

# Highly Accurate, Specific And Sensitive Quantitation By Droplet Digital PCR Reveals The Stability of Intrahepatic Hepatitis B Virus cccDNA

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## Research Article

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# Abstract

The persistence of covalently closed circular DNA (cccDNA) poses a major obstacle to curing chronic hepatitis B (CHB). Here, we used droplet digital PCR (ddPCR) for cccDNA quantitation. ddPCR measured a less than two-fold difference in the intrahepatic cccDNA content more accurately than conventional real-time PCR (qPCR), ( $R^2=0.9416$  and  $R^2=0.8963$ , respectively) and had also higher sensitivity and specificity than qPCR. The results of ddPCR correlated more closely with serum HB core-related antigen ( $R^2=0.9843$ ) than HB surface antigen (HBsAg) ( $R^2=0.9742$ ) in 24 HBV-infected human-liver-chimeric mice (PXB-mice). We demonstrated that the total cccDNA content did not change during liver repopulation, although the cccDNA content per hepatocyte was reduced in PXB-mice after entecavir treatment. In the 6 patients with HBV-related hepatocellular carcinoma, ddPCR detected cccDNA in both tumor and non-tumor tissues. In 13 HBeAg-negative CHB patients with pegylated interferon alpha-2a, cccDNA contents from paired biopsies were more significantly reduced in virological response (VR) than in non-VR at week 48 ( $p=0.0051$ ). Interestingly, cccDNA levels were the lowest in VR with HBsAg clearance but remained detectable after the treatment. Collectively, ddPCR revealed that cccDNA content is stable during hepatocyte proliferation and persists at quantifiable levels, even after serum HBsAg clearance.

## Introduction

Approximately 257 million people are estimated to be chronically infected with hepatitis B virus (HBV) worldwide and 887,000 deaths every year are attributed to complications of HBV infection [1]. Though nucleos(t)ide analogs (NAs) inhibiting viral DNA synthesis are approved antiviral therapies for chronic HBV (CHB) infection [2, 3], they cannot eliminate the virus due to the persistence of covalently closed circular DNA (cccDNA) in the hepatocytes.

Currently, intrahepatic cccDNA is detected by quantitative real-time PCR (qPCR) or Southern blotting (SB). However, the amount of cccDNA in human hepatocytes is rather low, limiting the use of SB [4, 5]. Because cccDNA is very stable, its reduction may be slow. Therefore, a more precise measurement is desirable to evaluate the impact of new anti-HBV drugs on cccDNA. Droplet digital PCR (ddPCR) provides an accurate copy number of a single gene sequence [4, 5]. Thus far, the quantitative accuracies of ddPCR and qPCR have not been systematically compared in detecting intrahepatic cccDNA. Meanwhile, clinical studies revealed the potential of serum hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels as surrogate biomarkers for intrahepatic cccDNA in CHB patients, but little information is available regarding the extent to which these serum markers reflect the cccDNA content in the liver in the absence of antibody responses [6–9]. Moreover, the impact of cell division on intrahepatic cccDNA content remains contentious, partly due to the lack of a highly quantitative methodology.

Here, we developed a new assay to quantitate cccDNA content using ddPCR. This ddPCR assay could measure cccDNA more accurately and with greater specificity and sensitivity than qPCR. The amount of cccDNA was stable during hepatocyte proliferation and PEG-IFN treatment *in vivo*.

## Materials And Methods

### Ethics statement.

This study was performed in accordance with the relevant national guidelines and regulations.

### Chimeric mice with human hepatocytes.

Severe combined immunodeficiency mice transgenic for the urokinase-type plasminogen activator gene ( $uPA^{wild/+}/SCID^{+/+}$  mice), with their livers replaced by human hepatocytes, (human-liver-chimeric mice) were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). These mice were infected with sera obtained from human hepatocyte chimeric mice previously infected with HBV subgenotype C2/Ce, as described in a previous report [10]. 24 HBV-infected chimeric mice were under various conditions at the time of autopsy, and seven mice showed low HBV infection efficacy due to ETV treatment during the acute phase of infection (Table S1). The animal protocol was approved by the Animal Welfare Committee of PhoenixBio Co., Ltd. (Permit Number: 1910) and carried out in compliance with the ARRIVE guidelines. More detailed procedures are given in the Supplementary Information.

### Patients.

A total of 6 HCC patients and 13 hepatitis B e antigen (HBeAg)-negative patients with CHB were enrolled in this study. Liver tumor tissues and the corresponding non-tumor tissues were collected from five patients with HBV infection (3 HBsAg-positive and 2 HBsAg-negative/anti-HBc-positive) and one patient without HBV infection (HBsAg-negative and anti-HBc-negative) at Tokushima University Hospital and Nagoya City University Hospital in Japan. Thirteen HBeAg-negative patients who received 48 weeks of PEG-IFN- $\alpha$ 2a (180  $\mu$ g/week) monotherapy at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand, were enrolled into this study, and paired liver tissues were collected before and after PEG-IFN treatment. Virological response (VR) was defined as an HBV DNA level below 2,000 IU/mL at 48 weeks after treatment. Thirteen patients with HBeAg-negative CHB who received 48 weeks of PEG-IFN monotherapy were categorized into 3 groups: non-VR ( $n = 6$ ), VR without HBsAg clearance ( $n = 4$ ) and VR with HBsAg clearance ( $n = 3$ ). At baseline, there was no significant difference in the levels of HBV DNA, HBsAg, or HBcrAg in the

serum, or the levels of total cccDNA and cccDNA per cell in the liver, among the 3 groups ( $p = 0.26473$ ,  $p = 0.64468$ ,  $p = 0.69439$ ,  $p = 0.30080$ , and  $p = 0.5565$ , respectively) (Table S2). This study was approved by the Institutional Review Boards at all three participating institutes: Nagoya City University Graduate School of Medical Sciences and Nagoya City University Hospital Institutional Review Board, Ethics Committee of Tokushima University Hospital and the faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the appropriate institutional ethics review committees of each institute. More detailed procedures are given in the Supplementary Information.

#### **Analysis of virological markers.**

Serum HBV DNA titers were measured in chimeric mice by real-time PCR, as described previously [11, 12]. Hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) were measured by chemiluminescent enzyme immunoassays using commercial kits (Fujirebio Inc., Tokyo, Japan), as described previously [10, 13]. HBV DNA titers were measured in patients using the Abbott Real Time HBV assay or the TaqMan polymerase chain reaction assay (COBAS TaqMan, Roche Molecular System). More detailed procedures are given in the Supplementary Information.

#### **Isolation of genomic DNA from the livers of chimeric mice.**

Genomic DNA was isolated from the livers of chimeric mice using the phenol/chloroform method, as described previously [14]. Protein-free DNA extraction from the mouse livers was carried out using a modified Hirt extraction procedure [15]. The genomic DNA and protein-free DNA were digested with DNase-free RNase and restriction enzyme (*Hind* III) before analysis. More detailed procedures are given in the Supplementary Information.

#### **Quantitation of HBV cccDNA and relaxed circular (RC) DNA by ddPCR.**

Total liver DNA treated with plasmid-safe ATP-dependent DNase (PSAD) (Epicentre, Madison, Wisconsin, USA) was used as template inputs for either qPCR or ddPCR amplification. Specific primers for cccDNA amplification were designed as shown in Fig. 1A, B. After generating reaction droplets, intrahepatic cccDNA was amplified using a C1000 touch™ Thermal Cycler (Bio-Rad, Hercules, California, USA). In some cases, intrahepatic cccDNA values were normalized by the cell number measured by the hRPP30 copy number variation assay (Bio-Rad, Pleasanton, California, USA) [16]. Of note, hRPP30 levels were separately determined using DNA that was not treated with PSAD, which digests genomic DNA. More detailed procedures are given in the Supplementary Information.

#### **Quantitation of HBV cccDNA by qPCR.**

Target intrahepatic cccDNA was amplified by the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). Serial dilutions of a plasmid containing an HBV monomer (pHBV: AB246337) served as quantitation standards. More detailed procedures are given in the Supplementary Information.

#### **Detection of HBV cccDNA by Southern blotting.**

Genomic DNA and protein-free DNA was isolated from the livers of chimeric mice infected with HBV. The DNAs were digested with the restriction enzyme, *Hind* III, that does not cleave HBV, and analyzed by Southern blotting. The signals were analyzed using an ImageQuant LAS 4000mini (GE Healthcare UK Ltd). To confirm the nature of cccDNA, the samples were subsequently digested with *Xho* I to linearize the DNA. Agarose gel electrophoresis and Southern blotting were performed as described previously [17].

## **Statistical Analysis.**

The coefficient of determination ( $R^2$ ) was assessed by linear regression analysis. The correlation between two continuous variables was analyzed using Pearson's correlation. The Mann-Whitney U-test was used to examine statistical difference between the VR and non-VR groups.

## **Results**

#### **Optimization and validation of ddPCR for the detection of HBV cccDNA.**

To establish an assay to detect HBV cccDNA using ddPCR technology, we used total DNA extracted from the livers of human-liver-chimeric mice that had been infected with HBV. Digestion of DNA by a restriction enzyme is believed to improve template accessibility and optimal annealing temperature. To test the effect of restriction enzyme digestion on the efficacy of cccDNA detection, temperature gradient ddPCR was performed in the presence of Hae III on the sample DNA treated with PSAD. *Xho* I, which does not recognize the cccDNA amplification region but cuts at one site in the HBV genome, was used for comparison. As shown in Supplementary Fig. S1A and B, Hae III digestion provided better separation of positive and negative signals, and the results were less affected by the temperature as the signal intensity of positive droplet populations started to

decrease at the annealing temperature below 58.5°C for Xho I and 55.3°C for Hae III. Based on these results, we decided to use Hae III digestion and set the annealing temperature at 61.2°C.

#### **ddPCR accurately measures less than two-fold differences in cccDNA content.**

To compare the sensitivity and accuracy of cccDNA detection between ddPCR and qPCR, we serially diluted total DNA extracted from the livers of HBV-infected chimeric mice from 20 to 0.156 ng/assay and conducted ddPCR and qPCR. As shown in Fig. 2A, the results of both ddPCR and qPCR displayed high goodness of fit ( $R^2 = 0.9995$  and  $R^2 = 0.9991$ , respectively). In addition, the results of ddPCR and qPCR exhibited a near-perfect correlation ( $R^2 = 0.9958$ ,  $p = 2.12 \times 10^{-70}$ ) (Fig. 2B). These results suggest that both ddPCR and qPCR are highly sensitive and sufficiently accurate to detect 2-fold differences of the intrahepatic cccDNA. In contrast, when the samples were diluted to various concentrations, ranging between 5 and 8 ng/assay, ddPCR but not qPCR was able to accurately measure the differences ( $R^2 = 0.9416$  and  $R^2 = 0.8963$ , respectively) (Fig. 2C). Collectively, these results suggest that both ddPCR and qPCR detect intrahepatic cccDNA with high sensitivity, but ddPCR is much more accurate than qPCR in measuring less than two-fold differences.

#### **Specific detection of cccDNA by ddPCR.**

The specificity of cccDNA detection was compared between ddPCR and qPCR. Serially diluted total DNA extracted from the sera of HBV-infected chimeric mice, from 0.32 to 0.04  $\mu\text{l}$ /assay, was analyzed by ddPCR and qPCR. Because total DNA in the sera of HBV-infected chimeric mice should contain a large amount of HBV DNA and a small amount of cccDNA, cccDNA-specific ddPCR of the serum should generate a very low signal. As references, the serum HBV DNA levels of the HBV-infected chimeric mice were measured using a primer-probe set that could detect both cccDNA and RC DNA (herein referred to "set 1") [12]. As shown in Fig. 2D, the results of both ddPCR and qPCR using the set 1 primer-probe set showed high concordance ( $R^2 = 0.9994$  and  $R^2 = 0.9906$ , respectively). In contrast, when the serially diluted serum samples were measured by the cccDNA specific primer-probe set with PSAD treatment, ddPCR was able to accurately measure cccDNA at a concentration of approximately 1/1000 of total DNA in all dilution series, ranging between 0.32 and 0.04  $\mu\text{l}$ /assay ( $R^2 = 0.9915$ ), but qPCR was unstable at low cccDNA concentrations ( $R^2 = 0.9259$ , Fig. 2D). These results suggest that ddPCR could detect intrahepatic cccDNA with higher specificity and sensitivity than qPCR.

The limits of quantitative value (LOQ) and detection value (LOD) for HBV-cccDNA-specific ddPCR were determined using serial dilutions of total DNA extracted from the livers of HBV-infected chimeric mice (Fig. 2E). When the coefficient of variation (CV) lower than 0.2 was used to set the LOQ, the LOQ was found to be 7.7 copies/0.625 ng assay. The HBV cccDNA-specific ddPCR but not qPCR detected 1 copy/0.039 ng assay. Therefore, we set the LOD at 1 copy/assay. Collectively, these results suggest the fine specificity of cccDNA-specific ddPCR.

Finally, we compared ddPCR and Southern blot (SB) analysis using DNA isolated from two chimeric mice in the chronic phase of HBV infection (Supplementary Fig. S2). As shown in the Supplementary Information, these values were closely correlated with the cccDNA content detected by the SB assay.

#### **The intrahepatic HBV cccDNA level correlates strongly with serum HBV parameters.**

To determine the extent to which the intrahepatic cccDNA level is reflected by the HBV markers in the serum, the cccDNA contents were measured in 24 HBV-infected chimeric mice under various conditions of infection, and then correlated with the serum HBsAg and HBcAg levels. As shown in Fig. 3A and B, the intrahepatic cccDNA levels were significantly correlated with the serum HBsAg level ( $R^2 = 0.9742$ ,  $P = 5.707 \times 10^{-7}$ ) and, even more closely, with the HBcAg level ( $R^2 = 0.9843$ ,  $P = 2.3065 \times 10^{-11}$ ), suggesting that both HBcAg and HBsAg are excellent surrogate serum markers of intrahepatic cccDNA. As shown in Fig. S3, the ETV-treated group had better correlations between the intrahepatic cccDNA levels and the serum markers, suggesting that the serum markers could accurately reflect the intrahepatic cccDNA levels, irrespective of the HBV DNA replication status.

#### **Hepatocyte proliferation has little impact on the total amount of cccDNA in the liver.**

Having established a highly quantitative cccDNA-specific ddPCR assay, we examined the impact of hepatocyte proliferation on the stability of cccDNA. All six chimeric mice undergoing hepatocyte repopulation were inoculated with HBV ( $1 \times 10^6$  copies per mouse) 4 weeks after transplantation, at which time the reconstitution rate was less than 30%, based on serum human albumin (hALB) levels (Fig. 4A) [18]. Serum HBV DNA levels were measured in all mice 4 weeks later, ranging between 1.7 to  $6.8 \times 10^5$  copies/ml (Fig. 4B). Two chimeric mice were sacrificed on week 4 and two of the remaining four were administered ETV orally at 0.2 mg/kg/day for 4 weeks. The other two mice served as controls. The HBV DNA levels had already decreased after one week of ETV treatment and continued to decrease during the treatment, reaching a nadir on week 10 (1.6  $\log_{10}$  copies reduction) (Fig. 4B). In the ETV-treated chimeric mice, serum HBsAg and HBcAg levels peaked at week 6 and week 5, respectively, and then decreased slightly (Fig. 4C and D). These markers continued to increase in the control group during the same period (Fig. 4B, C and D). Serum human albumin levels increased in both groups (Fig. 4E), reflecting liver repopulation, but the body weights did not change

(Fig. 4F). As shown in Fig. 4G and H, HBV RC DNA, ssDNA and cccDNA were readily detectable in the control mice at week 10, but not in the ETV treated mice, indicating the low sensitivity of SB.

We then analyzed the levels of intrahepatic cccDNA at weeks 4 and 10 after HBV infection (i.e. immediately before the ETV treatment (week 4) and two weeks after the termination of ETV treatment (week 10)) (Fig. 4I). Before ETV treatment, 96.0-142.0 copies of cccDNA were detected in 1,000 ng of total liver DNA. Interestingly, almost the same amounts of cccDNA were detected in the ETV-treated mice at week 10 after HBV infection, consistent with the serum HBcrAg and HBsAg levels. In contrast, cccDNA levels in the control mice increased markedly to 9,800 and 19,800 copies/1,000 ng, a greater than 100-fold increase in 6 weeks. To determine the amount of cccDNA/hepatocyte, intrahepatic hRPP30 was quantified at the corresponding time points. As shown in Fig. 4J, the hRPP30 level increased by approximately 2.6 and 2.3-fold in the ETV-treated and control mice, in 6 weeks. The results are consistent with the increase of serum hALB levels (Fig. 4E) and suggest the ongoing human hepatocyte repopulation of the chimeric mouse livers. Consequently, the cccDNA level per hepatocyte decreased from 0.016 and 0.024 copies/cell before ETV treatment to 0.011–0.012 copies/cell after ETV treatment (Fig. 4K), while it increased to 1.361 and 1.886 copies/cell in the control mice. Collectively, these results suggest that the total amount of cccDNA in the liver is not affected by hepatocyte repopulation, although the cccDNA level/hepatocyte appears to be reduced after ETV treatment.

#### **Intrahepatic HBV cccDNA in patients with HBV-associated hepatocellular carcinoma.**

To apply this ddPCR-based method to clinical samples, we measured the amount of intrahepatic cccDNA and RPP30 in tumor and non-tumor tissues from five HBV-related HCC patients using the ddPCR assay. Tissues from a non-HBV-related HCC patient were used as negative controls to determine the lower detection limit of intrahepatic cccDNA, which was estimated at less than 4 copies/1000 ng assay. As shown in Table 1, varying levels of intrahepatic cccDNA were detected in three HBsAg-positive patients among the five HBV-related HCC patients. In two HBsAg-positive, HBcrAg-positive patients (patients 3 and 4), the cccDNA levels were 511-1,095 copies/1000 ng in non-tumor tissues, and 37,280 – 48,590 copies/1000 ng in tumor tissues. In the HBsAg-positive, HBcrAg-negative patient (patient 2), cccDNA was detected weakly at 5.0 copies/1000 ng in tumor tissue, while it was undetectable in non-tumor tissue. In two HBV-related HCC patients whose HBV infections had resolved, cccDNA levels were below the detection limit. Intrahepatic RPP30 in all 6 HCC patients, including the negative control, were 36,100–109,500 and 51,650 – 104,000 copies/1000 ng in non-tumor and tumor tissues, respectively. These data showed that ddPCR could detect cccDNA in tumor and non-tumor tissues.

#### **The impact of PEG-IFN treatment of patients with HBeAg-negative CHB on intrahepatic cccDNA levels.**

To investigate the effect of PEG-IFN on intrahepatic cccDNA, we determined the levels of intrahepatic cccDNA in patients with HBeAg-negative CHB at baseline and 48 weeks after PEG-IFN treatment (Fig. 5, Supplementary Fig. S4 and Table S2). Thirteen patients with HBeAg-negative CHB who received 48 weeks of PEG-IFN monotherapy were categorized into 3 groups: non-VR, VR without HBsAg clearance and VR with HBsAg clearance. At baseline, there was no significant difference in the levels of HBV DNA, HBsAg, and HBcrAg in the serum, or the levels of total cccDNA and cccDNA per cell in the liver, among the three groups (Supplementary Table S2). As shown in Fig. S4 and Table S2, serum HBV DNA levels in the non-VR group either did not change or decreased temporarily at the end of PEG-IFN treatment, only to rebound thereafter. In the VR without or with HBsAg clearance groups, HBV DNA levels decreased continuously after PEG-IFN treatment (Supplementary Fig. S4). Serum HBsAg levels in the non-VR and VR without HBsAg clearance groups were  $2.8 \pm 1.1$  (1.32–3.26) and  $1.1 \pm 0.9$  (0.40–2.38) log IU/mL, respectively, at week 48, whereas HBsAg levels were undetectable at weeks 12 and 24 in the VR with HBsAg clearance group (Supplementary Fig. S4). Serum HBcrAg levels in the non-VR group could be quantitated ( $4.3 \pm 1.4$  (2.8–6.6) Log U/mL), while those in the VR without and with HBsAg clearance groups were mostly under the quantitation limit at week 48 (Supplementary Fig. S4). Interestingly, the levels of intrahepatic cccDNA and cccDNA per cell were significantly lower in the VR groups than the non-VR group at week 48 ( $p = 0.0160$  and  $p = 0.0432$ , respectively), associated with a significant reduction of cccDNA per cell ( $p = 0.0051$ ) (representative data shown in Fig. 5A-C; all data shown in Fig. 5D-F and Supplementary Table S2). These significant changes in cccDNA levels in the VR groups were associated with a higher peak of ALT than the non-VR group ( $p = 0.0165$ ) (Fig. 5A-G). In particular, in the VR with HBsAg clearance group, intrahepatic cccDNA levels at week 48 after PEG-IFN treatment were lower than those in the VR without HBsAg clearance group ( $p = 0.036$ ), in association with the higher ALT peak ( $p = 0.060$ ) (Fig. S5A-B), although HBcrAg levels in most VR patients were undetectable, due to the limited sensitivity. Remarkably, cccDNA levels could be readily quantitated even in patients (11 and 12) with HBsAg clearance after PEG-IFN treatment (Fig. 5C and Supplementary Table S2).

## **Discussion**

In this study, we developed a ddPCR assay for the quantitation of HBV cccDNA, with more sensitivity, specificity and accuracy than qPCR (Figs. 1 and 2). The specificity of ddPCR for cccDNA was validated by the SB assay results (Fig. 3). The intrahepatic cccDNA was closely correlated with both HBsAg and HBcrAg in the sera of HBV-infected chimeric mice (Fig. 3 and Supplementary Fig. S3). Notably, the total amount of cccDNA in the repopulating liver did not change in the absence of active HBV DNA replication during ETV treatment, although the cccDNA content/hepatocyte was reduced by hepatocyte proliferation (Fig. 4). To our knowledge, this is the first study indicating the stability of cccDNA in the proliferating liver, and this novel finding could be obtained because of the high accuracy of ddPCR. The ddPCR was also able to quantitate cccDNA in CHB patients

with HCC and those with HBsAg clearance after PEG-IFN treatment (Table 1). Importantly, ddPCR readily quantitated the intrahepatic cccDNA content, even after HBsAg clearance (Fig. 5).

It is generally believed that the amount of cccDNA should be reduced to a very low level to cure HBV infection. Thus, a reliable assay to measure cccDNA is required to evaluate the therapeutic efficacy of new anti-HBV treatments. ddPCR is a new technique with improved accuracy over qPCR [19]. Furthermore, ddPCR was reported to be less variable between experiments than qPCR, allowing long-term monitoring [20]. Several recent studies have applied the ddPCR technology to measure HBV cccDNA levels in cultured cells and the livers of CHB patients, and demonstrated the higher sensitivity of ddPCR than qPCR [21, 22, 23, 24]. In the current study, the novel ddPCR assay enabled more specific detection of small amounts of cccDNA, even in the presence of large amounts of HBV DNA, indicating more accuracy, specificity and sensitivity than qPCR (Fig. 2C, D and E). Combined with the previous finding that ddPCR is more reproducible than qPCR [20], ddPCR might be more appropriate for measuring cccDNA levels than qPCR in clinical settings, because most anti-HBV drugs do not directly target cccDNA and, therefore, require long-term treatment to achieve a meaningful cccDNA reduction.

The high accuracy, specificity and sensitivity of ddPCR allowed us to determine the impact of hepatocyte proliferation on cccDNA. Surprisingly, the total amount of cccDNA in the liver showed no reduction, even though the number of hepatocytes doubled in the absence of active HBV replication (Fig. 4), week 4 vs. week 10 with ETV). Because of the small number of mice involved, however, these results are not statistically significant. Nevertheless, the data suggest that cccDNA is extremely stable during hepatocyte repopulation. Our findings differ from those recently reported by Allweiss et al.; in their study, the total cccDNA levels in the liver appeared to be reduced by the proliferation of hepatocytes [25]. This discrepancy presumably reflects different experimental conditions. Our data suggest that hepatocyte death, not hepatocyte proliferation, is required to eliminate cccDNA. This notion is in line with the observations in NA treated woodchucks chronically infected with woodchuck hepatitis virus [26] and growing ducklings infected with duck HBV [21]. These observations support the hypothesis that some fraction of cccDNA was distributed to the daughter cells of infected hepatocytes (Fig. 6A). Alternatively, it is possible that the liver reconstitution after HBV infection mainly reflected the proliferation of uninfected hepatocytes, while the majority of HBV-infected hepatocytes did not proliferate during the same period of time (Fig. 6B). Further studies are required to distinguish these alternatives.

Secreted HBsAg and HBcrAg are considered good surrogate markers for intrahepatic cccDNA. Patients with low serum HBsAg and HBcrAg levels have been reported to have a low risk of relapse after cessation of NAs therapy [7, 27, 28, 29, 30]. Recent studies indicated that serum HBcrAg was a better surrogate marker of intrahepatic cccDNA than serum HBsAg [31, 32, 33]. Quantitation of cccDNA by ddPCR recapitulated those previous findings, as serum HBcrAg, but not HBsAg, was also significantly correlated with intrahepatic cccDNA in HBeAg-negative CHB patients ( $p = 0.00056$ , Supplementary Fig. S6). In human samples, HBcrAg was more closely correlated with cccDNA than HBsAg. Besides, a good positive correlation between serum HBsAg and cccDNA was obtained in chimeric mice with immunodeficiency (Fig. 3). This discrepancy presumably reflects the presence of anti-HBs in human samples. As expected, the more profound reduction of cccDNA in the VR than non-VR groups was associated with higher peak ALT activities after PEG-IFN treatment (Fig. 5), indicating a role of cell death in elimination of cccDNA. Interestingly, Lucifora et al. suggested that APOBEC3 family cytidine deaminase A3A mediates the IFN- $\alpha$  induced cccDNA degradation [34].

In conclusion, a sensitive, specific, highly quantitative ddPCR was developed for the measurement of cccDNA in the liver. This new method revealed that intrahepatic cccDNA is very stable during hepatocyte proliferation and persists at quantifiable levels, even after serum HBsAg clearance. The use of ddPCR will provide valuable information on the development of new drugs against CHB infection.

## Abbreviations

cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; ddPCR, droplet digital PCR; SB, Southern blotting; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; HCC, hepatocellular carcinoma; PEG-IFN, pegylated interferon alpha-2a; VR, virological response; HBV, hepatitis B virus; NAs, Nucleos(t)ide analogs; ETV, entecavir; qPCR, quantitative real-time PCR; uPA<sup>wild/+</sup>/SCID<sup>+/+</sup> mice, severe combined immunodeficiency mice transgenic for the urokinase-type plasminogen activator gene; human-liver-chimeric mice, the liver replaced with human hepatocytes; HBeAg, hepatitis B e antigen; PSAD, plasmid-safe ATP dependent DNase; hALB, human albumin; RCDNA, relaxed circular DNA; DRs, direct repeats; nt, nucleotide(s).

## Declarations

## Author contributions

S.H.: Conception, Data curation, Formal analysis, Validation, Investigation, Writing original draft, M.I.: Data curation, Formal analysis, Validation, Investigation, Writing, Reviewing and Editing the Manuscript; K.K.: Data curation, Formal analysis, Investigation, Reviewing and Editing the Manuscript; K.I.: Data curation, Investigation, Reviewing and Editing the Manuscript; N.C., Y.M., M.S., N.H.K., P.T.: Data curation Validation, Resources, Reviewing and Editing the Manuscript; H.M.: Conceptualization, Data curation, Validation, Methodology, Resources, Reviewing and

## Conflict of interest statement:

The authors declare no conflicts of interest associated with this manuscript.

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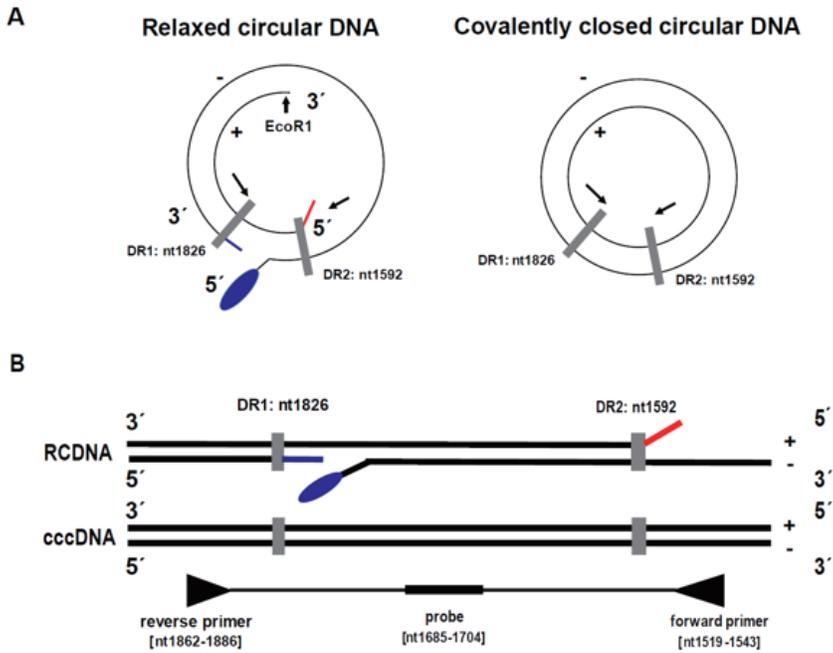
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## Tables

Table 1. Specific detection of HBV-DNA, cccDNA, HBsAg and HBcrAg in the sera and livers of 6 selected patients.

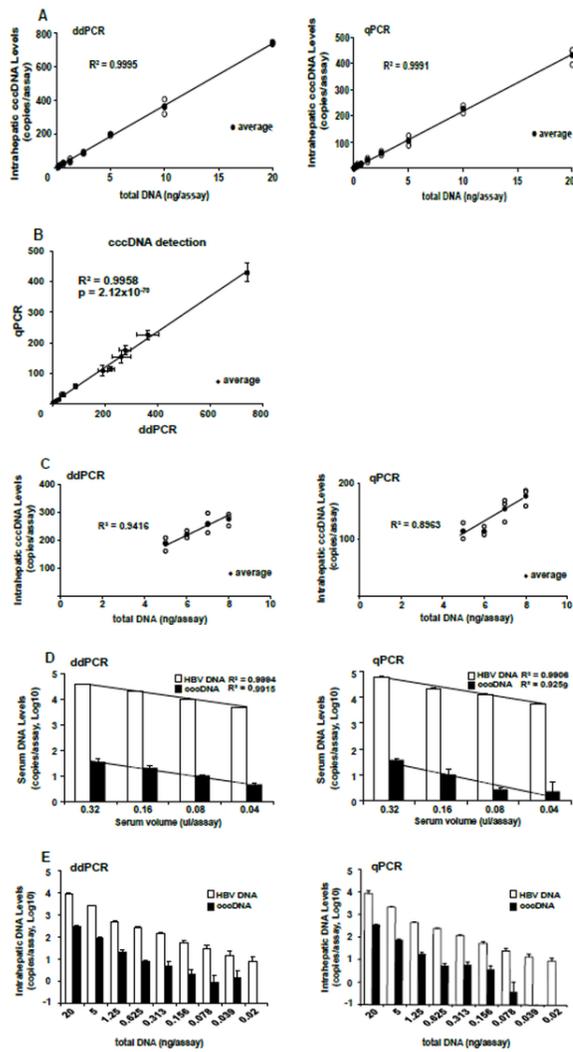
ID	Age	Sex	HBV DNA (log copies/mL)	Anti- HBc	HBsAg (IU/mL)	HBeAg/ anti- HBe	HBcrAg (LogU/mL)	Intrahepatic cccDNA (copies/1000 ng)		Intrahepatic RPP30 (copies/1000 ng)		Intrahepatic cccDNA (copies/cell)	
								Non- tumor	Tumor	Non- tumor	Tumor	Non- tumor	Tumor
1	60	M	-	+	-	-/-	< 3	2.8	0.6	82,450	51,650	0.00007	0.00002
2	71	M	-	+	9.2	-/+	< 3	1.2	5.0	36,100	79,400	0.00007	0.00013
3	59	M	4.1	+	>2,000	-/+	6.76–8.74	1,095	37,280	43,100	63,200	0.05081	1.17975
4	55	M	<2.1	+	814.1	-/+	4.6	511	48,590	109,500	104,000	0.00933	0.93442
5	70	M	-	+	-	-/-	< 3	0	0.6	62,800	60,500	-	0.00002
6	78	M	-	-	-	-/-	< 3	0	3.0	42,600	70,500	-	0.00009

## Figures



**Figure 1**

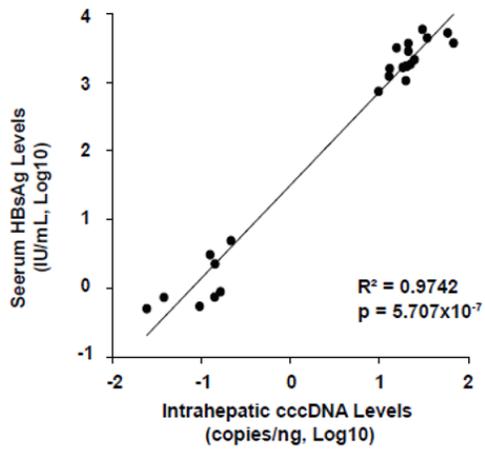
The structure of the HBV genome. (A) The HBV relaxed circular DNA (RC DNA) and cccDNA. There are two direct repeats (DRs) at nucleotides (nt) 1826 and 1592, and the numbering starts at the Eco RI site. RC DNA has a complete minus strand with a nine-base terminal redundancy (blue line) and a terminal protein polymerase attached to the 5–end (blue oval). The plus strand and has a 5′ end with an RNA primer (red line) but a variable 3′-end. PCR primers for amplification of cccDNA are targeted to opposite sides of the single-stranded gap regions of RC DNA. (B) The cccDNA-specific forward and reverse primers are 5′-ACGGGGCGCACCTCTCTTTACGCGG-3′ [nt 1519-1543] and 5′-CAAGGCACAGCTTGGAGGCTTGAAC-3′ [nt 1862-1886], respectively, and the probe: 5′-FAM-AACGACCGACCTTGAGGCAT-MGB-3′.



**Figure 2**

Comparison of ddPCR to real-time PCR for quantitation of cccDNA. (A) Intrahepatic cccDNA levels in the liver of chimeric mouse infected with HBV were measured using various amounts of DNA ranging from 20 to 0.156 ng/assay by ddPCR or real-time PCR. (B) The intrahepatic cccDNA levels measured by ddPCR and real-time PCR were correlated with each other. (C) Intrahepatic cccDNA levels from the liver of chimeric mouse with HBV infection were measured using various amounts of DNA, ranging from 8 to 5 ng/assay. (D) Total DNA isolated from the sera of chimeric mice was analyzed for HBV DNA and cccDNA. Serum HBV DNA levels were measured using a primer-probe set that detects both cccDNA and RCDNA. Serum cccDNA levels were determined using the cccDNA-specific primers and probe set by ddPCR or real-time PCR, with PSAD treatment. (E) Intrahepatic HBV DNA and cccDNA levels in the liver of chimeric mouse infected with HBV were measured using various amounts of DNA ranging from 20 to 0.02 ng/assay by ddPCR or real-time PCR. Each assay was conducted in triplicate. Target concentrations were repeatedly and independently tested three times.

### A Correlation between intrahepatic cccDNA level and serum HBsAg level



### B Correlation between intrahepatic cccDNA level and serum HBcrAg level

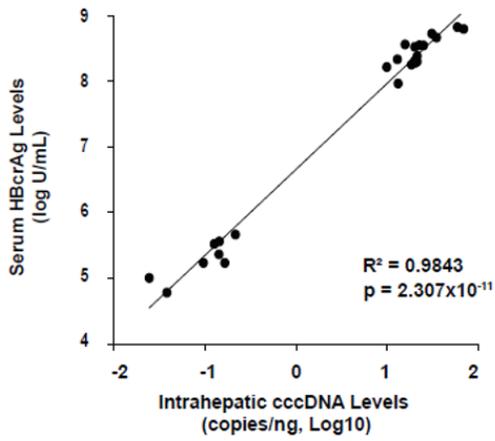
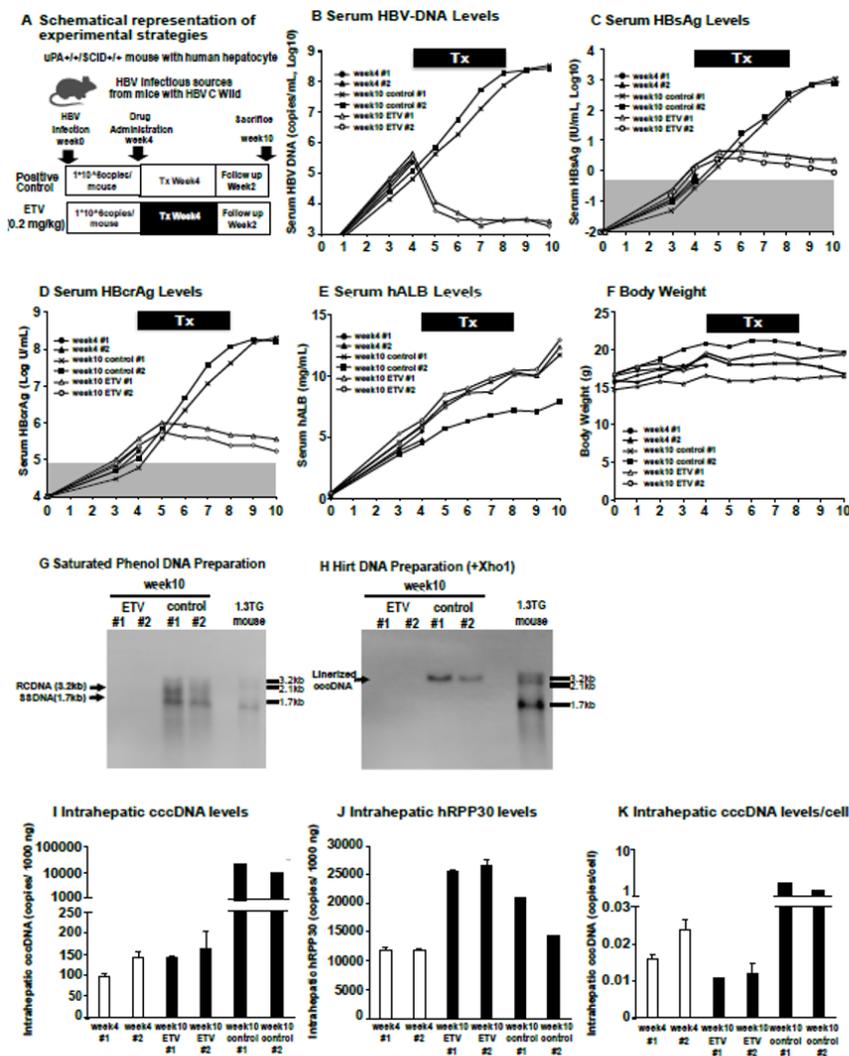


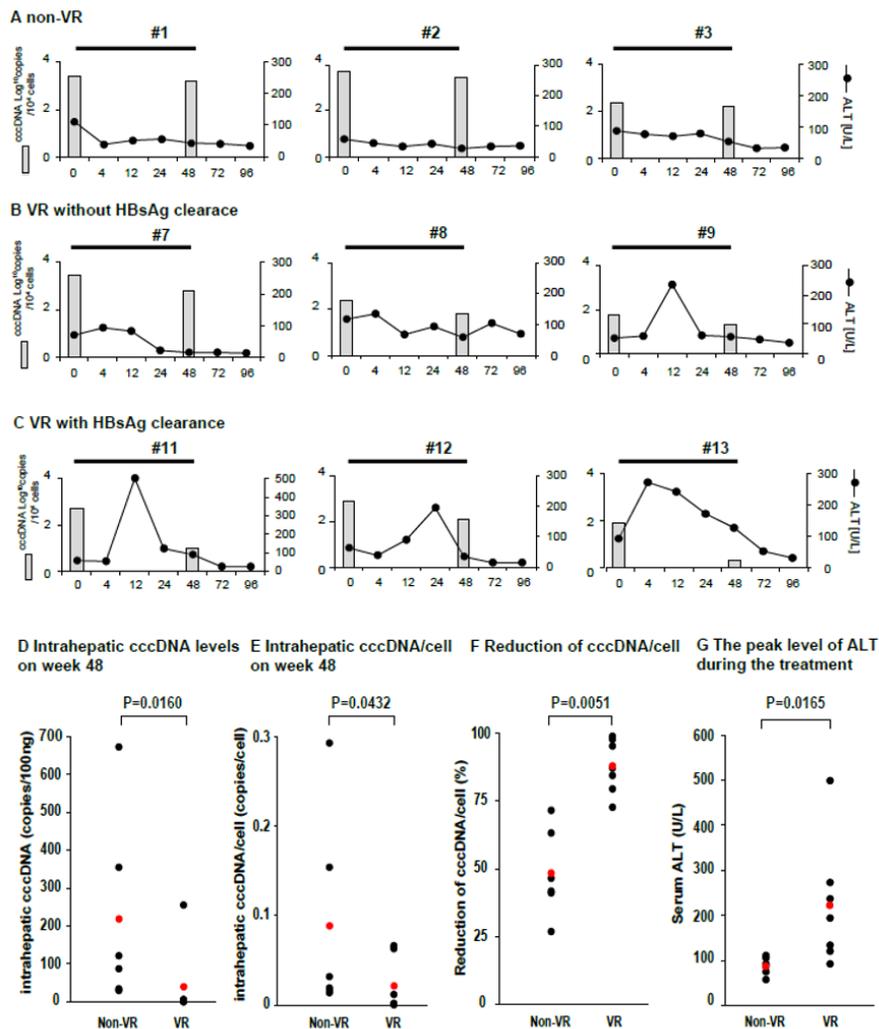
Figure 3

Correlation between intrahepatic cccDNA and serum HBV markers. The cccDNA contents in the liver of 24 HBV-infected chimeric mice under various conditions were measured and correlated with (A) serum HBsAg and (B) serum HBcrAg. Seven mice show distinctively low HBV infection rate as they were treated with ETV during acute phase of infection, while others were untreated or treated with ETV during chronic phase of infection. Characteristics of each mice were described in Table S1.



**Figure 4**

Kinetics of intrahepatic cccDNA in the chimeric mice during ETV treatment. (A) Schematic representation of the experiment. All six chimeric mice undergoing hepatocyte repopulation were inoculated with HBV. The two mice were treated with ETV (0.2 mg/kg/day) for 4 weeks and then followed for another 2 weeks after ETV withdrawal. The other two mice were treated with saline to serve as controls. (B) The serum HBV-DNA levels, (C) serum HBsAg levels, (D) serum HBcrAg levels, (E) human serum albumin and (F) their body weights were monitored during and after ETV treatment. Southern blot analysis of (G) HBV-DNA (RC DNA and ssDNA) and (H) cccDNA in the livers of HBV-infected chimeric mice, with or without ETV treatment, on week 10. These serum data (B, C and D) were associated with the levels of HBV-DNA replicative intermediates (RC DNA and ssDNA) and cccDNA in the livers of the ETV-treated and control groups by SB. (I) The intrahepatic cccDNA level of each chimeric mouse was determined by ddPCR. (J) Intrahepatic hRPP30 levels were determined by ddPCR. (K) Intrahepatic cccDNA levels/cell were calculated for each mouse. Values and error bars represent the means and standard deviations of two or more independent experiments.



**Figure 5**

Changes of intrahepatic cccDNA content and serum ALT in CHB patients with HBV infection by PEG-IFN treatment. Effect of PEG-IFN treatment on intrahepatic cccDNA/10<sup>4</sup> cells and serum ALT in CHB patients with (A) non-VR, (B) VR without HBsAg clearance and (C) VR with HBsAg clearance, during and after PEG-IFN treatment. These patients were treated with PEG-IFN monotherapy (180 µg/week) for 48 weeks and then followed for another 48 weeks after PEG-IFN withdrawal. (D) Each dot represents the average of duplicate tests for the copy number of intrahepatic cccDNA level in 100 ng total DNA of each patient. The Mann-Whitney U-test was used to examine statistical difference between the two groups. The mean values of the two groups were depicted by red dots, and were 217.7 (30.7-674.0) in the non-VR group and 39.8 (0.4-256.8) in the VR group. (E) Each dot represents the average of duplicate tests for the copy number of cccDNA per hepatocyte of each patients. The Mann-Whitney U-test was used to examine the statistical difference between the two groups. The mean values of the two groups were depicted by red dots, and were 0.088 (0.013-0.293) in the non-VR and 0.0206 (0.0002-0.0659) in the VR groups. (F) The reduction of cccDNA/cell (%) from baseline to the end of treatment was calculated in each patient, and the statistical difference was determined by the Mann-Whitney U-test. Red dots depict the mean values in the two group. (G) The peak levels of ALT during PEG-IFN treatment were 88.5 (58-111) in the non-VR and 222.0 (93-500) in the VR group. Comparison between two groups was performed by the Mann-Whitney U test.

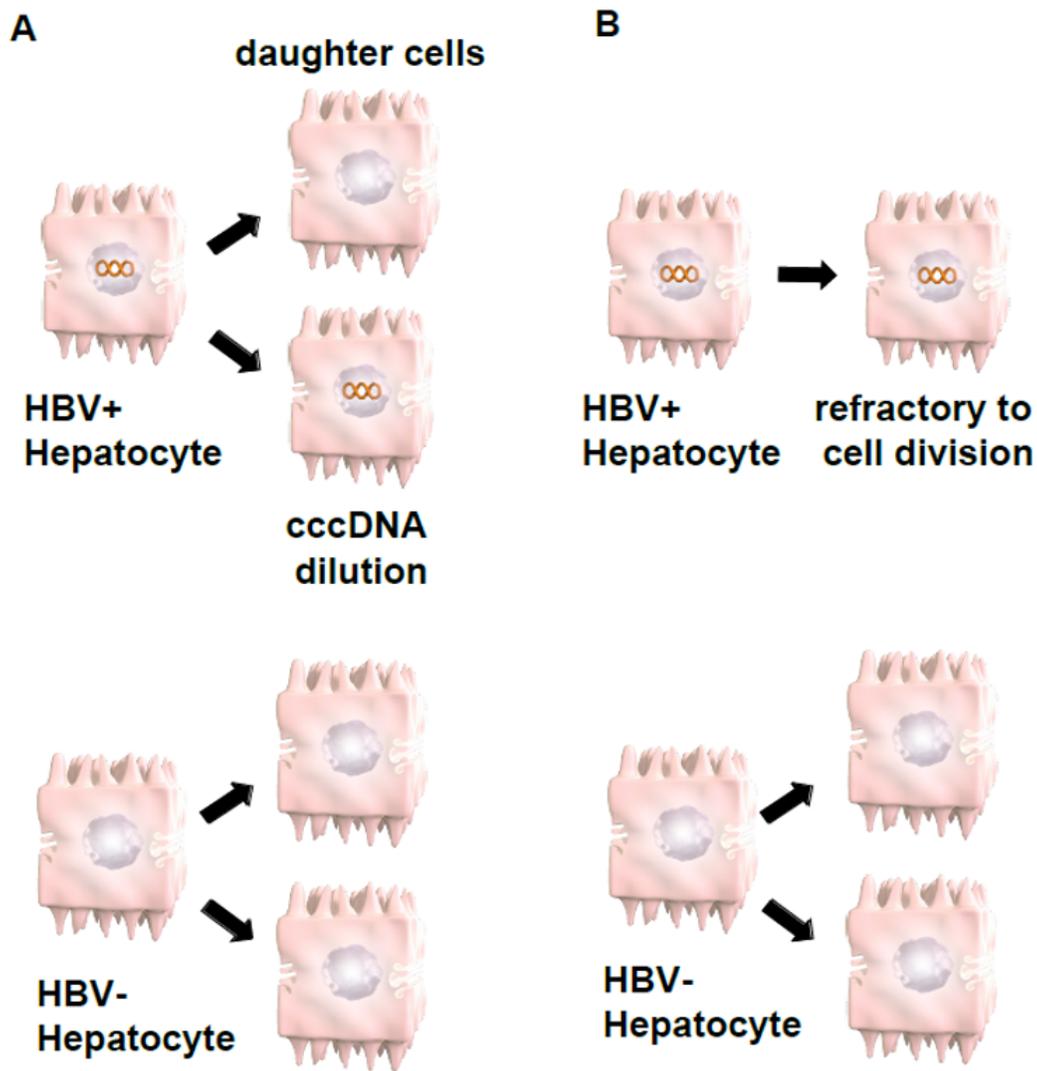


Figure 6

Persistence of intrahepatic cccDNA in dividing hepatocytes. Our in vivo study indicated that the total amount of intrahepatic cccDNA did not reduce despite the number of hepatocytes doubling in the absence of active HBV replication. It is possible that (A) cccDNA survives hepatocyte mitosis and passes to a daughter cell or (B) HBV infected hepatocytes are unable to divide and the liver was mainly reconstituted by the proliferation of uninfected hepatocytes.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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