

## ORIGINAL RESEARCH

## Transmission of HBV DNA Mediated by Ceramide-Triggered Extracellular Vesicles



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## SUMMARY

Extracellular vesicle is a nanovesicle that shuttles proteins, nucleic acids, and lipids, thereby influencing cell behavior. This article shows that ceramide-triggered extracellular vesicles work as DNA cargo for hepatitis B virus-DNA and are capable of transmitting to naive hepatocytes. Further, this article demonstrates that the transmission of hepatitis B virus-DNA via these extracellular vesicles is resistant to antibody neutralization.

**BACKGROUND & AIMS:** An extracellular vesicle (EV) is a nanovesicle that shuttles proteins, nucleic acids, and lipids, thereby influencing cell behavior. A recent crop of reports have shown that EVs are involved in infectious biology, influencing host immunity and playing a role in the viral life cycle. In the present work, we investigated the EV-mediated transmission of hepatitis B virus (HBV) infection.

**METHODS:** We investigated the EV-mediated transmission of HBV infection by using a HBV infectious culture system that uses primary human hepatocytes derived from humanized chimeric mice (PXB-cells). Purified EVs were isolated by ultracentrifugation. To analyze the EVs and virions, we used stimulated emission depletion microscopy.

**RESULTS:** Purified EVs from HBV-infected PXB-cells were shown to contain HBV DNA and to be capable of transmitting HBV DNA to naive PXB-cells. These HBV-DNA-transmitting EVs were shown to be generated through a ceramide-triggered EV production pathway. Furthermore, we showed that these HBV-DNA-transmitting EVs were resistant to antibody neutralization; stimulated emission depletion microscopy showed that EVs lacked hepatitis B surface antigen, the target of neutralizing antibodies.

**CONCLUSIONS:** These findings suggest that EVs harbor a DNA cargo capable of transmitting viral DNA into hepatocytes during HBV infection, representing an additional antibody-neutralization-resistant route of HBV infection. (*Cell Mol Gastroenterol Hepatol* 2017;3:272–283; <http://dx.doi.org/10.1016/j.jcmgh.2016.10.003>)

**Keywords:** HBV; Extracellular Vesicles; Transmission Pathway.

DNA.<sup>1,2</sup> Although an effective HBV preventive vaccine is available, HBV infection continues to be a major health concern.<sup>3</sup> Approximately 350 million people are chronically infected by HBV worldwide, and some of these infections will result in cirrhosis and hepatocellular carcinoma.<sup>2,4</sup>

There is growing evidence that HBV maturation and egress depend on intraluminal vesicles of maturing endosomes known as multivesicular bodies (MVBs).<sup>5–7</sup> HBV core and L envelope proteins have been shown to interact with the ubiquitin-interacting adaptor  $\gamma$ 2-adaptin and the Nedd4 ubiquitin ligase, possibly to regulate transport of the viral structures through the late endosomal pathway.<sup>8,9</sup> In addition, several studies have shown that Vps4A/B and Alix/AIP1 (a functional link between endosomal sorting complexes required for transport (ESCRT)-I and ESCRT-III in mammalian cells) contribute to HBV release.<sup>5,6</sup> Thus, HBV virions are thought to bud into late endosomes or MVBs by using ESCRT/Vps4B functions, subsequently exiting the cell by the extracellular vesicle (EV) pathway.

EVs fall into 2 general categories based on biogenetic definition.<sup>10</sup> One category generally is referred to as the *exosome*, which consists of 30- to 100-nm diameter nanovesicles that originate from the intraluminal vesicles of MVBs; the fusion of MVBs with the cell membrane results in the release of vesicles into the extracellular compartment.<sup>11</sup> The other category generally is referred to as *microvesicles*. Microvesicles generally are larger than exosomes (40- to 1000-nm diameter) and bud from the cellular membrane.<sup>10</sup> Historically, EVs were thought to function primarily in the

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**Abbreviations used in this paper:** anti-HBs, antibody to hepatitis B surface antigen; BSA, bovine serum albumin; ESCRT, endosomal sorting complexes required for transport; EV, extracellular vesicle; GEq, genome equivalent; HA, hemagglutinin; HBC, hepatitis B core; HBcAg, hepatitis B core antigen; HBIG, hepatitis B immune globulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; mRNA, messenger RNA; MVB, multivesicular body; PXB-cells, primary human hepatocytes derived from chimeric mice with human liver; nSMase, neutral sphingomyelinase; nts, nucleotides; PBS, phosphate-buffered saline; qPCR, quantitative real-time polymerase chain reaction; STED, stimulated emission depletion.

Most current article

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Hepatitis B virus (HBV) is an infectious agent that belongs to the *Hepadnaviridae* family, whose members are characterized by a partially double-stranded circular

removal of unnecessary proteins from the cell. More recent studies have shown that EVs are involved in multiple biological processes; under normal and pathologic conditions, EVs appear to shuttle their contents (including proteins, lipids, and nucleic acids) among various cells.<sup>12-14</sup>

Notably, recent studies have shown that EVs play an important role in infectious disease by mediating the transfer of pathogen-derived antigens and virulence factors.<sup>15</sup> EVs derived from virus-infected cells contain viral proteins and viral RNA; the contents of these vesicles are enclosed in a cellular membrane, thus enabling viruses to evade host immune response to herpes simplex virus,<sup>16,17</sup> human immunodeficiency virus,<sup>18,19</sup> Epstein-Barr virus,<sup>17</sup> and cytomegalovirus<sup>20</sup> infection. Furthermore, EVs from hepatitis A virus or hepatitis C virus-infected hepatocytes permit the respective viruses to invade and replicate within host hepatocytes.<sup>21,22</sup> However, the role of EVs in HBV infection still largely is unknown. Specifically, the lack of viral culture systems for HBV has hampered the evaluation of EV-mediated transmission of HBV infection.

In this study, we investigated EV-mediated transmission of HBV infection in a highly efficient HBV infectious culture system, for which we used primary hepatocytes derived from humanized chimeric mice with human livers. We further elucidated the involvement of ceramide-triggered EV production in HBV morphogenesis.

## Materials and Methods

### Ethics Statement

This study was performed in strict accordance with both the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the ethics committee of the Tokyo Metropolitan Institute of Medical Science (license number 15059).

### Primary Hepatocytes From Humanized Chimeric Mice: PXB-Cells

Primary human hepatocytes derived from chimeric mice with human liver (PXB-cells; PhoenixBio, Hiroshima, Japan) were cultured on type I, collagen-coated, 48-well plates using maintenance medium as previously described.<sup>23</sup> Culture fluids or cells from wells with the same treatment in a plate were pooled, and then used for analysis.

### Inocula Used for Infection of Primary Hepatocytes

Inocula of HBV genotype A or C were derived from serum obtained from humanized chimeric mouse at 8-10 weeks after infection by the respective virus.

### Infection of PXB-Cells by HBV

PXB-cells were inoculated with HBV at 5 genome equivalents (GEq) per cell. Inoculated cells were washed at 1 and 2 days after infection, and culture medium was collected every 5 days thereafter.

### HBV-DNA Quantification

DNA extraction from samples was performed using SMItest EX-R&D kits (Nippon Genetics, Tokyo, Japan) according to the manufacturer's instructions.

Quantification of HBV genomic DNA was performed by quantitative polymerase chain reaction (qPCR) using the Thunderbird Probe qPCR Mix (Toyobo, Osaka, Japan) CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). Amplification reaction mixtures (30  $\mu$ L) contained 5  $\mu$ L DNA solution, 200 nmol/L forward primer HB-166-S21 (nucleotides [nts] 166-186; 5'-CACATCAGGATTCCTAGGACC-3'), 200 nmol/L reverse primer HB-344-R20 (nts 344-325; 5'-AGGTTGGTGAGTGATTGGAG-3'), 300 nmol/L TaqMan (Thermo Fisher Scientific, Waltham, MA) probe HB-242-S26FT (nts 242-267; 5'-CAGAGTCTAGACTCGTGGTGGACTTC-3'), and 15  $\mu$ L of Thunderbird Probe qPCR Mix. Thermal cycling conditions were as follows: activation of uracil-N-glycosylase (UNG) by incubation at 50°C for 2 minutes; activation of Taq polymerase (Nippongene, Toyama, Japan) and inactivation of UNG by incubation at 95°C for 10 minutes; and amplification by 53 cycles of incubation at 95°C for 20 seconds and 60°C for 1 minutes. The standard curve for this quantification was calculated using a series of 10-fold dilutions (at defined concentrations) of a recombinant plasmid into which the HBV genome had been inserted.

### Indirect Immunofluorescence Analysis

Cells were fixed with 10% phosphate-buffered formalin (pH 7.4) for 10 minutes at room temperature. After washing 3 times with phosphate-buffered saline (PBS; pH 7.4), cells were permeabilized with 0.25% Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 10 minutes at room temperature. Viral antigen was stained with antibody to hepatitis B surface antigen (anti-HBs) rabbit serum (1:300) and Alexa-488-conjugated anti-rabbit IgG (Thermo Fisher Scientific). Nuclei also were stained using 4',6-diamidino-2-phenylindole.

### In Situ Hybridization

PXB-cells were fixed in 4% buffered paraformaldehyde (pH 7.5) for 1 hour at room temperature. After washing 3 times with PBS, the cells were treated with 0.25% Triton X-100 for 10 minutes at room temperature. To detect HBV DNA, in situ hybridization was performed as described previously using fragmented RNA probes synthesized from a full-length HBV genome.<sup>24</sup>

### EV Isolation

The culture fluids of PXB-cells were centrifuged at 2000  $\times$  g for 10 minutes at 4°C. To remove further cellular debris, the supernatants were centrifuged at 12,000  $\times$  g for 30 minutes at 4°C, and then filtered through a 0.22- $\mu$ m filter. The resulting filtered culture supernatant (designated as Sup in experiments) then was processed for isolation of EVs as follows. Sup was ultracentrifuged at 110,000  $\times$  g for 70 minutes at 4°C. The resulting supernatants were decanted and retained separately for subsequent use (see later) as the fraction without EVs (EV-). Meanwhile, the ultracentrifugation pellets were resuspended in PBS and subjected to a second round of ultracentrifugation

(110,000× g for 70 min at 4°C). The resulting supernatants were discarded, and the pellets were resuspended in PBS, yielding the EV fraction (EV+) used for subsequent analyses.

### RNA Extraction and Analysis

Total RNA was extracted from the PXB-cells and from the liver of humanized chimeric mouse using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Expression of messenger RNAs (mRNAs) in PXB-cells was determined by an Agilent Human Gene Expression 4 × 44k v2 microarray (Agilent Technologies, Santa Clara, CA). Expression of mRNAs in the liver of humanized chimeric mouse was determined by mRNA sequencing (Illumina HiSeq; Illumina, San Diego, CA). The expression of neutral sphingomyelinase (nSMase) 1, 2, and 3 were determined by TaqMan Gene Expression Assays (nSMase1, Hs00162006\_m1; nSMase2, Hs00920354\_m1; and nSMase3, Hs00215775\_m1; Thermo Fisher Scientific).

Total RNA was extracted from the culture fluid and EV fractions using SepaGene RV-R (Eidia, Tokyo, Japan), and reverse-transcribed using the High-Capacity Complementary DNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Viral complementary DNA then was quantified by qPCR as described earlier.

### Immunoprecipitation

Each aliquot (200 μL at  $3.0 \times 10^4$  viral DNA copies/mL) of Sup, EV-, and EV+ fractions from primary human hepatocytes was mixed with an equal volume of PBS containing 1% bovine serum albumin (BSA) and 5 μg of one of the following: anti-CD9 antibody (M-L13; Becton, Dickinson and Company, Franklin Lakes, NJ), anti-CD63 antibody (MEM-259; Abcam, Cambridge, UK), anti-CD81 antibody (JS-81; Becton, Dickinson and Company), or anti-hemagglutinin (HA) tag antibody (12CA5(2); BioVision, San Francisco, CA), or with diluted serum (1:300) from a normal (HBV-naive) or HBs-immunized mouse. The resulting mixture was incubated for 16 hours at 4°C. Each fluid + antibody mixture then was combined with 50 μL of a 50% slurry of protein G-Sepharose 4 Fast Flow (GE Healthcare, Chicago, IL) and incubated with rotation at 4°C for 2 hours. The mixtures were centrifuged at  $2300 \times g$  for 20 seconds at 4°C, and the supernatants were discarded. The pellets were washed twice with 1% BSA in 10% Dulbecco's modified Eagle medium. The pellets then were resuspended in 10% Dulbecco's modified Eagle medium containing 1% BSA and used for quantification analysis of HBV DNA.

### Treatment With GW4869

PXB-cells were infected with HBV. At 22 days post-infection, the cells were incubated with culture medium containing GW4869 (Merck Millipore, Billerica, MA) at the indicated concentrations. After 4 days' incubation, the culture fluid and cells were collected, pooled, and used for analysis of the HBV-DNA titer.

### Inoculation of HBV From Each Fraction to PXB-Cells

The Sup was collected from the HBV-infected PXB-cells. The EV+ and the EV- fractions were prepared as described

earlier. PXB-cells were inoculated with 5 GEq/cell of HBV derived from the Sup, EV+, or EV- fractions.

### Neutralization Test

For time-course analysis, a volume of the supernatant containing  $2 \times 10^6$  HBV-DNA copies was incubated with 1 IU hepatitis B immune globulin (HBIG; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) in 500 μL of culture medium for 1 hour at room temperature. Naive PXB-cells were cultured with the HBIG-pretreated culture supernatant for 2 or 24 hours. After the incubation period, the cells were collected, pooled, and used for analysis.

For neutralization analysis of Sup, EV-, and EV+ fractions, HBV-vaccinated human serum or AB human serum (Pel-Freez Biologicals, Rogers, AR) diluted 1:10 in culture medium was mixed with an equal volume of Sup, EV+, or EV- containing  $2.0 \times 10^6$  viral DNA copies. These mixtures were incubated at 37°C for 1 hour, and then 250 μL of each mixture was used to inoculate a PXB-cell culture ( $4.0 \times 10^5$  cells, 5 GEq/cell). For analysis of viral entry, at 3 hours after infection, the cells were washed 5 times with PBS and then collected for analysis. For long-term analysis, the cells were washed at 1 and 2 days postinfection and the culture fluid was harvested every 5 days thereafter. Collected culture fluids were pooled and used for qPCR analysis.

### Scanning Electron Microscopy

Samples for scanning electron microscopy were prepared by spotting of microvesicles (suspended in 0.1% PBS) onto poly-L-lysine-coated (Sigma-Aldrich, St. Louis, MO) coverslips. The samples were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer fixative solution for 30 minutes at room temperature and then washed 3 times with 0.1 mol/L phosphate buffer solution. The samples were immersed in a 1% (wt/vol) OsO<sub>4</sub> solution for 1 hour at 4°C, then dehydrated in a graded ethanol series and transferred to t-butyl alcohol. The samples were dried in a freeze dryer (Hitachi ES-2020; Hitachi High-Tech Fielding Corporation, Tokyo, Japan), coated with OsO<sub>4</sub> for 1 minute using an osmium plasma coater (NL-OPC80; Nippon Laser and Electronics Laboratory, Aichi, Japan), and examined using a Hitachi S-4800 field emission scanning electron microscope at an accelerating voltage of 10 kV.

### Stimulated Emission Depletion Microscopy

Samples of the culture fluid or EVs were fixed with 4% paraformaldehyde on poly-L-lysine-coated coverslips for 30 minutes at room temperature. The samples were washed twice with PBS; incubated with 50 mmol/L NH<sub>4</sub>Cl for 10 minutes at room temperature; washed twice with PBS; permeabilized with 50 μg/mL digitonin for 10 minutes at room temperature; washed twice with PBS; treated with 0.25% Triton X-100 for 10 minutes at room temperature; washed twice with PBS; and blocked with blocking buffer (1% BSA and 1 mmol/L EDTA in PBS) at 4°C overnight. The slides then were incubated with primary antibodies for 2 hours at room temperature. The primary antibodies were used at the following concentrations or dilutions: anti-CD81

antibody and biotin-conjugated anti-CD81 antibody, 2  $\mu\text{g}/\text{mL}$ ; anti-hepatitis B core antigen (anti-HBc) mouse serum, 1:100; anti-HBs rabbit serum, 1:600. The slides were washed 5 times with PBS containing 0.05% Tween 20 and then incubated with 40  $\mu\text{g}/\text{mL}$  secondary antibody (Alexa 488- or Alexa 555-conjugated anti-mouse, anti-rabbit, or streptavidin; Thermo Fisher Scientific) for 2 hours at room temperature. After washing, the specimens were mounted using Prolong Diamond (Thermo Fisher Scientific) and examined using a TCS SP8 stimulated emission depletion (STED)  $3\times$  microscope (Leica Microsystems, Wetzlar, Germany).

### DNase I Treatment

The DNase I reaction mixture consisted of 26  $\mu\text{L}$  of Sup, EV-, or EV+ fractions (or extracted DNA as a control), 1  $\mu\text{L}$  of DNase I (10 U/mL; GE Healthcare), and 3  $\mu\text{L}$  of DNase I buffer. The mixture was incubated at 37°C for 10 minutes. The reaction mixture then was incubated at 75°C for 10 minutes to heat-inactivate the DNase I. DNA extraction from samples was performed using SMItest EX-R&D kits (Nippon Genetics) according to the manufacturer's instructions, and viral DNA was quantified by qPCR as described earlier.

### Statistical Analysis

Statistical analyses were performed with Prism software (version 6.0; GraphPad, GraphPad Software, La Jolla, CA). Statistical significance was determined by using 2-tailed 1-way analysis of variance with a post hoc Bonferroni or Mann-Whitney *U* test. *P* values less than .05 were considered significant.

## Results

### Primary Hepatocytes Derived From Humanized Chimeric Mice Support HBV Propagation

Several in vitro studies have reported that HBV can infect the primary hepatocytes derived from human and tree shrew, or any of several cell lines.<sup>25</sup> Here, to evaluate EV-mediated HBV transmission, we used primary hepatocytes derived from humanized chimeric mice with human livers (PXB-cells).<sup>23</sup> First, we examined whether PXB-cells supported HBV propagation. As indicated in the schematic protocol (Figure 1A), supernatant was collected for 42 days after infection with HBV; both genotypes A and C were tested (separately). HBV DNA in the resulting culture supernatants was measured by real-time qPCR. Both genotypes of HBV propagated well in PXB-cells and the viral DNA level in culture fluid reached approximately  $10^7$ – $10^8$  copies/mL (on day 42) (Figure 1B). Furthermore, immunofluorescent analysis and in situ hybridization analysis showed that HBV infected approximately 75% of the PXB-cells on day 41 (Figure 1C).

Next, we tested if supernatant from HBV-infected PXB-cell culture showed infectivity for naive PXB-cells. The time-course analysis of the supernatants obtained from the supernatant-infected cells showed that HBV-DNA levels increased to approximately  $10^7$ – $10^8$  copies/mL on day 37

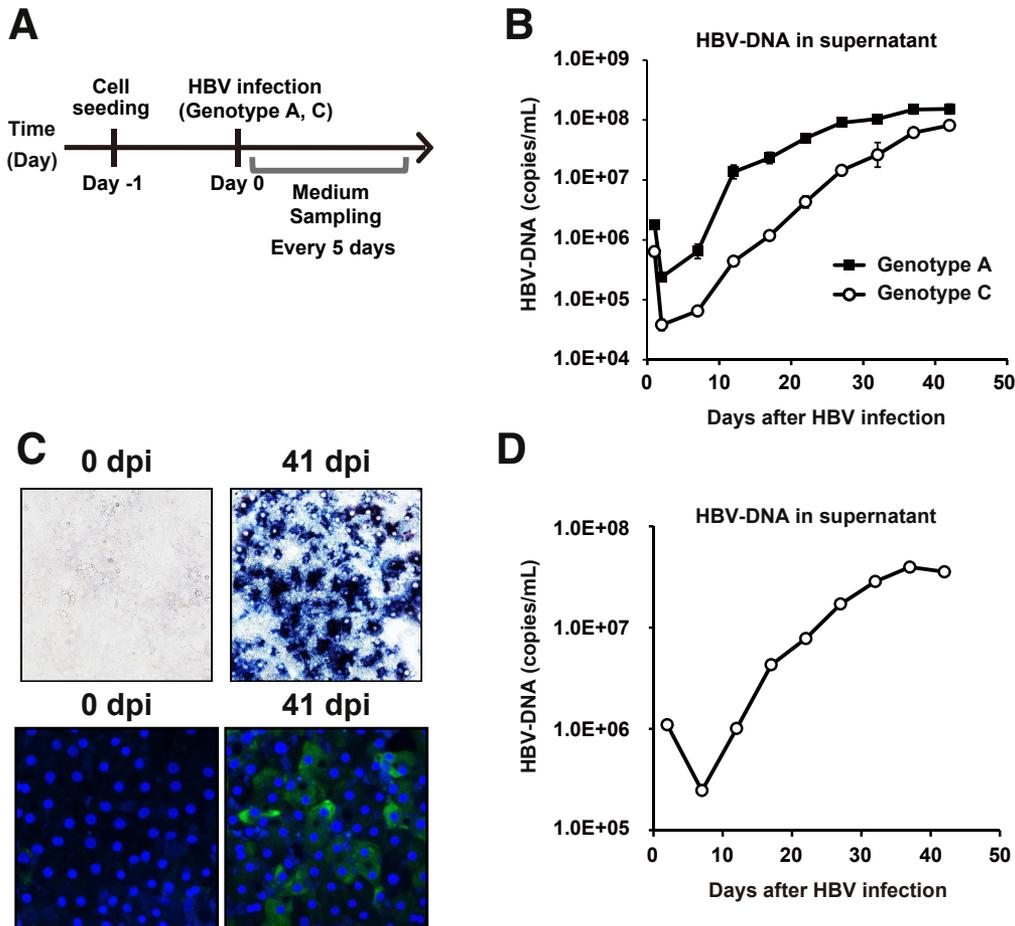
(Figure 1D). These results indicated that HBV infected and replicated in PXB-cells, and that the supernatant of HBV-infected PXB-cells showed the same infectivity of naive PXB-cells as the serum of HBV-infected humanized chimeric mice. We next used this system to examine EV-mediated HBV transmission.

### EVs Isolated From HBV-Infected Primary Hepatocytes Contain HBV DNA

Recent studies have shown that EVs from hepatitis C virus-, cytomegalovirus-, and Epstein-Barr virus-infected cells contain RNA and proteins for the respective viruses.<sup>17</sup> However, it still is not known whether EVs can harbor a viral DNA cargo. To test this possibility, we assessed whether EVs from the culture supernatant of HBV-infected PXB-cells contain HBV DNA. qPCR analysis of the EV+ fraction isolated from HBV-infected PXB-cells showed that  $62.5\% \pm 39.8\%$  of HBV DNA in the culture supernatant was carried in the EV fraction (Figure 2A). The concentrations of HBV RNA in the Sup and in the EV- fraction were  $0.021 \pm 0.015$  and  $0.018 \pm 0.016$  of those of HBV DNA in each fraction, respectively, whereas the corresponding ratio in the EV+ fraction was  $0.0018 \pm 0.0004$  (Figure 2B). We next immunoprecipitated the Sup with antibodies (anti-CD9, anti-CD63, and anti-CD81) against representative exosome membrane proteins and then determined HBV-DNA levels in the immune-precipitation samples. The HBV-DNA levels of specimen recovered from immune-precipitation with anti-CD9, CD63, and CD81 antibodies were  $0.84 \pm 0.18$ ,  $2.08 \pm 1.77$ , and  $2.17 \pm 0.64$ , respectively, of that recovered from immune-precipitation with control antibody (Figure 2C). These observations are consistent with microarray and RNA sequencing data (Figure 2D and E), indicating that CD81 (but not CD9) is highly expressed in HBV-infected PXB-cells and livers. Next, to analyze what fraction of HBV virions (Dane particles) and EVs carrying HBV DNA are contained in the 3 fractions (Sup, EV-, and EV+), we immune-precipitated the 3 fractions with antibodies against HBs and CD81, and then determined HBV-DNA levels in the immune-precipitation samples. In specimens recovered from immune-precipitation with anti-HBs antibody, specimens derived from EV- were enriched for HBV DNA, whereas HBV DNA was not detected in specimens derived from EV+ (Figure 2F). In contrast, specimens recovered from immune-precipitation of EV+ with anti-CD81 antibody were enriched for HBV DNA compared with those derived by anti-CD81 immune-precipitation of Sup or EV- (Figure 2F). Considered together, the results of this experimental series suggested that EVs derived from HBV-infected PXB-cells carry viral DNA as a cargo, but that these particles apparently are depleted for HBV RNA.

### GW4869 Treatment Suppresses the Production of HBV-DNA-Containing EVs

Next, we investigated the mechanisms that generate HBV-DNA-containing EVs. Two major pathways have been



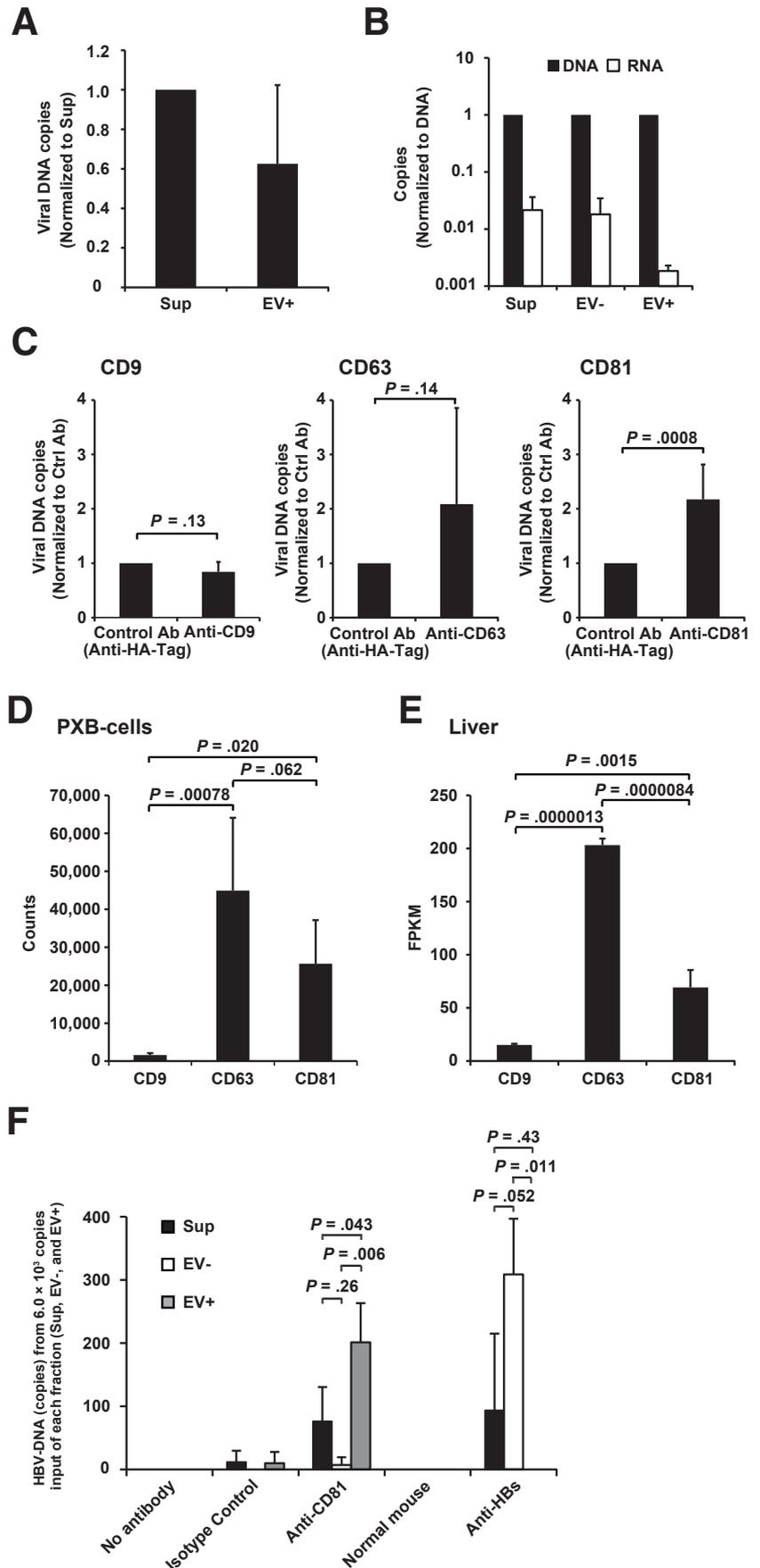
**Figure 1. Primary hepatocytes derived from humanized chimeric mice with human livers support HBV propagation.** (A) Experimental schedule of HBV infection of PXB-cells. (B) Time-course studies of the PXB-cells inoculated with chimeric mouse serum samples positive for HBV genotypes A or C. HBV-DNA levels in supernatants were determined by qPCR ( $n = 3-4$  per group at each time point). (C) Detection of viral DNA by in situ hybridization (upper) and of HBs antigen by immunofluorescent assay (lower) from HBV-infected PXB-cells at 41 days postinfection (dpi). Upper: Viral DNA, blue. Lower: HBs antigen, green; nuclei, blue. (D) Time-course studies of the PXB-cells inoculated with supernatants from HBV-infected PXB-cells ( $n = 3$ ). (B and D) Data were pooled from 3 or 4 independent experiments with 1 plate per experiment. (C) The data were reproducible in 3 independent experiments with 1 plate per experiment, and the figure shown here is a representative one. (B and D) Values are presented as means  $\pm$  SDs.

implicated in exosome generation: one is an ESCRT-mediated process,<sup>11,26</sup> and the other is a ceramide-triggered process.<sup>27</sup> In other work with HBV-infected humanized chimeric mice (Figure 3A and B), we observed that serum and liver HBV-DNA levels correlated with accumulation of the transcript encoding nSMase2, a key enzyme for ceramide-mediated exosome generation. On the basis of those results, we hypothesized that the production of HBV DNA from HBV-infected hepatocytes depends on the ceramide-mediated exosome pathway. To test this prediction, we treated HBV-infected PXB-cells with GW4869, an nSMase inhibitor that is known to inhibit ceramide biosynthesis.<sup>27,28</sup> Treatment with GW4869 attenuated HBV-DNA levels in culture supernatants, but not in hepatocytes (Figure 3C and D). These results suggested that inhibition of ceramide biosynthesis suppresses the production of HBV-DNA-containing EVs. In confirmation of this hypothesis, we observed that GW4869 treatment suppressed the number of EVs visible in electron

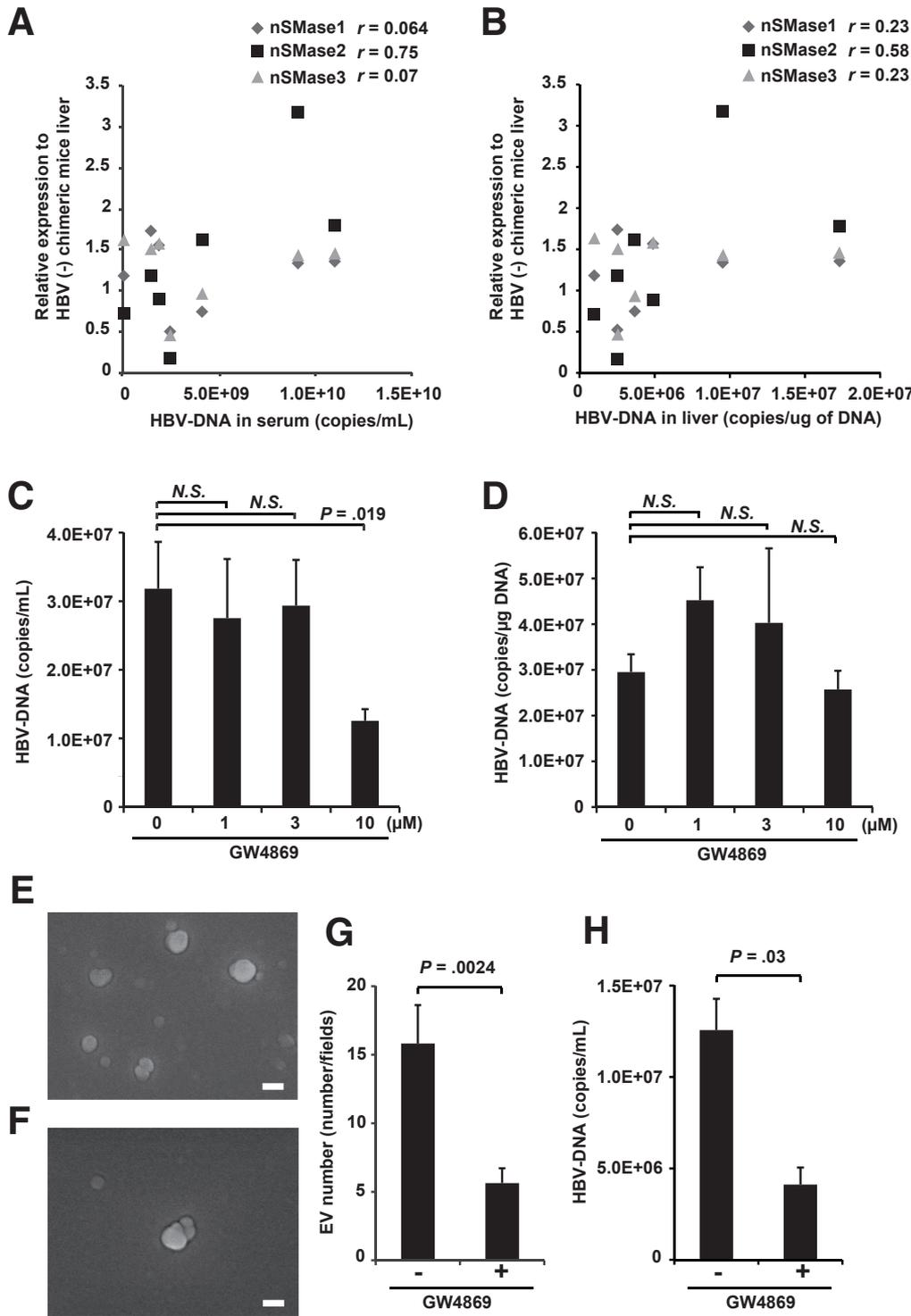
microscopic images of purified EVs isolated from HBV-infected PXB-cells (Figure 3E and F). In addition, GW4869 treatment resulted in decreased HBV-DNA levels in the purified EV fraction when normalized per milliliter of the original culture supernatant, consistent with the decrease of the number of EVs (Figure 3G and H). Considered together, these results indicated that HBV-DNA-containing EVs are produced via the ceramide-triggered exosome pathway.

### STED Microscopic Analysis Shows That EVs Incorporate CD81 and HBcAg Protein

To clarify whether or not the HBV-DNA-containing EVs possess viral proteins, we examined the interaction of hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) with EVs labeled with anti-CD81 antibody. The interaction was examined using STED microscopy, which permits the detection of co-located EVs and viral



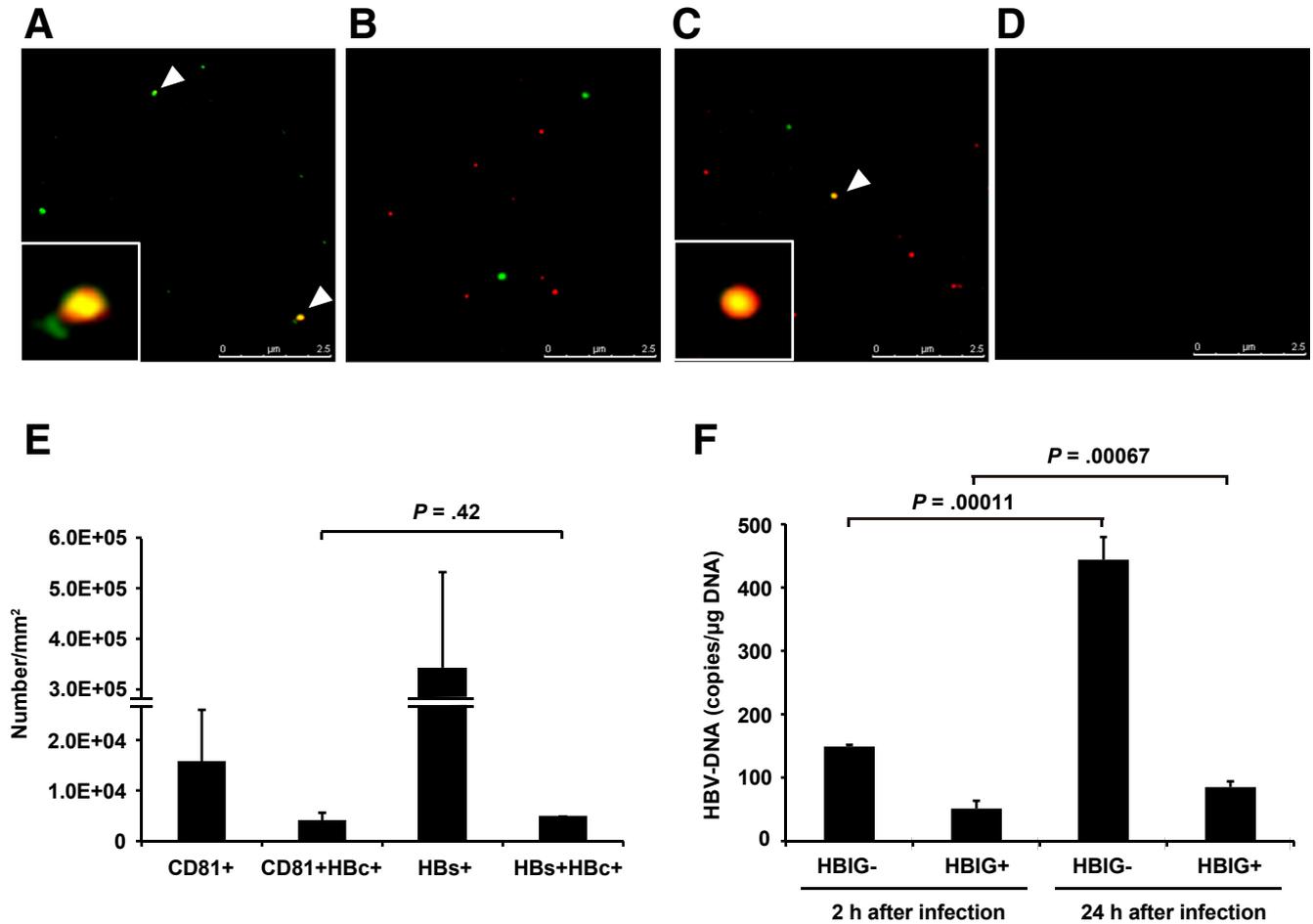
**Figure 2. Characterization of extracellular vesicles derived from HBV-infected hepatocytes.** (A) The ratio of viral DNA in EV fractions to viral DNA in Sup (n = 3). (B) The ratio of HBV-DNA:HBV-RNA copies in Sup, the EV- fraction, and the EV+ fraction (n = 3). (C) HBV-DNA levels (determined by qPCR) after immune-precipitation of fractions with each antibody. Values are normalized to HBV DNA of control antibody (Control Ab) in the respective experiment (n = 3). (D) Expression of mRNAs encoding CD9, CD63, or CD81 in PXB-cells as determined by an Agilent Human Gene Expression 4 × 44k v2 microarray. (E) Expression of mRNAs encoding CD9, CD63, or CD81 in liver from humanized chimeric mouse as determined by mRNA sequencing (Illumina HiSeq). (F) HBV-DNA levels (determined by qPCR) in Sup, EV- fraction, and EV+ fraction after immune-precipitation of each fraction containing 6.0 × 10<sup>3</sup> viral DNA copies with each antibody (n = 3). Data were pooled from (A–D, and F) 3 independent experiments with 1 plate per experiment, or (E) from 3 individual animals. Values are shown as means ± SDs. Ctrl, control; HA-Tag, hemagglutinin tag.



**Figure 3. GW4869 treatment suppresses the secretion of HBV-DNA-containing EVs.** (A and B) The correlation between nSMase 1, 2, and 3 and (A) serum or (B) liver HBV DNA in HBV-infected humanized chimeric mice (n = 7). Each symbol represents a distinct animal. To measure the strength of the association, the Pearson correlation coefficient was calculated. (C and D) HBV-DNA levels (determined by qPCR) in (C) culture supernatant and (D) PXB-cells after GW4869 treatment (n = 3). (E and F) Electron microscopic images of purified EV fraction isolated from HBV-infected PXB-cells treated with (E) vehicle or (F) GW4869. White bar, 100 nm. (G) The number of purified EV and (H) copy number of HBV DNA in EV fraction isolated from HBV-infected PXB-cells with (+) or without (-) GW4869 treatment (10  $\mu$ mol/L) (n = 3). Data were pooled from (A and B) 7 individual animals, (C, D, and H) 3 independent experiments with 1 plate per experiment, or from (G) 3–4 fields per experiment, or (E and F) data were representative of 3 independent experiments with 1 plate per experiment. Values are shown as (C, D, and H) means  $\pm$  SDs or (G) SEM.

proteins. Imaging analysis with STED microscopy showed that CD81 co-localized with HBcAg but not with HBsAg (Figure 4A and B). Furthermore, the frequency of HBcAg+ CD81+ spots was 25% that of CD81+ spots, a value comparable with that of HBcAg+ HBsAg+ spots (Figure 4E). These results suggested that HBV-DNA-containing EVs possess HBcAg, but not HBsAg. This result motivated us to

examine the effect of anti-HBs neutralizing antibody on HBV entry and replication. We performed a time-course analysis of PXB-cells inoculated with HBV that had been pretreated with HBIG (Figure 4F). Neutralizing antibodies decreased intracellular HBV-DNA level by 70% compared with HBV-DNA levels in nontreated hepatocytes 2 hours after infection. Furthermore, in the presence of neutralizing



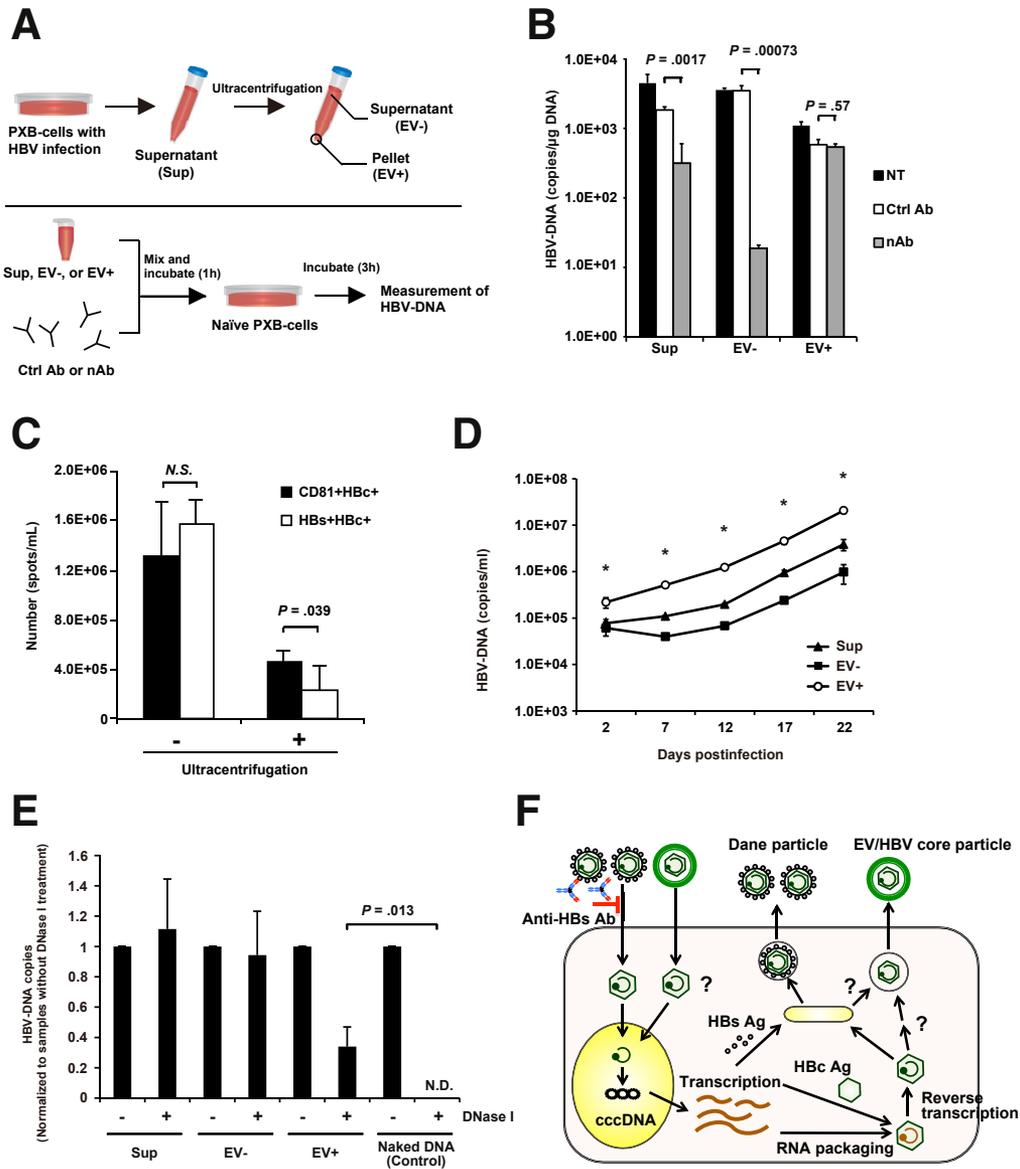
**Figure 4. Characterization of HBV-containing infectious EVs.** (A–D) STED microscopic images of supernatant of HBV-infected PXB-cells. Scale bars: 2.5 µm. (A) Green, CD81; red, HBcAg; arrowhead, CD81+HBcAg+ spot; inset, enlarged image of CD81+HBcAg+ spot; (B) green, CD81; red, HBsAg; (C) green, HBcAg; red, HBsAg; arrowhead, HBcAg+HBsAg+ spot; inset, enlarged image of HBcAg+HBsAg+ spot; and (D) isotype control. (E) Number of CD81+, CD81+HBcAg+, HBsAg+, and HBcAg+HBsAg+ spots observed from 20 × 20 µm fields. Values are shown as means ± SD for values normalized per mm<sup>2</sup> of 3 independent determinations. (F) Intracellular HBV-DNA levels of primary human hepatocytes at 2 or 24 hours after HBV infection (n = 3). HBIG- and HBIG+ indicate that the cells were inoculated without or with (respectively) HBIG pretreatment. (A–D) The data were reproducible in 3 independent experiments with 1 plate per experiment, and the figures shown here are representative images from one such experiment. Data were pooled from (E) 3 independent experiments with 1 field per experiment, or (F) 1 plate per experiment. Values are shown as means ± SDs.

antibodies, HBV-DNA levels in nontreated hepatocytes at 24 hours after infection increased 3-fold compared with HBV-DNA levels at 2 hours after infection, and increased 1.6-fold even in the HBIG-treated hepatocytes. These results suggested that HBV DNA is transmitted and replicates even in the presence of neutralizing antibodies.

**EV Fractions Transmit HBV DNA to Primary Hepatocytes via a Mechanism That Is Resistant to the Effects of Neutralizing Antibodies**

Next, we focused on the transmission of HBV mediated by EVs. Specifically, we isolated purified EVs from the culture supernatant of HBV-infected PXB-cells, and then used the EV+ fraction to infect naive PXB-cells; this procedure is shown schematically in Figure 5A. qPCR analysis confirmed

that this procedure yielded detectable levels of HBV DNA at 3 hours after infection by the EV+ fraction (Figure 5B). We noted, however, that HBV-DNA levels still were approximately 10-fold lower after infection with EV+ compared with levels obtained after infection with Sup or EV- fractions. We inferred that the higher HBV levels observed upon infection with the Sup and EV- fractions reflected the presence of HBV virions in the Sup and EV- fractions, and that these virions were removed from the EV+ fraction via ultracentrifugation. To clarify the effect of ultracentrifugation on HBV virions and HBV-DNA-containing EVs, we examined the number of CD81+HBcAg+ and HBsAg+HBcAg+ particles using STED microscopy before and after ultracentrifugation. As shown in Figure 5C, the number of HBcAg+HBsAg+ spots showed a larger depletion than that of the HBcAg+CD81+ spots. That is, the number of



**Figure 5. HBV-DNA-containing EVs show infectivity.** (A) Depiction of the infection experiment procedure. (B) HBV-DNA levels in cells at 3 hours postinfection with the indicated HBV fraction. As indicated, each fraction was either not pretreated (NT) or pretreated with control antibody (Ctrl Ab) or with neutralizing antibody (nAb) (n = 3). (C) Numbers of HBcAg+CD81+ and HBcAg+HBsAg+ spots before (-) and after (+) ultracentrifugation by STED microscopic images (n = 3). (D) HBV-DNA levels in culture fluid at each time point after infection with the indicated HBV fraction (n = 3). \*HBV-DNA level of cells infected with the EV+ fraction was significantly higher than those of cells infected with the Sup or EV- fraction. (E) Ratio of HBV-DNA levels in the respective samples treated with and without DNase I (n = 3). Samples consisted of Sup, EV-, or EV+ fractions, or of naked (control) DNA. N.D., not detected. (F) Proposed mechanisms of HBV propagation and secretion. In all cases, data were pooled from 3 independent experiments with 1 plate per experiment, and values are shown as means ± SDs.

HBcAg+CD81+ spots was 2.6-fold that of HBcAg+HBsAg+ spots after ultracentrifugation, suggesting an enrichment for HBV-containing EVs compared with HBV virions. Next, to show that infection by the EV+ fraction reflected an EV-specific role, we investigated whether the transmission of HBV was inhibited by the presence of neutralizing antibodies—that is, whether pre-incubation with virus (virion)-specific antibodies interfered with the infection process. For virus entry analysis, the cells were collected at 3 hours postinfection and the viral DNA in cells was analyzed. As shown in Figure 5B, HBV-DNA levels after

infection with either Sup or EV- were inhibited significantly in the presence of neutralizing antibodies. In contrast, the use of neutralizing antibodies did not decrease HBV-DNA levels after infection with the EV+ fraction. In addition, we analyzed the replication ability of HBV-containing EVs. PXB-cells were infected with Sup, EV-, or EV+ to determine whether HBV DNA transmitted by EVs supported viral replication in the transmitted hepatocytes. Culture fluid from HBV-infected PXB-cells was collected for 22 days postinfection, and the titer of viral DNA was quantified. The viral DNA titer from EV+-infected PXB-cells increased

progressively over the course of the experiment; a similar pattern was observed after infection with the other fractions (Figure 5D). These results showed that the HBV DNA derived from EV was replicative. Finally, to test whether viral DNA is encapsidated or just attached to the membrane of the EVs, we analyzed the DNase I resistance of the HBV DNA carried by the EV+ fraction. As Figure 5E shows, control DNA (naked DNA) subjected to DNase I treatment was not detected by qPCR. In contrast, approximately 40% of HBV DNA in the EV+ fraction was resistant to DNase I treatment. This result indicated that at least 40% of HBV DNA was encapsidated in EVs.

The results of this experimental series indicated that EVs can transmit HBV infection even in the presence of neutralizing antibodies, that is, via a non-virion-mediated process (Figure 5E).

## Discussion

EVs are presumed to function primarily in intercellular communication. These vesicles can influence the immune system; act as signaling complexes; transfer receptors from one cell to another; and convey specific mRNAs, microRNAs, and proteins between cells, thereby impacting numerous physiological processes.<sup>12–14</sup> EVs also facilitate viral transport, spread cell damage, and stimulate malignant transformation.<sup>17</sup> However, the role of EVs in HBV infection still largely is unknown. The lack of a viral culture system for HBV has hampered the evaluation of EV-mediated transmission of HBV infection.

Several HBV culture systems have been used in previous *in vitro* HBV analyses.<sup>25</sup> Primary human hepatocytes and primary tree shrew hepatocytes are susceptible to HBV infection, but the limited availability of, and genetic variation among, such primary cells makes such culture systems difficult to use.<sup>29</sup> Recent work has shown that sodium taurocholate cotransporting polypeptide-overexpressing cell lines show susceptibility to HBV infection and support HBV propagation.<sup>30</sup> However, the efficiency of HBV infection and production in this cell line is reduced compared with that in primary hepatocytes. In other work, Ishida et al<sup>23</sup> reported that PXB-cells, which are primary hepatocytes from chimeric mice, show high susceptibility to HBV infection and support persistent infection and viral production at high titers (approximately 10<sup>6</sup> copies/mL in the supernatant). Furthermore, these cells are more readily available, and show less genetic variation, than primary human or tree shrew hepatocytes. Because PXB-cells are primary hepatocytes, the characteristics of these cells are assumed to better resemble those of human hepatocytes compared with hepatoma cell lines. Taking these advantages into consideration, we propose that the PXB-cell culture system currently is the best model for analysis of the clinical effects of HBV.

By using this HBV infectious culture system, we showed that the EV fraction derived from HBV-infected PXB-cells contains abundant HBV DNA (representing 60% of all HBV DNA in the culture supernatant), but is relatively depleted for HBV RNA. We further showed that HBV-DNA-containing EVs are capable of HBV transmission to naive PXB-cells.

Several previous studies have reported that viral RNA is transmitted via EVs,<sup>21,22</sup> but this report shows that EVs carry DNA as cargo and are capable of transmitting viral DNA into hepatocytes.

Two major pathways have been implicated in exosome generation: one is an ESCRT-mediated process,<sup>11,26</sup> and the other is a ceramide-triggered process.<sup>27</sup> HBV core and L envelope proteins have been shown to interact with the ubiquitin-interacting adaptor,  $\gamma$ 2-adaptin, and the ubiquitin ligase, Nedd4, in a process thought to regulate transport of viral structures through an ESCRT-mediated process.<sup>8,9</sup> In contrast, a role for ceramide in HBV transmission has not been reported. In the present study, we showed that GW4869, an inhibitor of ceramide-triggered EVs, suppresses HBV DNA in the cell supernatant but not in cells. Furthermore, GW4869 treatment decreases EV production. The decrease of EV number in the supernatant was comparable with the decrease in the level of HBV DNA in the EV fraction (when values were normalized per milliliter of supernatant), suggesting that GW4869 suppresses the production of HBV-DNA-containing EVs. Thus, HBV-DNA-containing EVs appear to be generated through the ceramide-triggered pathway, although the role of the ESCRT pathway in the production of HBV-containing infectious EVs remains to be elucidated. The combination of the present and previous work shows that HBV exploits both sorting pathways, with ESCRT-mediated processes used in the generation of Dane particles and ceramide-triggered processes in the generation of HBV-DNA-transmitting EVs.

We have shown here that the transmission of HBV DNA to naive PXB-cells occurs via EV-enclosed HBV DNA, and that this transmission is resistant to the effects of neutralizing antibodies. This observation is consistent with the lack of HBsAg in HBV-DNA-transmitting EVs, as confirmed by STED microscopic analysis. It has been reported that 10%–30% of newborns from HBsAg/HBeAg-positive mothers cannot be protected by passive/active vaccination alone and become chronic HBV carriers.<sup>3</sup> It also has been reported that asymptomatic occult HBV infections are frequent even in individuals who have protective levels of anti-HBs antibodies.<sup>3</sup> These reports implied that some virus evades antibody neutralization. Our findings suggest that HBV-containing infectious EVs may contribute to transmission even in the presence of antibody neutralization, separate from the effect of HBV *S* gene escape mutants.<sup>3</sup>

We showed that even in the presence of neutralizing antibody, 30% of the HBV inoculum still was capable of infecting naive PXB-cells (Figure 4F), a process thought to be mediated by HBV-DNA-transmitting EVs. Our DNase I treatment experiment showed that although the viral DNA of Sup and EV were not degraded by the DNase I treatment, approximately 60% of the viral DNA of EV+ was degraded and 40% of the viral DNA was resistant (Figure 5E). This result was consistent with the low efficiency of the attachment of EV+ to PXB-cells (Figure 5B). Given that the HBV EVs in the Sup fraction were completely resistant to DNase I treatment, we postulate that the HBV EVs in the EV+ fraction may be damaged by the ultracentrifugation step, and that this damage may affect the efficiency of EV attachment to the hepatocytes.

After the inoculation of PXB-cells with EV+, the viral DNA level of the culture fluid showed a gradual increase. This observation suggested that HBV DNA transmitted by EVs replicates in hepatocytes. Interestingly, although the EV+ fraction showed a low efficiency of attachment to hepatocytes, the viral DNA level in the culture fluid of PXB-cells infected with EV+ was significantly higher than the viral DNA level in the culture fluids of cells inoculated with Sup or EV-. EV is known to contain various host factors (eg, microRNAs), and these factors may enhance the initial replication of HBV. Indeed, some microRNAs are reported to enhance the replication of HBV.<sup>31,32</sup> To clarify this point, further studies focusing on HBV-DNA replication of EV will be needed.

In summary, we have shown, using primary hepatocytes derived from humanized chimeric mouse, that HBV DNA-containing EVs are generated through ceramide-triggered vesicle formation, and that these EVs are infectious to primary hepatocytes. Furthermore, HBV transmission by these EVs is resistant to antibody neutralization because these vesicles lack HBs antigen, as shown by STED microscopic analysis. These results suggest the existence of a separate pathway for transmission of HBV; distinct strategies may be needed to address HBV infection by this pathway.

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**Conflicts of interest**

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