 cells for HCV genome replication, protein synthesis and infectious progeny production (Fig. S2E; Fig. 1E and 1F).

Although these observations suggest that syntenin is not directly involved in the HCV life cycle, we noticed that the intracellular level of E2 in syntenin-expressing cells was significantly lower than that in naïve cells and control cells stably expressing green fluorescence protein (GFP) (Fig. 1E; Fig. S2D). In contrast, the intracellular abundance of core and NS5A was not affected by syntenin overexpression (Fig. 1E).

Syntenin overexpression enhances E2 secretion through exosome-like vesicles without affecting the production of infectious HCV particles

Cooperating with syndecan and Alix, syntenin has recently emerged as major player in the biogenesis of exosomes and its overexpression is sufficient to induce highly efficient exosome production through the MVB.[19,20] Intracellular E2 levels declined at a much faster rate in Huh7.5.1-syntenin cells than in GFP expressing cells (Fig. S3) and we hypothesized that more efficient exosome production in Huh7.5.1-syntenin cells might contribute to this observation. To test this hypothesis, we tested

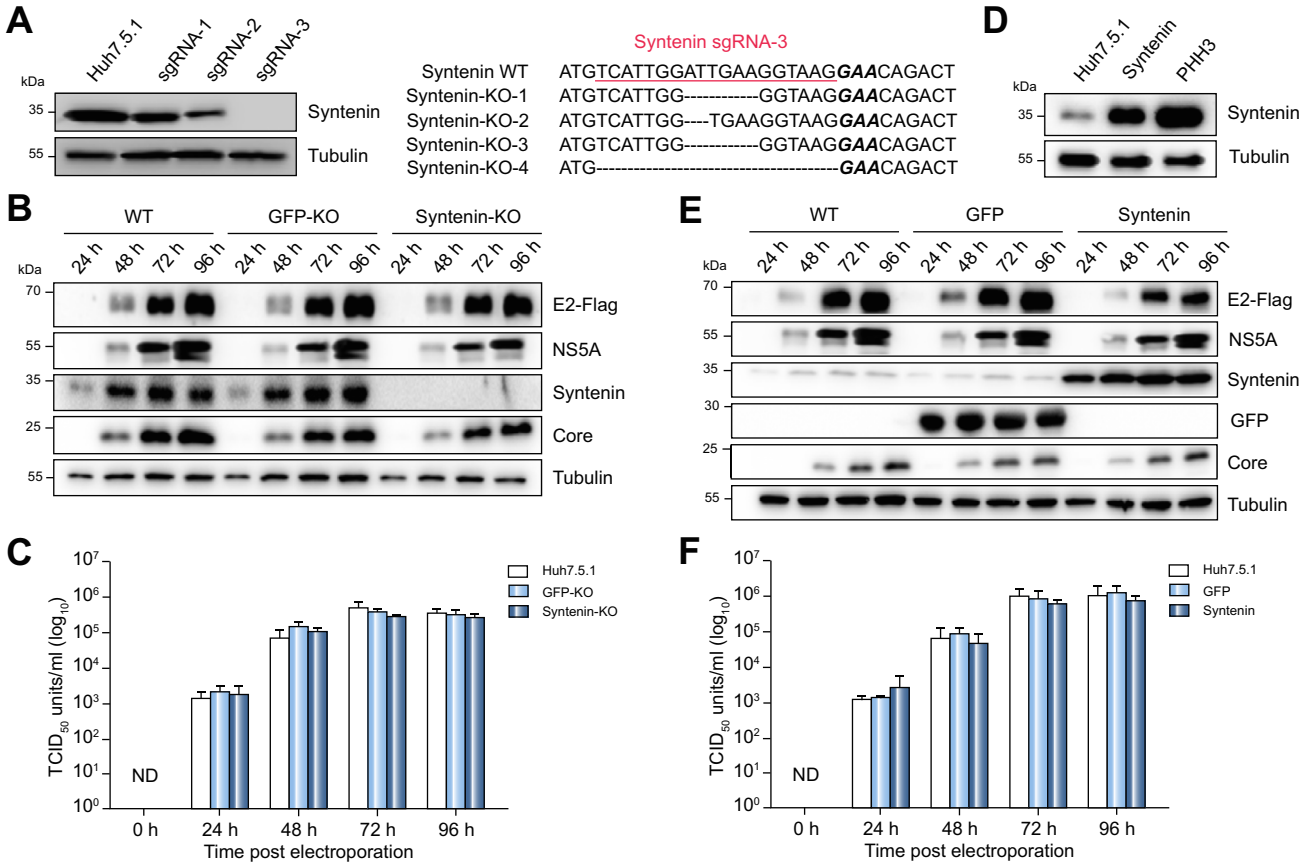

Fig. 1. Impact of syntenin knockout and overexpression on HCV protein expression and viral progeny production. (A) Syntenin knockout Huh7.5.1 cells were generated by using the CRISPR/Cas9 system introduced into cells by lentiviral transduction. A Western blot analysis of transduced cell pools and the parental cell line is shown on the left. The panel to the right displays the deletions identified in cell clones transduced with the sgRNA-3 construct. Sequences spanning the syntenin target site in individual knockout cell clones were amplified by PCR. The nucleotide sequence of 4 individual knockout clones was compared with wild-type syntenin in Huh-7.5.1 cells. The target sequence of the most effective sgRNA is underlined. (B and C) Huh7.5.1 (WT), the negative control cell line (GFP-KO) and syntenin-KO cells (cell pool from sg-RNA3) were electroporated with Jc1-E2^{Flag} RNA; culture supernatants and cells were collected at given time points post electroporation. (B) Total cell lysates were analyzed by SDS-PAGE and Western blot using antibodies specified on the right of each panel. (C) Virus titers were determined using the $TCID_{50}$ assay. (D) Syntenin-expressing Huh7.5.1 cells were generated by lentiviral transduction. Expression of the proteins in cell pools was determined by Western blot. Tubulin served as loading control. PHH3: total cellular proteins from PHHs (patient number 3). (E and F) Huh7.5.1 cells (WT), GFP (negative control) and syntenin-expressing cells were electroporated with Jc1-E2^{Flag} RNA. Culture supernatants and cells were collected at given time points post electroporation; followed by similar (E) western blot and (F) $TCID_{50}$. Data represent means with standard deviation from 3 independent assays. GFP, green fluorescence protein; HCV, hepatitis C virus; KO, knockout; N.D., not detected; sgRNA, single guide RNA; $TCID_{50}$, 50% tissue culture infectious dose; WT, wild-type. (This figure appears in colour on the web.)

the impact of syntenin overexpression on exosome production. As shown in Fig. 2A and 2B, overexpression of syntenin led to significantly increased production of extracelluar vesicles. Since HCV (Jc1-E2^{Flag}) facilitated straightforward E2-complex purification and the addition of the flag-tag to E2 did not change E2 secretion (Fig. S4A), we chose Jc1-E2^{Flag} for the following experiments. Next, we had to rule out a possible impact of syntenin overexpression on HCV virion production. Jc1-E2^{Flag} virions were produced in Huh7.5.1 cells ectopically expressing GFP or syntenin, and secreted virions were subjected to density gradient analysis to determine the biophysical and biochemical properties of released virus particles. We found that syntenin expression levels did not change the density distribution of infectious HCV virions, suggesting that syntenin does not affect HCV-LVP production (Fig. S4B). Moreover, neither the amount of virus particles nor the titer of infectious virions was altered (Fig. S4C and 4D), suggesting that reduced intracellular E2 amounts might be due to enhanced secretion of E2-containing extracellular vesicles.

To address this assumption, intra- and extracellular E2-containing complexes of cells transfected with the Jc1-E2^{Flag} genome were characterized. We first conducted E2-Flag-specific immune-precipitation using total cell lysates in the presence of 1% Triton X-100 and found that E2 coprecipitated core, but not syntenin (Fig. 2C), arguing against strong protein–protein interactions between syntenin and E2. When we performed E2-Flag-specific capture of extracellular E2-containing structures (including virions), we found that E2-Flag-affinity purification pulled down comparable amounts of core protein and apoE, most likely reflecting virus particles (Fig. 2D; Fig. S4C,D). Although these observations corroborated the lack of a syntenin effect on the HCV life cycle, we noted that syntenin overexpression led to higher abundance of E2, Alix, HSP70, CD63 and syntenin in the E2-Flag containing immune complexes (Fig. 2D; Fig. S5A,B). These results suggested that, in addition to being incorporated into infectious HCV particles, E2 might reside on the surface of extracellular vesicles carrying exosome markers.

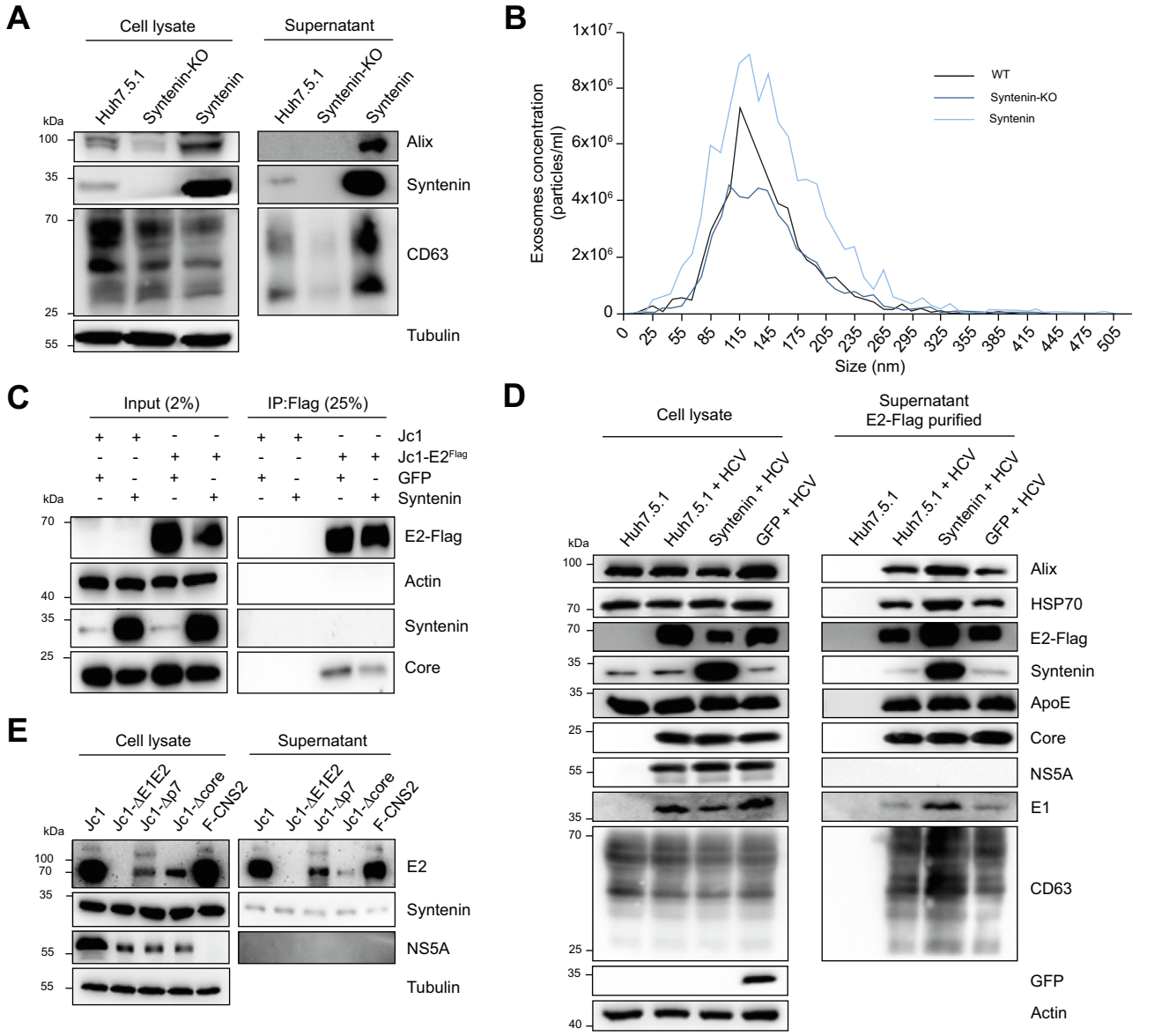

Fig. 2. Ectopic expression of syntenin increases production of extracellular vesicles and E2 secretion independent from infectious HCV production. (A) Exosome samples were prepared using sequential centrifugation. Intracellular and exosomal content of Alix, syntenin and CD63 were detected by western blot. (B) Exosome samples were subjected to nanoparticle tracking analysis. (C) GFP and syntenin-expressing Huh7.5.1 cells were electroporated with Jc1 or Jc1-E2^{Flag} RNA. After 72 h, cells were harvested, lysates were used for flag-specific pull-down and captured immune complexes were analyzed by Western blot using antibodies specified on the right (right panel). Total cell lysates used for the immunoprecipitation were analyzed in parallel (left panel). Numbers above the panels indicate the percentage of the respective sample loaded onto the gel. (D) Infectious HCV (Jc1-E2^{Flag}) contained in supernatant of Huh7.5.1-GFP or Huh7.5.1-syntenin cells was concentrated by ultrafiltration, subjected to flag-affinity purification, and analyzed by western blot (right panel). Total cell lysates were analyzed in parallel. (E) Huh7.5.1 cells were electroporated with Jc1 or deletion mutants specified on the top. After 96 h, cells and supernatant were harvested for western blot. Corresponding cell lysates were analyzed in parallel. Detected proteins are specified on the right of each panel. GFP, green fluorescence protein; HCV, hepatitis C virus.

Intra- and extracellular E2 levels were compared in Huh7.5.1 cells electroporated with assembly defective HCV genomes (Jc1-ΔE1E2, Jc1-Δp7 and Jc1-Δcore) and a cell line stably expressing an HCV polyprotein fragment from core to NS2 (CNS2). This revealed that E2 secretion efficiency is not significantly different from the HCV wild-type (Fig. 2E, Fig. S5C.D). Collectively, these data suggest that syntenin overexpression enhances the secretion of E2, independently of infectious HCV virion assembly.

Next, we applied centrifugation methods and an exosome release inhibitor, GW4869, to verify HCV E2 secretion through exosomes. Using supernatant of Jc1-E2^{Flag} transfected cells we found that Flag-E2 is not present in 10 K (10,000 × g) pellets (ectosomes) but is in 100 k (100,000 × g) pellets (exosomes) (Fig. 3A). GW4869 treatment significantly reduced E2^{Flag} secretion (Fig. 3B; Fig. S6A,B) without affecting the production of infectious HCV progeny (Fig. S6C). Since syntenin-enhanced E2 secretion did not increase infectivity and RNA amounts in E2^{Flag} preparations, we hypothesized that RNA might not be a major component of E2 and syntenin-containing extracellular vesicles. To test this assumption, we conducted attachment assays using Huh7.5.1 cells and affinity purified HCV particles released from Huh7.5.1-GFP cells or Huh7.5.1-syntenin cells. Attachment

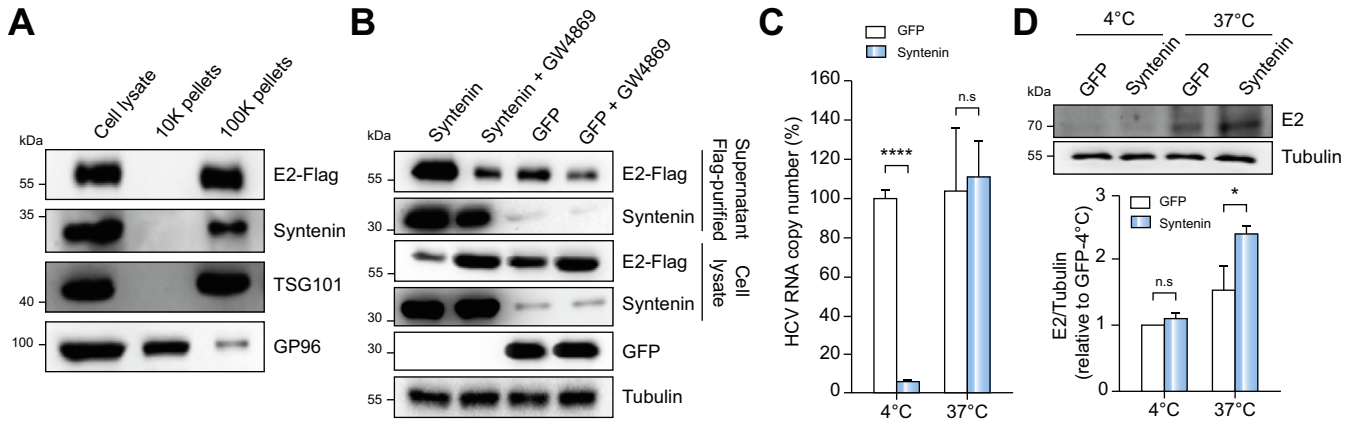

Fig. 3. HCV infected cells produce exosomes carrying E2. (A) Huh7.5.1 cells were electroporated with the Jc1-E2^{Flag} genome. After 96 h, supernatants were harvested and subjected to differential centrifugation at 300×g for 10 min, 2,000×g for 10 min, 10,000×g for 40 min (10 k pellets) and 100,000×g for 90 min (100 k pellets). The pellets were lysed and analyzed by western blot. Detected proteins are specified on the right of each panel. (B) GW4869 inhibits secretion of E2 and syntenin. GFP or syntenin overexpressing Huh7.5.1 cells were electroporated with Jc1-E2^{Flag}; 48 h post electroporation cells were treated or not with GW4849 (10 μM) for 24 h. Cells and culture supernatants were collected for western blot. (C) Viruses released from Huh7.5.1-GFP or Huh7.5.1-syntenin cells were affinity purified and pre-chilled to 4 °C. Cell attachment was performed on ice for 2 h. Thereafter, cells were washed 5 times with cold PBS, followed by cell lysis using TRIzol and RNA extraction for RT-qPCR. (D) After attachment at 4 °C or internalization at 37 °C, E2 levels in total cell lysates were analyzed by western blot (top), which followed E2 quantification (bottom). Data represent means with standard deviation from 3 independent assays. GFP, green fluorescence protein; HCV, hepatitis C virus; RT-qPCR, quantitative reverse transcription PCR.

assays were conducted at 4 °C to avoid uptake and to just measure binding of purified E2 structures to the cells. As shown in Fig. 3C, when using RNA-equivalent amounts of inocula, the attachment efficiency of E2^{Flag} preparations from Huh7.5.1-syntenin cells was significantly reduced compared to that of E2^{Flag} preparations from Huh7.5.1-GFP control cells. In contrast, E2 attachment was not significantly different (Fig. 3D). This result supported the notion that most of the E2^{Flag} containing structures did not contain HCV RNA. Moreover, immunofluorescence analysis demonstrated that most areas of E2 and syntenin co-localization were devoid of viral RNA (Fig. S7). Collectively, these data suggest that syntenin overexpression increases the secretion of E2 through enhanced exosome production.

Syntenin promoted E2-coated exosomes are formed independently of HCV replication and they do not contain core protein

To further corroborate the assumption that syntenin overexpression induces efficient secretion of E2-containing extracellular vesicles independently of HCV assembly and release, we established cell lines stably expressing syntenin or GFP as control and an HCV polyprotein fragment (CNS2) encompassing Core, E1, Flag-E2, p7 and NS2 (Fig. 4A; Fig. S8A). Culture supernatants of these cells, or naïve Huh7.5.1 cells with or without HCV infection, were harvested and used for Flag-affinity purification. As shown in Fig. 4B and Fig. S8B, syntenin overexpression significantly increased the abundance of Alix, HSP70, CD63 and syntenin in E2-containing extracellular structures whereas core protein was absent, corroborating the hypothesis that E2 is released on the surface of extracellular vesicles. Flag-E2 containing structures released from Huh7.5.1 cells expressing syntenin and CNS2 were further subjected to immune-EM analysis. Results showed that these membranous structures carry E2 and CD63 on their surface (Fig. 4C).

With the aim of confirming that the released structures were extracellular vesicles, we purified the structures using sequential ultracentrifugation (Fig. 4D and Fig. S8C). In the culture supernatant of cells overexpressing syntenin, larger amounts of E2^{Flag} were captured and immune complexes also contained large amounts of Alix and hsp70. Release of these exosome markers was not affected by the CNS2 HCV polyprotein as analogous amounts were found in immune complexes isolated from Huh7.5.1-syntenin cells in the absence of HCV proteins. Importantly, stable knockdown of TSG101, a protein that is essential for exosome biogenesis,[31] significantly decreased E2 abundance in exosome pellets (Fig. 4E). These E2-containing extracellular vesicles had a density of 1.121 g/ml (Fig. S9A), consistent with them being exosomes. Exosomes with similar density (1.123 g/ml) were detected in HCV preparations from Huh7.5.1-syntenin cells (Fig. S9B), while infectivity peaked at a density of 1.08 (Fig. S4B). Collectively, these data corroborate the assumption that E2-coated exosomes are produced independently of HCV virion production and that E2-coated exosomes secreted from Huh7.5.1-syntenin cells represents a major subviral particle population.

Impact of syntenin on HCV sensitivity to antibody neutralization

Having found that E2-coated exosomes are assembled and released in parallel to HCV virions, we hypothesized that these exosomes might possess E2 epitopes similar to those of E2 incorporated into viral particles. In this case, E2-coated exosomes might sequester HCV-neutralizing antibodies and contribute to escape from the humoral immune response. Therefore, we performed neutralization assays using the broadly HCV-neutralizing monoclonal antibody AR3A[32] and HCV (JcR2a and Jc1)[25] produced in Huh7.5.1-GFP and Huh7.5.1-syntenin cells, respectively. As shown in Fig. 5A and 5B, HCV released from Huh7.5.1-GFP cells was efficiently neutralized by the AR3A antibody, whereas HCV derived from Huh7.5.1-syntenin cells was much less susceptible to neutralization. At an antibody concentration of 0.4 μg/ml, the infectivity of HCV produced from Huh7.5.1-syntenin cells was not significantly affected. In contrast, at the same concentration, the AR3A antibody blocked up to 90% of the infectivity of HCV derived from Huh7.5.1-GFP cells. Consistently, addition of

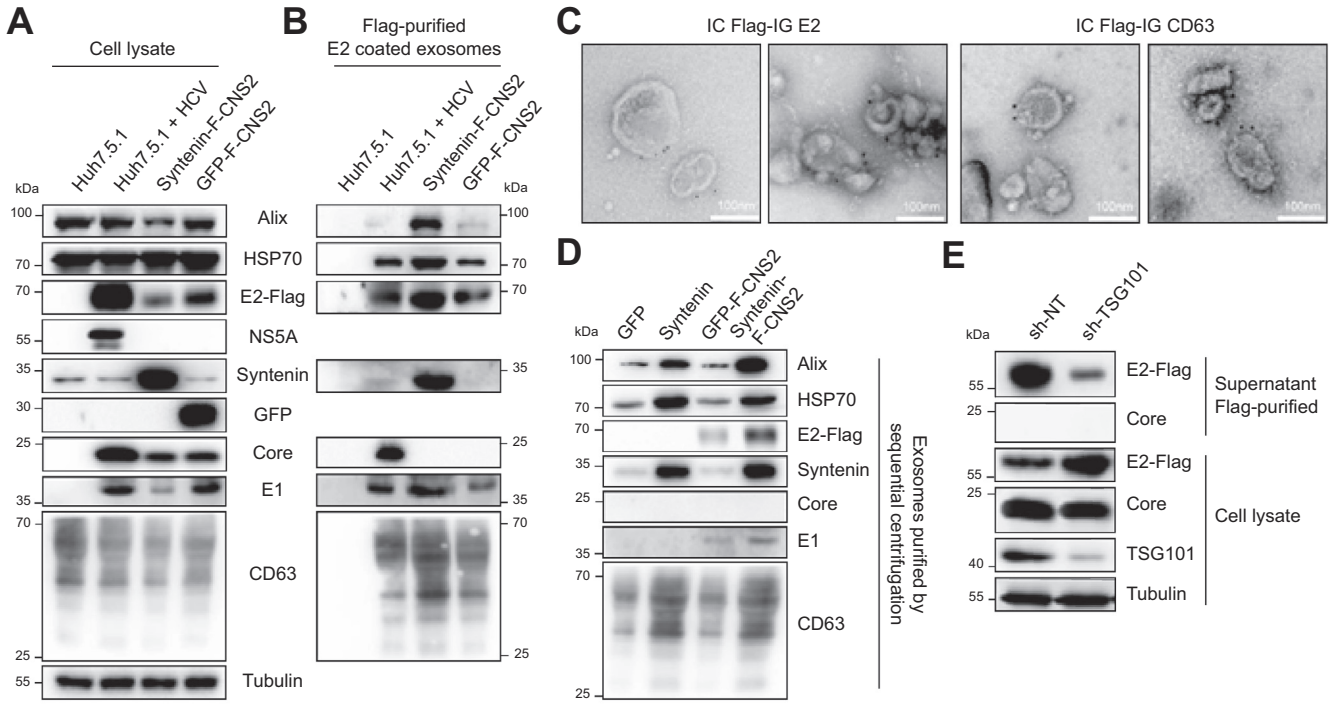

Fig. 4. Efficient E2 secretion in syntenin-containing exosomes is independent of HCV replication and virus production. (A) Generation of GFP- and syntenin-Huh7.5.1 cell pools stably expressing CNS2 (with $E2^{Flag}$). Total cell lysates were subjected to western blot analysis using antibodies specified on the right. Naïve Huh7.5.1 cells electroporated or not with the full-length Jc1-$E2^{Flag}$ genome (HCV) served as reference. (B) $E2^{Flag}$-containing immune complexes isolated from supernatants of cells specified on the top were analyzed by western blot using antibodies with given specificity. (C) Immuno-EM analysis of affinity purified extracellular vesicles released from Huh7.5.1 cells expressing syntenin and F-CNS2. Representative images of Flag-E2 and CD63 labeling are shown. Primary antibodies were visualized with gold-conjugated secondary antibody (10 nm diameter gold particles). (D) Exosomes from Huh7.5.1 cells expressing GFP, syntenin, GFP-F-CNS2 and syntenin-F-CNS2 were enriched by sequential centrifugation. Proteins contained in exosome preparations were determined by western blot using antibodies specified on the right of each panel. (E) Stable knockdown of TSG101 was performed in Huh7.5.1 cells expressing F-CNS2. Cells and supernatant derived E2-exosomes were collected for western blot. Detected proteins are specified on the right of each panel. CNS2, core to NS2; EM, electron microscopy; GFP, green fluorescence protein; HCV, hepatitis C virus.

E2-coated exosomes from Huh7.5.1-syntenin-CNS2 cells and Huh7.5.1-syntenin-E2 to HCV infection desensitized AR3A antibody neutralizing potency significantly (Fig. 5C). Moreover, Huh7.5.1-syntenin cells derived JFH1 chimeric viruses from other genotypes, including H77 (1a), Con1 (1b), S52 (3a), all demonstrated a relatively reduced susceptibility to AR3A and AP33[33] neutralization (Fig. 5D).

Knowing that syntenin expression levels determine E2-coated and syntenin-containing exosome production from HCVcc, we further evaluated E2, core, syntenin, CD63 and Alix levels in 74 serum samples (genome copy number > $5^{*}10^{5}$) from patients with chronic HCV genotype 1b infection (Fig. S10A). The results showed that syntenin level in sera is strongly correlated to the levels of other exosome markers, suggesting syntenin level is a reliable indicator of serum exosome abundance (Fig. S10B, 10C). Following this, we found that syntenin level is correlated with E2 level, whereas core level is not (in Fig. 6A, Fig. S10D), which is in agreement with cell culture derived observations. We hypothesized that E2-exosome abundance in sera reflected by syntenin level could be responsible for weakening the potency of neutralizing antibody in these sera. Therefore, we tested the neutralization activity of these sera against Con1-chimeric virus and analyzed the correlations between serum neutralizing capability and E2/syntenin. The results demonstrated that higher E2/syntenin levels correlated with less serum neutralizing capability against homologous chimeric HCV (Fig. 6B, 6C). In addition, we selected 3 serum samples (H19, H70, H73) with a high level of E2/syntenin and another 3 (H49, H63, H74) with low E2/syntenin abundance to perform cross-genotype neutralization assays using JFH1 chimeric virus. Similarly, cross-genotype neutralizing activity of serum samples with lower E2/syntenin was stronger than those with higher E2/syntenin (Fig. 6D-F). At last, we produced Con1 (Fig. 6G) and J6 (Fig. 6H) chimeric viruses from both Huh7.5.1-syntenin and Huh7.5.1-GFP cells and found that virus derived from syntenin overexpressing cells with higher E2-exosome production is also more resistant to chronic-phase patient serum (H27 with moderate level of E2/systenin). Collectively, these data showed that both sera containing E2-exosome and HCVcc derived E2-exosome could assist HCV escape from patient-derived neutralization.

The syntenin level in serum does not necessarily reflect that in liver. Thus, we further validated these findings in PHHs. Due to a low yield of HCV infectivity from these cells, infection by HCV (Jc1-$E2^{Flag}$)[24] was conducted after syntenin depletion was achieved by lentiviral transduction of syntenin-specific short hairpin RNAs (shRNAs) (Fig. 7A). As shown in Fig. 7B, 2 days after lentiviral transduction, syntenin abundance in PHHs was reduced. We then infected these cells with Jc1 at a multiplicity of infection of 5. Viruses released from these cells were collected for antibody neutralization assays 48 h later. In agreement with the findings obtained in Huh7.5.1 cells, HCV produced from control PHHs was more resistant to AR3A antibody neutralization than HCV derived from syntenin-knockdown PHHs (Fig. 7C). Up to 80% of HCV infectivity from syntenin-depleted PHHs

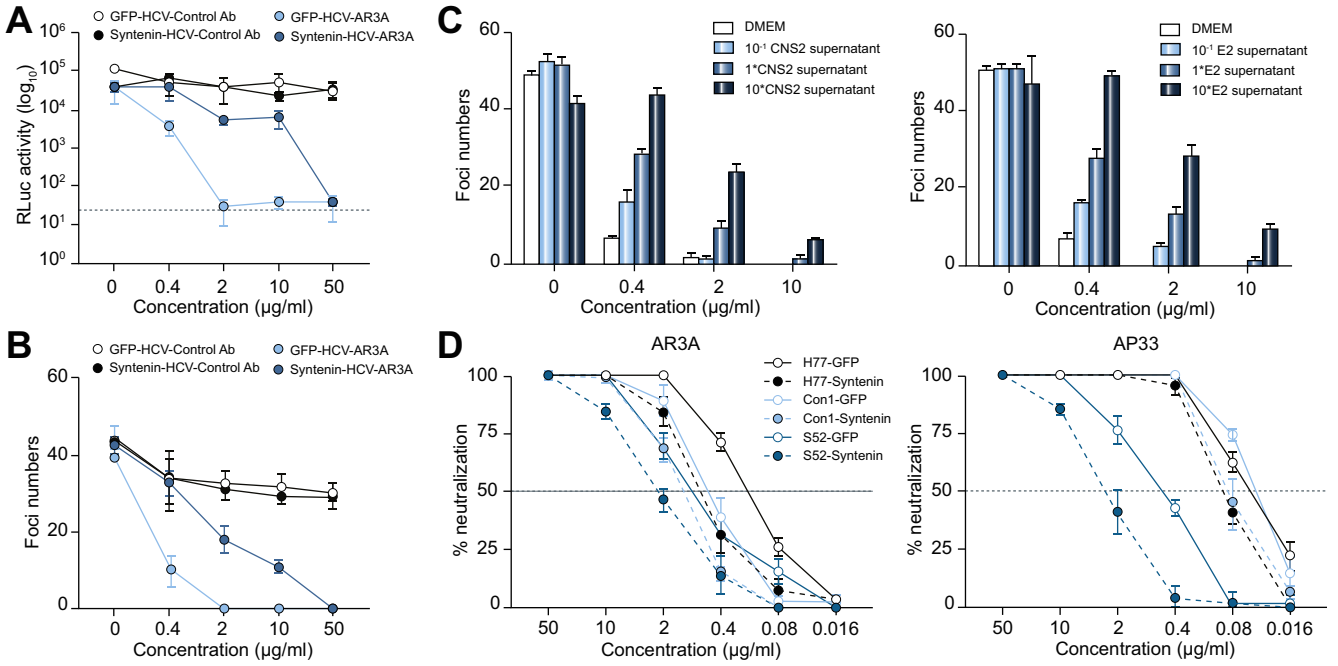

Fig. 5. HCV produced from Huh7.5.1-syntenin cells is less susceptible to neutralization with an E2-specific antibody. (A) HCV particles released from JcR2a-transfected Huh7.5.1-GFP and Huh7.5.1-syntenin cells were subjected to neutralization. After prior treatment with given concentrations of the E2-specific antibody AR3A or an irrelevant control antibody for 1 h, samples were used to infect naïve Huh7.5.1 cells. After a 4 h-incubation period inocula were removed, fresh medium was added, and cells were harvested 72 h later. HCV replication was quantified by luciferase assay. (B) HCV particles released from Jc1-transfected Huh7.5.1-GFP and Huh7.5.1-syntenin cells were subjected to the analogous neutralization assay. Viral titers were quantified. (C) HCV particles released from Jc1-transfected Huh7.5.1 cells were mixed (volume ratio 1:1) with 10-fold diluted (10^{-1}), undiluted (1*),or 10-fold concentrated (10*) culture supernatant from CNS2 (left) or E2 (right) expressing Huh7.5.1 cells. The subsequent neutralization assay was performed with the AR3A antibody. (D) H77, Con1 and S52 chimeric HCV particles were produced from Huh7.5.1-GFP and Huh7.5.1-syntenin cells. AR3A (left) and AP33 (right) monoclonal antibodies were used for neutralization assay. 72 h post infection, foci numbers were counted and the percentages of neutralization are shown. Data represent means and standard deviation from 3 independent assays. CNS2, core to NS2; GFP, green fluorescence protein; HCV, hepatitis C virus.

was neutralized by the AR3A antibody at a concentration of 0.002 ug/ml. At the same concentration, the AR3A antibody did not affect infectivity of HCV produced in non-targeting shRNA transduced control PHHs. Taken together, these results suggest that syntenin enhanced the production of E2-coated exosomes, lowering the susceptibility of HCV to neutralization by E2-specific antibodies.

Discussion

Taking advantage of HCV E2-specific affinity purification and ultracentrifugation methodologies, important insights into the morphological and biochemical traits of HCV virions have been gained.[6,7,17,25,34] Previous EM analysis demonstrated the presence of E2 decorated extracellular vesicles. However, the biochemical insight of these vesicles and their function has not been investigated. In this study, we identified that syntenin is a determinant of E2-coated exosome production in HCV infected cells (Fig. 8). We believe that these E2-coated exosomes help HCV escape from E2-specific antibody neutralization for the following reasons: i) viral infectivity of HCV produced from syntenin-depleted cells was more sensitive to antibody neutralization; ii) E2-coated exosomes from Huh7.5.1 cells helped HCV infection in the presence of neutralizing antibody; iii) lower serum neutralizing capability was correlated with higher E2/syntenin levels from serum samples. Notably, the correlation between E2 levels and syntenin levels is relatively moderate. E2-coated exosomes can only be produced from liver, whereas exosome abundance in serum samples may be affected by other sources. This could explain why the correlation between serum neutralizing capability and E2 levels was more significant.

Although neither depletion, nor overexpression of syntenin in Huh7.5.1 cells or PHHs affected the HCV life cycle, syntenin expression was found to enhance E2 secretion as revealed by decreased intracellular and increased extracellular E2 amounts. The production of infectious virions does not depend on syntenin expression level and ectopic expression of syntenin does not alter the biochemical and biophysical properties of infectious virions. Additionally, E2-coated exosomes lacking core protein and viral RNA are released from Huh7.5.1 cells expressing syntenin and CNS2. Moreover, in contrast to the high enrichment of virions by E2-affinity purification, CD63 affinity purification precipitated less than 0.01% of input virions (data not shown). With these observations, we propose that syntenin expression-enhanced E2 secretion is independent of HCV assembly. Exosomes seem to be involved in HCV life cycle in many ways.[23,35–37] It has been reported that exosomes carrying HCV RNA trigger innate immune responses in plasmacytoid dendritic cells and induce monocyte differentiation.[35,36] Exosomes might transmit replication competent sub-genomic RNA to spread infection,[23,37] although this transmission route is not robust compared to cell free virus infection. Our findings identified that syntenin modulated E2-coated exosomes in parallel with the production of infectious HCV particles.

Ectopic expression of syntenin in Huh7.5.1 cells, at a level comparable to that in PHHs, enhanced HCV E2 secretion and profoundly reduced intracellular E2 abundance. However, knockout of syntenin did not increase intracellular E2 amounts.

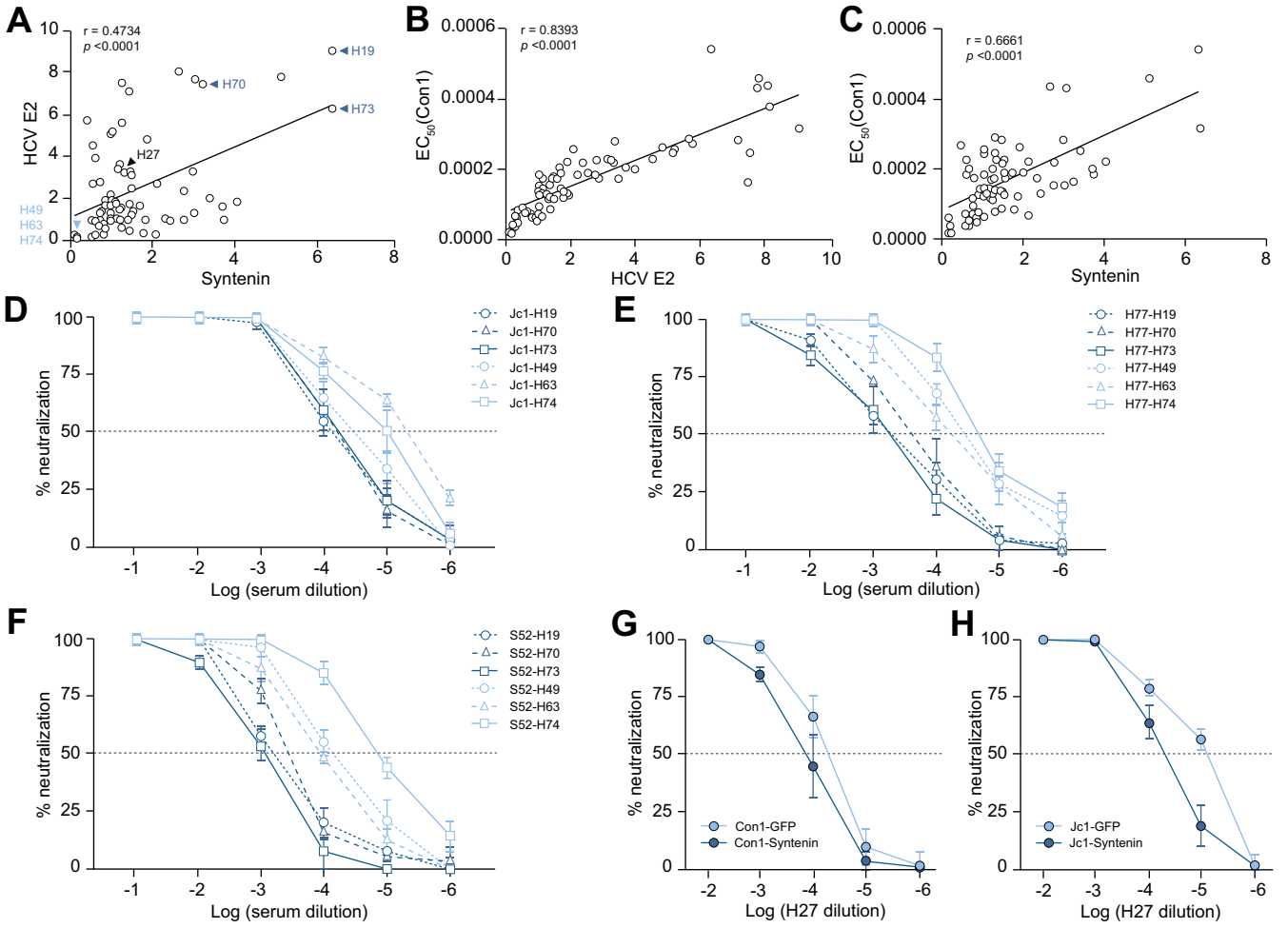

Fig. 6. High E2/syntenin level in chronic-phase patient sera correlates with reduced serum neutralizing activity. (A) Syntenin, E2 and core levels in 74 serum samples (genome copy number >5*10^5) from chronic genotype 1b hepatitis C patients were evaluated by quantification of western blot (Fig. S10). (B, C) Correlation between E2 levels *vs.* syntenin level in serum samples. Correlation between serum neutralizing capability *vs.* E2/syntenin level in serum samples. (D-F) J6, H77 and S52 chimeric HCV particles were produced from Huh7.5.1 cells. Neutralization assays were performed using 2 groups of chronic-phase patient serum samples (H19, H70 and H73 with high level of E2/syntenin and H49, H63, H74 with low E2/syntenin abundance). 72 h post infection, foci numbers were counted and percentages of neutralization were shown. (G,H) Con1 and J6 chimeric HCV particles were produced from Huh7.5.1-GFP and Huh7.5.1-syntenin cells. Chronic-phase patient serum (H27) was used for the neutralization assay. Data represent means and standard deviation from 3 independent assays. GFP, green fluorescence protein; HCV, hepatitis C virus.

We speculate that far less E2 is secreted via exosomes (supported by constitutive syntenin expression in Huh7.5.1 cells) than as a component of infectious HCV particles. This could also explain why the amount of syntenin and other exosome markers is much lower than apoE in E2-affinity purified HCV preparations from naïve Huh7.5.1 cells.

Membrane cargo protein sorting to syntenin-containing exosomes is mediated by various mechanisms involving ESCRT, tetraspanins, syndecan and lipid-dependent routes.[21] Interestingly, it has been shown that human CD81 can reinforce HCV envelope protein secretion through exosomes in Chinese hamster ovary cells,[38] suggesting that CD81-enhanced exosome biogenesis or tetraspanin-mediated sorting mechanisms might be involved in E2 loading onto syntenin-containing exosomes. During HCV entry, the CD81 large extracelluar loop interacts with the E2 ectodomain.[39,40] However, it is unclear whether CD81-enhanced E2 secretion via exosomes is mediated by the same interaction or an interaction between transmembrane regions of these 2 proteins within membrane microdomains. Further experiments are warranted to assess the contribution of tetraspanin-mediated and other sorting mechanisms to E2-exosome formation. E1/E2 heterodimers are present on the surface of both HCV-LVPs and exosomes. Exosomes carrying E1/E2 heterodimer or only E2 can similarly desensitize AR3A neutralization, suggesting that the AR3A epitope is not altered by the presence of E1. But we could not rule out the possibility that other E2 epitopes may be affected by E1.

HCV utilizes different strategies to escape neutralization by antibodies. It has been shown that serum components like high-density lipoprotein attenuate antibody-mediated neutralization of HCV in a hyper variable region 1 (HVR1) dependent manner.[41–43] During HCV-LVP assembly and maturation, apolipoprotein E might assist neutralization escape via masking the HCV-LVP surface.[15,44,45] In addition to HVR1-mediated shielding and apolipoprotein E masking strategies, we have shown that HCV infected cells secret E2-coated exosomes that contribute to viral escape from neutralization by both E2 specific antibody and chronic-phase patient serum, which poses another challenge for rational HCV vaccine development.

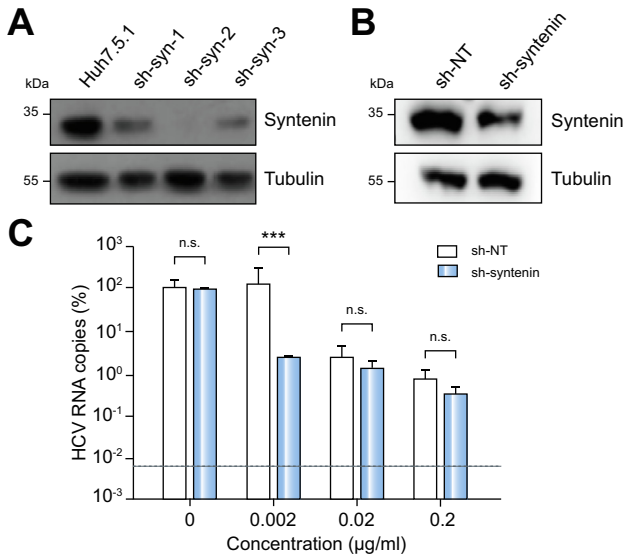

Fig. 7. Lower syntenin expression in PHHs sensitize HCV to antibody neutralization. (A) Knockdown efficiency of 3 different syntenin-specific shRNAs. Three independent syntenin-specific shRNAs were transduced into Huh7.5.1 cells using the lentiviral vector pLKO.1. After 2 passages in puromycin-containing medium, to select for stably transduced cells, they were lysed and western blot analysis was performed to determine knockdown efficiency. Tubulin served as loading control. (B) PHHs were transduced with lentiviruses encoding syntenin-specific shRNA and 48 h later cells were analyzed by western blot. Tubulin served as loading control. (C) PHHs transduced with lentiviruses specified on the top right for 48 h were infected with HCV (Jc1; MOI = 5) and 2 days later culture supernatants were harvested. An E2-specific antibody (AR3A) was added to PHH derived HCV and 1 h later naïve Huh7.5.1 cells were inoculated with the virus-antibody mixture. Viruses were washed off after 4 h and fresh medium was added to the cells. After 72 h, cells were collected for total RNA extraction. HCV genome copy numbers were quantified by RT-qPCR. Data represent means with standard deviation from 3 independent assays. GFP, green fluorescence protein; HCV, hepatitis C virus; MOI, multiplicity of infection; PHH, primary human hepatocyte; sh-NT, non-targeting shRNA; shRNA, short hairpin RNA; RT-qPCR, quantitative reverse transcription PCR.

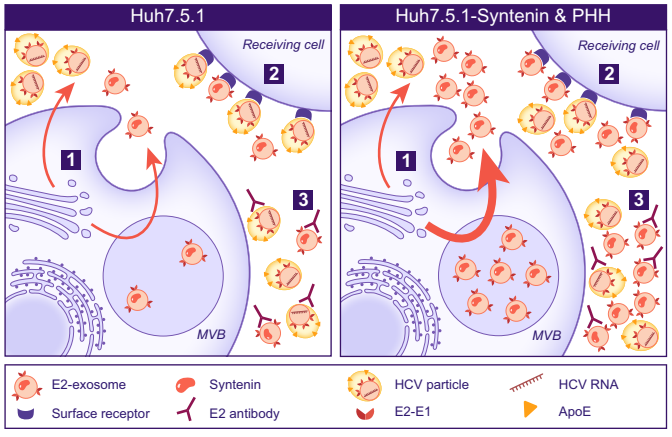

Fig. 8. Working model of syntenin-assisted E2-secretion on exosomes. In naïve Huh7.5.1 cells (syntenin low) secretion of E2-coated exosomes is limited, whereas in Huh7.5.1-syntenin cells and PHHs (syntenin high) secretion of these vesicles is high. These E2-decorated vesicles might sequester neutralizing antibodies, thus promoting neutralization escape of infectious HCV particles. 1, HCV virions and E2 exosome assembly and release; 2, E2-coated exosomes affect HCV virion attachment; 3, E2-coated exosomes reduce HCV sensitivity to neutralizing antibody. HCV, hepatitis C virus; PHH, primary human hepatocyte.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

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

Authors' contributions

L. D., W. J., Y. C. and G. L. designed experiments. L. D., W. J.,X. W., A. M., M. H., Y. C., Y. Y., J. Y., Z. T. and J. N. performed experiments. L. D., X. P., R. B., and G. L. analyzed data. L. D., R. B. and G. L. wrote the manuscript.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.03.006.

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