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Research Article

Precore mutation enhances viral replication to facilitate persistent infection especially in HBeAg-negative patients

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ABSTRACT

Naturally occurred precore (PC, G1896A) and/or basal core promoter (BCP, A1762T/G1764A) mutations are prevalent in chronic HBV-infected patients, especially those under HBeAg-negative status. However, the replicative capacity of HBV with PC/BCP mutations remains ambiguous. Herein, meta-analysis showed that, only under HBeAg-negative status, the serum HBV DNA load in patients with PC mutation was 7.41-fold higher than those without the mutation. Both PC mutation alone and BCP + PC mutations promoted HBV replication in cell and hydrodynamic injection mouse models. In human hepatocyte chimeric mouse model, BCP + PC mutations led to elevated replicative capacity and intrahepatic core protein accumulation. Mechanistically, preC RNA harboring PC mutation could serve as mRNA to express core and P proteins, and such pgRNA-like function favored the maintenance of cccDNA pool under HBeAg-negative status. Additionally, BCP + PC mutations induced more extensive and severe human hepatocyte damage as well as activated endoplasmic reticulum stress and TNF signaling pathway in livers of chimeric mice. This study indicates that HBeAg-negative patients should be monitored on HBV mutations regularly and are expected to receive early antiviral treatment to prevent disease progression.

1. Introduction

Chronic hepatitis B virus (HBV) infection remains a public health issue worldwide with estimated 296 million people currently infected and 1.5 million new infections occurring annually (WHO, 2022).

Once HBV infection is established, the double-strand covalently closed circular DNA (cccDNA) is the template for all HBV transcripts (Wang et al., 2020). Of them, the 3.5 kb pregenomic RNA (pgRNA) plays an integral role in viral life cycle by serving as mRNA for structural core (HBV core antigen, HBcAg, HBc) and polymerase (P) proteins translation, as well as the template for reverse transcription to the 3.2 kb relaxed circular DNA (rcDNA). The other 3.5 kb mRNA, precore RNA (preC RNA) encodes the precore protein (p25), which is processed by removing its N-terminal 19 residues to produce p22cr in endoplasmic reticulum (ER), and subsequently generates mature e antigen (HBV e antigen, HBeAg, p17) by cleavage of C-terminal end in Golgi apparatus. Though not essential for viral replication, HBeAg facilitates the establishment of chronic infection by suppressing host immune response against HBV (Kuipery et al., 2020).

Nevertheless, HBeAg is the first viral protein to vanish during the natural course of the disease. HBeAg loss or seroconversion has been generally considered as the relief of the disease characterized by HBV DNA decline, ALT normalization and histological improvement, but seldom of HBeAg-negative patients achieve elimination of virus and spontaneous HBV surface antigen (HBsAg) loss. In some cases, viral rebound occurs or even worse, patients experience a quicker progression

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to cirrhosis and hepatocellular carcinoma after a few decades (Alexopoulou and Karayiannis, 2014). Therefore, it is urgent to clarify the underlying mechanisms relevant to viral persistence under HBeAg-negative status.

G1896A is one of the most common mutations in precore (PC) region of HBV genome, which induces a premature stop codon and abrogates HBeAg expression (Lau et al., 2021). Explosive studies have been focused on the role of G1896A mutation in viral replication since the first report in late 1980s. However, conflicting results still exist in either clinical studies (Lau et al., 2021; Nakashima et al., 2004; Pivert et al., 2015; Ouneissa et al., 2012) or *in vitro* experiments (Samal et al., 2015; Koumbi et al., 2016; Zhao et al., 2023). In addition, A1762T/G1764A, the double mutations located in the basal core promoter (BCP) region, have also attracted great concern. The prevalence of G1896A and/or A1762T/G1764A mutations increases progressively with age, whether in HBeAg-positive or -negative patients (Lau et al., 2021). More than that, it was reported previously by us that HBeAg-negative patients with A1762T/G1764A mutations usually had higher HBV DNA load compared to those without mutations (Yang et al., 2022). Mechanistically, BCP mutations not only selectively upregulate the level of pgRNA to promote HBV replication but also create an additional functional binding site of hepatocyte nuclear factor 1α and mitigate its suppressive effect on HBV (Yang et al., 2023). Though the impact of G1896A and A1762T/G1764A combined mutations was also addressed previously, the conclusion was controversial (Jammeh et al., 2008; Bhukya et al., 2021). And the exact effects of the G1896A and combined mutations on HBV replication remain a conundrum.

Herein, meta-analysis was performed to investigate the association of G1896A mutation with HBV DNA level under different HBeAg status. Moreover, we explored the effects and the potential mechanisms of G1896A mutation alone (hereinafter also referred as PC mutation) or in combination with A1762T/G1764A mutations (BCP + PC mutations) on HBV replication. Eventually, the infectivity, replication capacity and pathogenicity of HBV carrying BCP + PC mutations were examined in human hepatocyte chimeric mice.

2. Materials and methods

2.1. Search strategies

We searched Pubmed, Embase and Cochrane library up to June 18, 2022. The retrieval field was title/abstract (and keyword). The strategies were combination of several words as follows: (hepatitis B OR HBV) AND ((precore OR pre-core) AND (mutant* OR variant* OR mutation*) OR G1896A) (Supplementary Table S1). Manual search was also conducted to ensure comprehensive retrieval. The protocol was registered in the International Prospective Register of Systematic Reviews database (PROSPERO ID: CRD42022352392).

2.2. Selection criteria

We included the observational studies, such as cross-sectional, cohort and case-control studies, which enrolled untreated HBsAg-positive patients without other hepatitis viruses or human immunodeficiency virus infection. In addition, studies should provide data on serum HBV DNA load (log_{10} copies/mL) with or without PC mutation according to HBeAg status and sample size of each study was expected to exceed 30.

2.3. Data extraction and risk of bias assessment

Two reviewers independently extracted data using pre-designed electronic forms. The forms consisted of first author, year of study, country, HBV genotype, gender, age, number of patients, the load and detection method of HBV DNA. Subsequently two reviewers used modified Risk Of Bias In Non-randomized Studies -of Exposure (ROBINS-E) to assess the risk of bias in these observational studies. The discrepancies between two reviewers were discussed until agreement was reached.

2.4. Statistical analysis of meta-analysis

STATA V12.0 (StataCorp, Texas, USA) was used for meta-analysis. I^2 was used to measure the overall heterogeneity across included studies. $I^2 > 50\%$ suggested high heterogeneity and the random-effect model was chosen. Instead, we chose the fixed effect model. The weighted mean difference (WMD) with 95% confidence interval (95% CI) was calculated. $P < 0.05$ was considered statistically significant. Impact of publication bias was estimated using the Egger's test.

2.5. Plasmids

Plasmids prcccDNA (genotype D) and pCMV-Cre were gifted by Prof. Qiang Deng of Fudan University (Li et al., 2018). As Qi et al. (2014) reported, plasmid prcccDNA is constructed by engineering a loxP-chimeric intron into a monomeric HBV genome in a precursor plasmid. After cotransfection with pCMV-Cre, prcccDNA would be excised by Cre/loxP-mediated DNA recombination in the nuclei of hepatocytes to generate a 3.3 kb rcccDNA, which was heat-stable and epigenetically organized as a minichromosome. This system could avoid the interference of duplicated regulatory elements and the heterologous promoters like the commonly used 1.0 ×, 1.2 × or 1.3 × HBV constructs, with purpose to well mimic cccDNA. Vector (pKF3H), plasmid p25-WT expressing full-length precore protein (genotype D), and p25-Cp_{AUA} which was constructed by substitution of Cp starting codon by AUA in p25-WT, were gifted by Prof. Jutao Guo of Blumberg Institute (Liu et al., 2021). PC or BCP + PC mutations were introduced into the prcccDNA plasmid by homologous recombination using Basic Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China) and designated as prcccDNA-PC and prcccDNA-BCP + PC, respectively. Similarly, PC mutation was introduced into the p25-WT and p25-Cp_{AUA} to generate p25-PC and p25-Cp_{AUA}-PC, respectively. Plasmids p25-WT-P-Flag and p25-PC-P-Flag were generated by adding sequence of truncated P (terminal protein and spacer domains) followed by a 3 × Flag to p25-WT and p25-PC. Plasmids prcccDNA-WT-P-Flag, prcccDNA-PC-P-Flag and prcccDNA-WT-P-Luci, prcccDNA-PC-P-Luci were generated by directly inserting sequence of 3 × Flag or Luciferase between spacer and reverse transcriptase domain in prcccDNA-WT and prcccDNA-PC.

2.6. Cell lines and transfection

The human hepatoma cell line HepG2 was purchased from the American Type Culture Collection, and Huh7 was obtained from Shanghai Cell Bank, Chinese Academy of Sciences. HepG2-NTCP was HepG2-derived cell line stably expressing human NTCP (Michailidis et al., 2017). All cell lines were cultured in Dulbecco's modified Eagle's medium (Corning, California, USA) supplemented with 10% fetal bovine serum (Gibco, California, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco). The cells were seeded into Collagen I from rat tail (Corning)-coated plates for 16 h and transfected with desired plasmids using Lipofectamine 2000 (Invitrogen, California, USA). The supernatants and cells were collected at the indicated time points post transfection for specific analysis as described below.

2.7. Chemiluminescence detection of HBsAg, HBeAg and large HBs (L-HBs)

The levels of HBsAg, HBeAg and L-HBs in the supernatant were measured by the commercial chemiluminescence detection kit (New Industries Biomedical Engineering, Shenzhen, China) following the manufacturer's instructions.

2.8. Extraction and quantification of HBV DNA and mRNA by real-time quantitative polymerase chain reaction (qPCR)

Supernatant HBV DNA was prepared using a Viral DNA/RNA Kit (TransGen Biotech), quantified with 2 × RealStar Power SYBR qPCR Mix (GenStar, Beijing, China) and calculated by standard curves. HBV-specific primers flanking the chimeric insertion in prcccDNA were used to quantify HBV DNA in culture supernatants/sera of transfected models (Supplementary Table S2). After PCR amplification, the product of nascent HBV DNA is 156 bp while that of plasmid prcccDNA is 2336 bp. Therefore, HBV DNA can be determined using qPCR without interference of plasmid prcccDNA. The primers used to detect HBV DNA in culture media/sera of infected models were listed in Supplementary Table S2.

Total cellular/hepatic RNA was extracted with TRIzol reagent (Vazyme, Nanjing, China). Subsequently RNA was reverse transcribed to cDNA using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme). Then the mRNA levels of indicated genes were detected by qPCR with 2 × RealStar Power SYBR qPCR Mix (GenStar). The primer pairs were listed in Supplementary Table S2.

2.9. Western Blot

Cells were lysed in 1 × Lysis buffer with proteinase inhibitor at 4 °C for 30 min. The supernatants of cell lysates were collected, added with loading buffer and incubated at 100 °C for 10 min. Then proteins of different molecular weight were separated by SDS–PAGE and transferred onto PVDF membrane (Invitrogen). The membrane was blocked with 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween 20) at room temperature for 1–2 h and hybridized with primary antibodies overnight at 4 °C. Second antibodies were fluorescent-conjugated or HRP-linked anti-rabbit/mouse IgG. The proteins were finally visualized by Odyssey infrared Imager (LI-COR Biosciences, Nebraska, USA) and Tanon-5200 Chemiluminescent Imaging System (Tanon Science & Technology, Shanghai, China). The primary antibodies were as follows: anti-β-Tubulin (Applygen, Beijing, China), anti-HBc (gifted by Prof. Ningshao Xia of Xiamen University), anti-HBs (Abcam, Cambridge, UK), anti-Flag (Sigma, Missouri, USA). Due to the overlapped amino acid sequences of p22cr with HBc, the antibody of HBc could also detect p22cr via cross-reaction.

2.10. Luciferase reporter assay

For determining the expression of P protein, prcccDNA-WT-P-Luci or prcccDNA-PC-P-Luci system was co-transfected with Renilla luciferase vectors into HepG2 cells. Luciferase activity in cytoplasmic extracts was detected 36 h post transfection using Dual Luciferase Reporter Assay Kit (Promega, Wisconsin, USA). The result was presented as a ratio of firefly/Renilla luciferase activity and normalized to wildtype (WT) group.

2.11. Particle gel assay

At 72 h after transfection, HepG2 cells were lysed with core lysis buffer for 30 min at room temperature. The cell lysates were centrifugated. Then the supernatants were collected and added with DNA buffer. The mixed supernatants were fractionated by electrophoresis through nondenaturing 1.8% agarose gels and transferred to a nitrocellulose membrane by dipping in with TNE buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA). The membrane was hybridized with anti-HBc after fixing and blocking. Second antibody was HRP-linked anti-mouse IgG and HBV capsids were visualized by Tanon-5200 Chemiluminescent Imaging System (Tanon Science & Technology).

2.12. Northern Blot and Southern Blot

Intracellular HBV DNA and RNA were detected by Southern Blot and Northern Blot as described previously (Liu et al., 2021; Guo et al., 2017). Briefly, total intracellular RNA was extracted with TRIzol reagent (Invitrogen) while intracellular HBV DNA was extracted with core lysis buffer, proteinase K, and phenol–chloroform orderly from transfected Huh7 cells. HBV DNA and RNA were resolved by electrophoresis in 1.5% agarose gel and transferred onto a nitrocellulose membrane. The membrane was probed with α-32P-UTP labeled minus-strand specific full-length HBV riboprobe.

2.13. Immunoprecipitation-qPCR

Harvested supernatants from transfected HepG2 cells were added onto 30% sucrose solution and concentrated by ultracentrifugation at 200,000 ×*g* for 15 h at 4 °C. The viral particles were resuspended with appropriate volume of TNE buffer. The viral suspensions were precleared by addition of Dynabeads Protein G (Invitrogen). After the removal of beads, anti-HBsAg (Abcam) and anti-preS2 antibodies (Abcam) were added at a ratio of 1:2 and incubated overnight at 4 °C. Dynabeads Protein G were added again and incubated at 4 °C for 4 h. The beads were washed with PBS for 8 times, resuspended in core lysis buffer and digested by proteinase K (20 mg/mL) at 50 °C for 1 h. Finally, HBV virion DNA was extracted by phenol-chloroform and determined by qPCR as described above.

2.14. HBV infection of HepG2-NTCP cells

HepG2-NTCP cells were seeded into collagen-I from rat tail-coated plates for 12 h, and then pretreated with DMEM supplemented with 3% FBS, 1% non-essential amino acids (NEAA) and 2% DMSO for 24 h. Viral particles, which were precipitated from harvested supernatant of transfected HepG2 cells, were dissolved in DMEM containing 3% FBS, 2% DMSO, 1% NEAA, 8% PEG8000 and infected HepG2-NTCP cells at a multiplicity of infection (MOI) of 200. The inoculums were removed at 24 h post infection and the cells were washed with PBS for 5 times before cultured with DMEM containing 3% FBS, 1% NEAA and 2% DMSO. HBsAg, HBeAg and HBV DNA in culture media were detected as described above at indicated time points.

2.15. Animals

Hydrodynamic tail-vein injection (HDI) of HBV expression plasmids into C57BL/6J mice. C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. 10 μg prcccDNA-WT or -PC or -BCP + PC and 10 μg pCMV-Cre were dissolved in a volume (mL) of PBS equivalent to 10% body weight (g) of the C57BL/6J mice and were injected within 5–8 s. Sera of mice were collected and detected at indicated points.

Construct of human hepatocyte chimeric mice and HBV infection. Human hepatocyte chimeric mice (named as Hu-URG) were gifted by Beijing Vitalstar Biotechnology Co., Ltd. Briefly, 8-week-old male URG mice were injected intraperitoneally with doxycycline (DOX) to induce liver injury. Then primary human hepatocytes were delivered through the spleen vein to construct Hu-URG mice. The postoperative mice were continuously fed DOX for more than 8 weeks. Hu-URG mice were subsequently infected by tail-vein injection with 1.0×10^9 copies/mL WT or BCP + PC mutant viral suspension (genotype D, 200 μL per mouse). Sera and liver samples of mice were collected at pre-designed points.

All operations on animals followed internationally recognized guidelines. This study was approved by the Institutional Review Board of Peking University.

2.16. Droplet digital PCR

To assess the level of intrahepatic replication-competent HBV DNA (repDNA, defined as cccDNA and rcDNA), genome DNA was extracted from liver tissues of infected Hu-URG mice by DNeasy Blood & Tissue Kit (Qiagen, Duesseldorf, German) according to manufacturer's directions.

For HBV cccDNA quantitation, intrahepatic total DNA was treated with T5 Exonuclease (NEB, MA, USA) for 1 h at 37 °C to digest single-strand DNA and linear double-strand DNA. Primers targeting the HBV DNA gap region and a fluorescence hybridization probe were used to detect the viral cccDNA and repDNA. Species-specific primers (identify human genome but not mouse genome) targeting RNase P and a fluorescence hybridization probe were used to determine the number of human hepatocytes. The primer pairs and probe were listed in Supplementary Table S2. Reaction mix was comprised of ddPCR Supermix (no dUTP) (Bio-Rad, California, USA), primers, probe and total/digested DNA sample. Reaction droplets were generated by QX200™ Droplet Generator (Bio-Rad). Intrahepatic HBV cccDNA or repDNA was amplified using T100™ Thermal Cycler (Bio-Rad). After amplification, positive and negative droplets were quantified by a QX100™ Droplet Reader (Bio-Rad) using QuantaSoft™ analysis software version 1.7.4 (Bio-Rad). Intrahepatic HBV cccDNA and repDNA values were normalized to the number of human hepatocytes.

2.17. Hematoxylin & eosin (H&E) and immunohistochemistry (IHC) staining

Liver samples were fixed with 4% paraformaldehyde for 24 h and embedded with paraffin. Paraffin sections were used either for H&E or IHC staining techniques. H&E-stained liver sections were reviewed and percentage of hepatocyte degeneration was estimated by a pathologist (Dr. Huange Li of Fudan University), who was blinded to grouping of mice. For IHC staining, the primary antibody used was anti-HBcAg (Maixin Biotech, Fuzhou, China), anti-HBsAg (Maixin Biotech) and anti-8OHdG (Santa Cruz Biotechnology, Texas, USA). Ten fields of IHC-stained liver sections were selected randomly for each sample and Image J pro plus (Media Cybernetics, Maryland, USA) was used for quantification of IHC staining signals. The ratio of integrated option density (IOD) to area represented the intrahepatic expression of HBcAg or HBsAg.

2.18. RNA sequencing analysis

Total RNA of the liver tissues was extracted using Trizol reagent and RNA sequencing was supported by Majorbio (Majorbio, Shanghai, China). Reads were aligned against human genomic reference and mouse genomic reference. $P < 0.05$ was used for filtering differentially expressed genes.

2.19. Statistical analysis of experiments

For statistical analysis, unpaired two-tailed student's t-test and one-way ANOVA were performed using GraphPad Prism V7.0 (GraphPad Software, California, USA). Quantitative data are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s.: not statistically significant.

3. Results

3.1. HBeAg-negative patients with PC mutation have higher HBV DNA level than those without mutation while the opposite is true for HBeAg-positive patients

The selection process of meta-analysis was presented in Supplementary Fig. S1. Finally, six studies containing 783 HBeAg-positive patients and another seven studies enrolling 1002 HBeAg-negative patients were included (Supplementary Table S3) (Rodriguez-Frias et al., 2006; Choi et al., 2009; Kawabe et al., 2009; Qin et al., 2009; Yang et al., 2009; Zhang et al., 2009; Moradzadeh et al., 2013; Yim et al., 2015; Geng et al., 2016; Pan et al., 2017; Yan et al., 2018). The risk of bias of these observational studies was provided in Supplementary Table S4. The publication of bias was not significant ($P = 0.331$). The results showed that irrespective of HBeAg status, there was no significant difference in HBV DNA levels between patients with and without PC mutation (WMD = −0.24; 95% CI, −0.81 to 0.34; $P = 0.421$; Fig. 1). However, after stratifying the patients by HBeAg status, serum HBV DNA load of patients with PC mutation was found 0.87 $\log_{10}$ copies/mL (7.41-fold) higher than those without the mutation under HBeAg-negative status (WMD = −0.87; 95% CI, −1.56 to −0.18; $P = 0.014$); oppositely, in HBeAg-positive patients, it was 0.37 $\log_{10}$ copies/mL (0.43-fold) lower than those without PC mutation (WMD = 0.37; 95% CI, 0.08 to 0.66; $P = 0.012$). The substantial heterogeneity was not reduced even after considering of HBeAg status, and it might be caused by variations in HBV genotype and disease state of chronic HBV-infected patients among these studies.

3.2. PC mutation enhances HBV replication in the cell transfection model

In order to determine the precise mechanisms of PC mutation in HBV replication, prcccDNA-PC system was transiently transfected into human hepatoma cells. As shown in Fig. 2A, PC mutation eliminated HBeAg expression but elevated HBV DNA and HBsAg levels in the culture supernatant. Consistently, Western Blot analysis demonstrated that PC mutation slightly enhanced the intracellular core and HBs expression (Fig. 2B). Meanwhile, no discernible difference was observed in terms of intracellular total 3.5 kb mRNA and preC RNA levels between the PC and WT group (Fig. 2C). These results suggested that PC mutation might promote HBV replication at the post-transcriptional level, and the underlying mechanisms needed to be further explored.

3.3. PC mutation enables preC RNA to express core and P proteins

Considering that premature termination of translation might endow the downstream initiation codon to translate, a serial of plasmids was constructed to explore the effect of PC mutation on preC RNA translation (Supplementary Fig. S2). Firstly, the abolishment of HBeAg expression was confirmed by p25-Cp_{AUA}-PC with initiation codon of core protein mutated, and it also proved that the false positivity of HBeAg in p25-PC group was likely owing to the cross-reaction with HBcAg of the commercial detection kit (Fig. 3A). Interestingly, Western Blot and particle gel assay revealed that PC mutation led to the disappearance of HBeAg precursor p22cr, the appearance of core protein (also referred as p21) and capsid formation in p25-PC group (Fig. 3B), as well as the enhanced core protein expression in the cccDNA-mimicking prcccDNA system (Fig. 3D).

Generally, core proteins are excessive to the need of HBV replication. Since the binding of newly translated P protein with pgRNA initiates the encapsidation and subsequent reverse-transcription, it prompted us to examine whether PC mutation could enable preC RNA to translate P protein. In order to conveniently detect P protein and avoid the potential interference with the transcriptional co-regulator HBx, sequence of truncated P (encoding terminal protein and spacer domains of P protein) followed by a 3 × Flag was added into p25 plasmid to construct p25-WT-P-Flag and p25-PC-P-Flag, respectively. Meanwhile, sequence of 3 × Flag or Luciferase was directly inserted between the spacer and reverse transcriptase domain in prcccDNA (prcccDNA-WT-P-Flag and prcccDNA-PC-P-Flag; prcccDNA-WT-P-Luci and prcccDNA-PC-P-Luci, respectively) to further validate the conjectures (Supplementary Fig. S2). As expected, PC mutation vanished supernatant HBeAg levels and increased the intracellular expression of P protein in p25-P-Flag, prcccDNA-P-Flag (Fig. 3C and D) and prcccDNA-P-Luci systems (Fig. 3E and F). Overall, these data demonstrated PC mutation enabled preC RNA to perform pgRNA-like function, expressing core and P proteins, which in turn facilitated HBV replication.

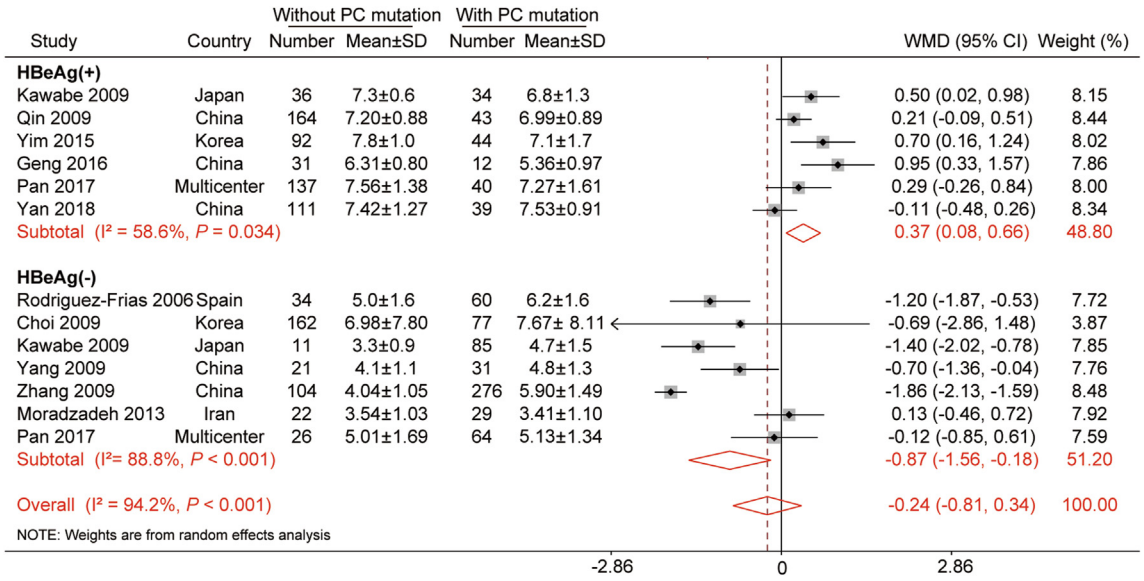

Fig. 1. WMD for HBV DNA level with or without PC mutation under different HBeAg status.

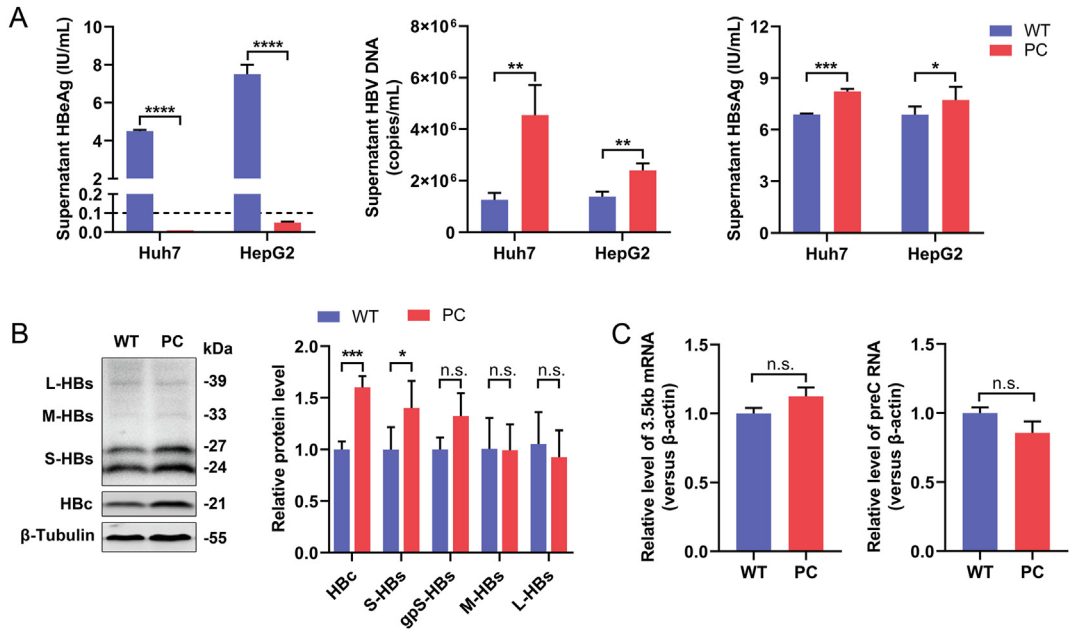

Fig. 2. PC mutation enhances HBV replication in the cell transfection model. prcccDNA-WT/PC and pCMV-Cre were co-transfected into Huh7 and HepG2 cells. **A** Supernatant HBeAg, HBsAg and HBV DNA levels in Huh7 and HepG2 cells. Western Blot of HBc, HBs (**B**) and preC RNA, 3.5 kb mRNA levels (**C**) in transfected HepG2 cells. L-HBs: large hepatitis B surface antigen; M-HBs: middle hepatitis B surface antigen; S-HBs: small hepatitis B surface antigen. Lower limit of detection was shown as dotted lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; n.s., not statistically significant.

3.4. PC combined BCP mutations promote HBV replication in cell transfection and HDI mouse models

Next, the effect of PC and BCP combined mutations on HBV replication was explored. Expectedly, BCP + PC mutations did enhance HBV replication, as indicated by relatively higher levels of HBV DNA and replicative intermediates in the transfected cell models (Fig. 4A and B), likely also through selectively upregulating pgRNA while down-regulating preC RNA (Fig. 4C, Supplementary Fig. S3), in addition to the post-transcriptional function of PC mutation.

To assess the replicative fitness of these mutants *in vivo*, prcccDNA/pCMV-Cre plasmids encoding either WT or mutant HBV genomes were delivered into C57BL/6J mice through HDI (Supplementary Fig. S4). Serum HBeAg level was increased firstly and then decreased in WT group while it was persistently negative in PC or BCP + PC group (Fig. 4D). Corresponding to results *in vitro*, PC or BCP + PC mutations both elevated serum HBV DNA on day 3 post HDI (Fig. 4D). To sum up, these results indicated that PC and BCP combined mutations promoted HBV replication via various mechanisms in *in vitro/vivo* transfection models.

3.5. PC mutation alone or combined with BCP mutations enhance viral replication in HepG2-NTCP cells

To further investigate the replicative capacity of PC or BCP + PC mutants, HBV DNA titers of concentrated supernatants of transfected HepG2 cells were measured by qPCR. Then HepG2-NTCP cells were

inoculated with viral particles of the same genome equivalent at a MOI of 200 and cultured for 9 days (Fig. 5A). Expectedly, HBeAg was not detectable in mutant groups while sustainably positive in WT group, and HBsAg secreted from mutant-infected cells was at lower amounts compared to that from WT strain-infected cells (Fig. 5B). However, the amount of HBV DNA in the supernatants of HepG2-NTCP cells infected with mutants was less than those infected with WT strains at day 3 post infection, but it surpassed at day 9 post infection (Fig. 5B).

To address relatively lower levels of HBV DNA in PC or BCP + PC groups at initiation of infection, above results were carefully screened. As shown in Fig. 2A, supernatant HBsAg was increased but not in proportion to the fold change of HBV DNA in PC group when compared to that of WT group. More than that, intracellular L-HBs was not elevated in parallel with small HBs (Fig. 2B). BCP and PC combined mutations even caused a decrease of HBsAg in the supernatant, though the intracellular HBs showed no significant change (Fig. 5C and D). Since HBsAg is essential for progeny virion production and infectivity, the impact of mutations on infectious progeny virion production was investigated. It has been reported that the pre-S1 domain of L-HBs is a key determinant for HBV entry, mediating viral interaction with the Na^+-taurocholate cotransporting polypeptide on hepatocytes (Yan et al., 2012). We further detected the level of L-HBs to determine the infectivity of viral particles in the supernatant of transfected HepG2 cells. The L-HBs levels were significantly lower in supernatants of both PC and BCP + PC groups (Fig. 5E). The yield of virions in supernatant of transfected HepG2 cells was also determined by immunoprecipitation with anti-preS2/anti-HBsAg antibodies and subsequent qPCR. Though total HBV DNA and HBV virion DNA levels were elevated in mutant groups, particularly in BCP + PC group (Fig. 5F), the production efficiency of infectious virions, defined as ratio of virion DNA to total HBV DNA, was significantly decreased (Fig. 5G). The above results demonstrated that PC or BCP + PC mutations not only primarily increased non-infectious naked capsids but also reduced the infectivity by down-regulating the composition of L-HBs on virions.

3.6. BCP + PC mutations contribute to maintenance of cccDNA pool in human hepatocyte chimeric mice

To determine the viral infectivity and replicative capacity of cccDNA *in vivo*, the human hepatocyte chimeric mice were infected with WT or BCP + PC mutant viral particles of the same genome equivalent via tail-vein injection (Fig. 6A). Notably, HBV DNA level of WT group increased more rapidly between weeks 2–8 but was finally caught up by BCP + PC group (Fig. 6B), agreed with the observations in HepG2-NTCP cells. Additionally, while HBeAg and HBsAg increased between weeks 2–12 post infection and peaked at week 12 in WT group, serum HBeAg was negative and HBsAg was constantly lower and peaked at week 16 in BCP + PC group (Fig. 6B).

As one mouse with high replication phenotype in BCP + PC group was dead at week 16 post infection, the rest were sacrificed at week 20 post infection. Intrahepatic cccDNA and replication-competent HBV DNA (repDNA, defined as cccDNA and rcDNA) in BCP + PC mutant-infected mice showed an increased trend at week 20 post infection (Fig. 6C). As expected, cccDNA carrying BCP + PC mutations possessed greater replicative capacity, as assessed by ratio of repDNA and cccDNA (Fig. 6D). Furthermore, IHC staining showed that HBcAg was exhibited a nuclear and cytoplasmic pattern in both groups. Coincidentally, the HBcAg-positive intensity and field were significantly enhanced in BCP + PC group (Fig. 6E). However, IHC staining of HBsAg displayed a

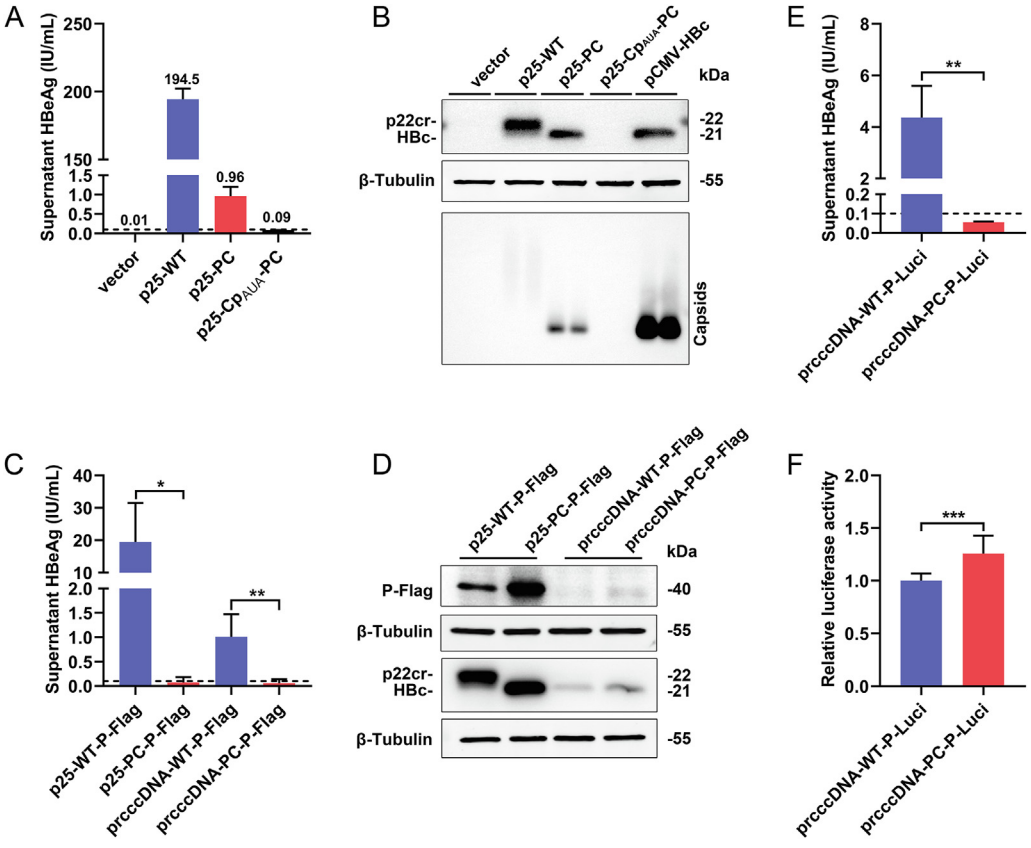

Fig. 3. PC mutation enables preC RNA to express core and P proteins. The wildtype (WT) or mutated p25, p25-P-Flag, prcccDNA-P-Flag, prcccDNA-P-Luci system was transfected into HepG2 cells. (**A**, **C**, **E**) Supernatant HBeAg levels in different systems. **B** Western Blot for p22cr, HBc (upper panel) and particle gel assay for capsids in p25 system (lower panel). **D** Western Blot for p22cr, HBc, P-Flag in p25-P-Flag and prcccDNA-P-Flag systems. **F** P proteins examined by luciferase reporter assay in the prcccDNA-P-Luci system. Lower limit of detection was shown as dotted lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not statistically significant.

cytoplasmic predominant pattern and no significant change of HBsAg-positive intensity and field was observed in BCP + PC group (Supplementary Fig. S5). In summary, BCP + PC mutations increased replicative capacity of cccDNA, contributing to maintenance of cccDNA pool likely through intracellular replenishment in this animal infection model.

3.7. BCP + PC mutations induce more severe liver injury in human hepatocyte chimeric mice

To test whether PC and BCP combined mutations exert direct cytopathic effect in human hepatocyte chimeric mice absent of a mature immune system, H&E staining was performed and it revealed that infection with HBV harboring BCP + PC mutations induced more extensive and severe degeneration of hepatocytes, as well as cytoplasmic vacuolation (Fig. 7A). Next, transcriptomic analysis was carried out using RNA sequencing to differentiate the molecular characteristics in livers infected with WT and BCP + PC mutant strains. Gene set enrichment analyses implied that BCP + PC mutants might exacerbate hepatocyte injury by inducing endoplasmic reticulum stress (ERS), unfolded protein response (UPR) (Fig. 7B), and activating tumor necrosis factor (TNF) pathway (*CASP3*, *AKT3*, *ATF6B*, *CEBPB*, *SOCS3*) (Fig. 7E, Supplementary Fig. S6). We also found that the intrahepatic mRNA level of glucose-regulated protein 78 (GRP78), X-box binding protein 1 (XBP1), activating transcription factor 4 (ATF4), C/EBP-homologous protein (CHOP), growth arrest and DNA damage 34 (GADD34), ER sulfhydryl oxidase 1 (ERO1A and ERO1B) were potently increased in chimeric mice infected with BCP + PC mutants, suggesting the enhanced ERS and reactive oxygen species (ROS) production (Fig. 7C). Moreover, IHC staining of 8-hydroxyguanine (8-OHdG), a marker of oxidative DNA damage, demonstrated the presence of 8-OHdG–positive cells in BCP + PC groups, whereas few were detected in the WT group (Fig. 7D). Intriguingly, in BCP + PC group, interferon (IFN) α and γ signaling pathways were suppressed, paralleled with significantly downregulated expression of *IFIT2*, *TNFAIP2*, *STAT2*, *MX1*, *SRI* in human hepatocytes (Supplementary Fig. S7). Regarding to the mouse gene expression profile, enrichment was found associated with cell cycle-related pathways and hippo signaling pathway in BCP + PC group (Fig. 7F, Supplementary Fig. S8). This result might indicate a compensatory proliferation of mouse hepatocytes, in response to the damage of human hepatocyte infected by HBV with BCP + PC mutations. Taken together, BCP + PC mutations exerted direct cytopathic effect to induce severe human hepatocyte injury.

4. Discussion

It was estimated that almost 30% of chronic HBV-infected patients had dominant PC mutants (Lau et al., 2021). However, the replicative capacity of PC mutants remains ambiguous. Since HBeAg status is strongly correlated with HBV DNA levels, meta-analysis was conducted to investigate the relationship of PC mutation with serum HBV DNA level under different HBeAg status. Importantly, studies only enrolling treatment-naïve patients were included to avoid antiviral treatment affecting frequency of PC mutation and HBV DNA level. And this meta-analysis demonstrated that HBeAg-negative patients with PC mutation had a higher serum HBV DNA level than those without the mutation, but it was converse in HBeAg-positive patients. Besides, it was observed that irrespective of HBeAg status, the difference of HBV DNA between patients with and without the mutation was disappeared. We speculated that prior studies drew contradictory conclusions due to the lack of concern about labeling patients with HBeAg status (Nakashima et al., 2004; Pivert et al., 2015; Ouneissa et al., 2012).

To prove the enhanced replication capacity of HBV carrying the PC mutation, using unit-length HBV constructs (genotype D), which could well mimic cccDNA *in vitro* and *in vivo*, we proposed that PC mutation might enable preC RNA to perform pgRNA-like function (expressing core and P protein), which favored capsid formation and pgRNA encapsidation, consequently promoting HBV replication. Therefore, we speculated that the HBV constructs with duplicated regulatory elements and the heterologous promoters might mask the effects of PC mutation on HBV replication, leading to the inconsistent reports in previous studies (Samal et al., 2015; Koumbi et al., 2016). But it can't be completely ruled out that pgRNA-like mRNA could be transcribed from nt1818/1819 on p25

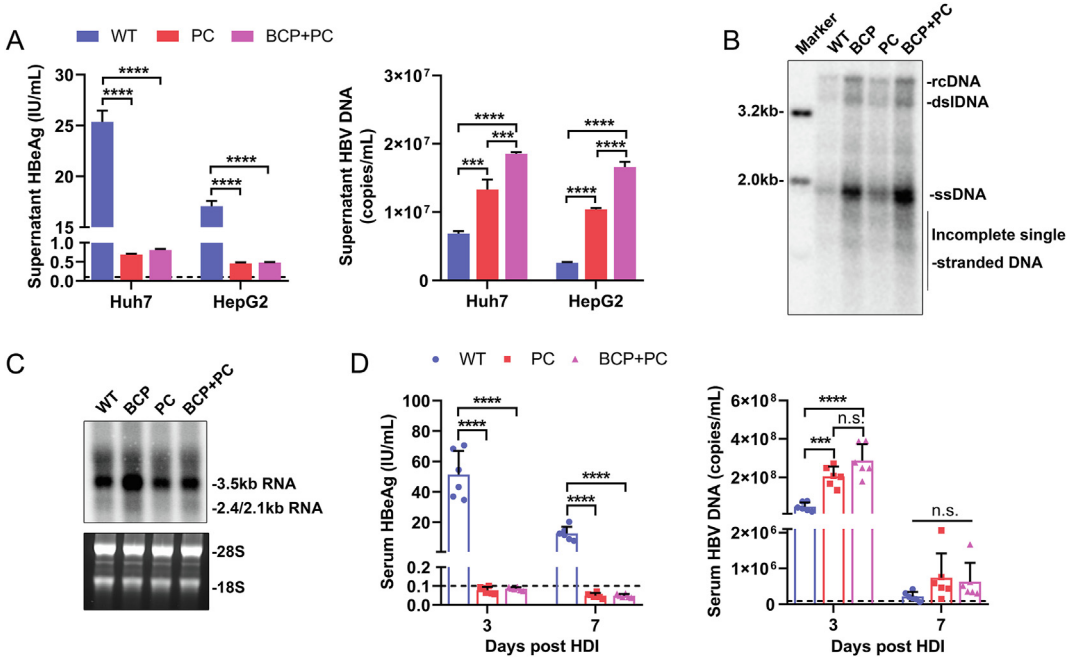

Fig. 4. PC combined BCP mutations promote HBV replication in cell transfection and HDI mouse models. prcccDNA systems were transfected into Huh7 or HepG2 cells. **A** Supernatant HBeAg and HBV DNA levels in transfected Huh7 and HepG2 cells. Southern Blot of intracellular HBV DNA (**B**) or Northern Blot of intracellular HBV RNAs (**C**) in transfected Huh7 cells. prcccDNA systems were delivered into C57BL/6J mice (n = 6/group) by HDI. **D** Serum HBeAg and HBV DNA levels at day 3 and 7 post HDI. HDI: hydrodynamic injection. Lower limit of detection was shown as dotted lines. ***, $P < 0.001$; ****, $P < 0.0001$; n.s., not statistically significant.

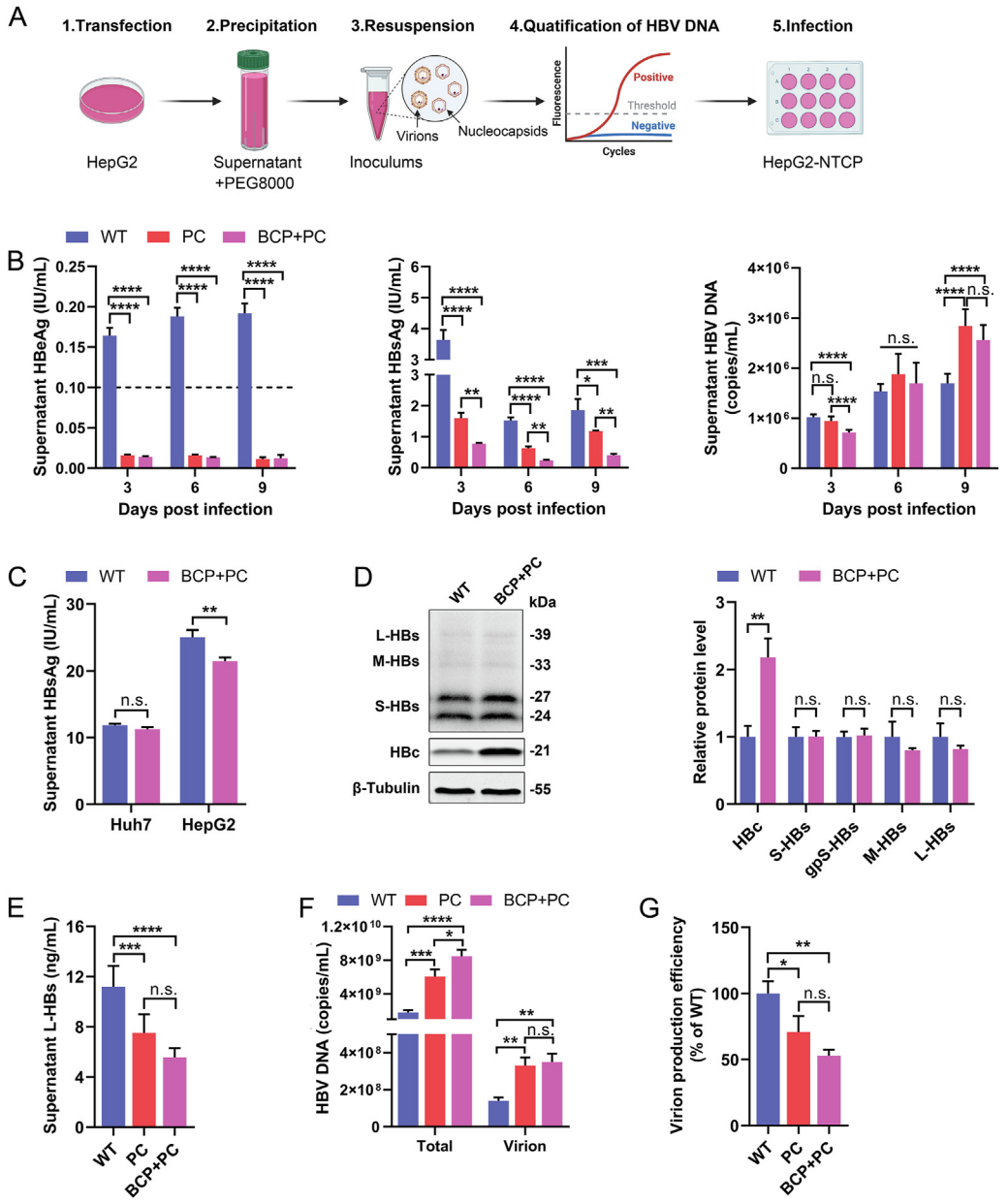

Fig. 5. PC mutation alone or combined with BCP mutations enhance viral replication in HepG2-NTCP cells. **A** HepG2-NTCP cells were inoculated by concentrated WT and mutant viral particles of the same genome equivalent at a multiplicity of infection of 200 as schematic. **B** HBeAg, HBsAg and HBV DNA from culture media of infected HepG2-NTCP cells at day 3, 6, 9 post infection. prcccDNA systems were transfected into Huh7 or HepG2 cells. **C** Supernatant HBsAg level in transfected Huh7 and HepG2 cells. Western Blot of HBc, HBs (**D**) and supernatant L-HBs (**E**) in transfected HepG2 cells. **F** Total HBV DNA detected directly by qPCR and HBV virion DNA determined by immunoprecipitation-qPCR. **G** Virion production efficiency was defined as a percentage of HBV virion DNA and standardized by WT group. L-HBs: large hepatitis B surface antigen; M-HBs: middle hepatitis B surface antigen; S-HBs: small hepatitis B surface antigen. Lower limit of detection was shown as dotted lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; n.s., not statistically significant.

plasmids. Whether PC mutation results in an increase of these pgRNA-like mRNA in p25 systems warrants further exploration. In addition, we observed significantly reduced preC RNA/total 3.5 kb mRNA ratio in PC group, which might attribute to nonsense-mediated decay, a mechanism commonly present in eukaryotic cells (Karousis and Muhlemann, 2019).

Moreover, PC and BCP combined mutations occurred increasingly with age in chronic HBV-infected patients. Of note, the well-known mechanism of BCP mutations (upregulate pgRNA and downregulate preC RNA) in HBV replication was contradictory with that of PC mutation (enable preC RNA to translate viral proteins). Herein, our results showed the coexistence of mutations still enhanced viral replication in both cell transfection and HDI mouse models.

Furthermore, human hepatocyte chimeric mice, which supported the complete viral life cycle and chronic infection *in vivo*, were infected with WT and BCP + PC mutant viral particles of the same genome equivalent. As expected, though the proportion of virions in inoculums was lower at infection initiation, the serum HBV DNA of BCP + PC group was early lower but subsequently caught up, in line with the observation in HepG2-NTCP cells. The intrahepatic repDNA, cccDNA and ratio of repDNA to cccDNA showed an increased trend in BCP + PC mutant-infected mice. Moreover, as HBeAg-negative mutants have the selective advantage

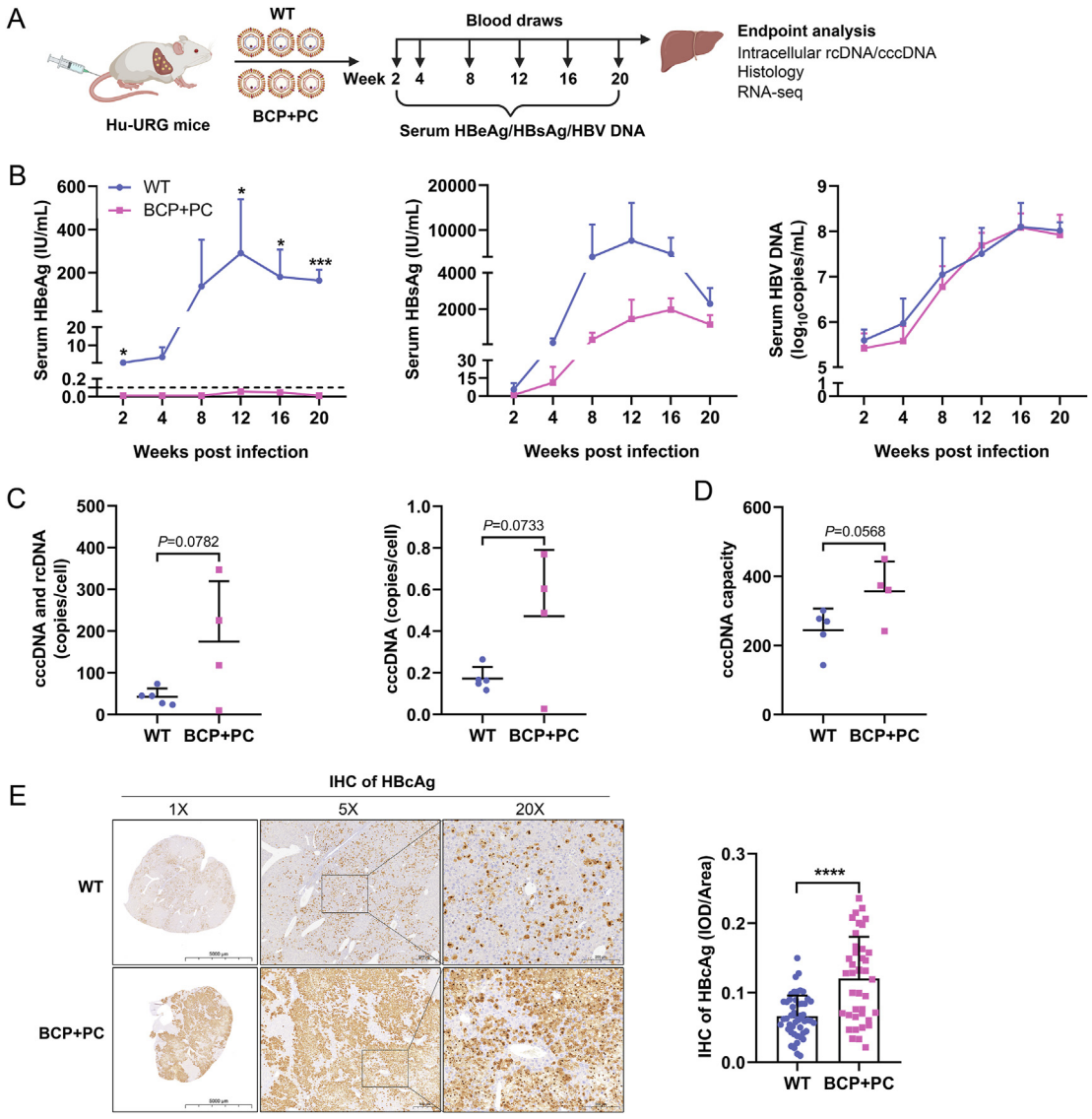

Fig. 6. BCP + PC mutations contribute to maintenance of cccDNA pool in human hepatocyte chimeric mice. **A** Schematic of HBV infection in chimeric mice (n = 5/group) (Created with BioRender.com). One mouse in BCP + PC group was dead at week 16 post infection. Liver samples were obtained from chimeric mice infected with WT strains (n = 5) and BCP + PC mutants (n = 4) at week 20 post infection. **B** Serum HBsAg, HBeAg and HBV DNA of chimeric mice at the indicated time points post infection. **C** Intrahepatic cccDNA and repDNA of chimeric mice analyzed by droplet digital PCR. **D** cccDNA capacity was defined as ratio of repDNA/cccDNA. **E** Representative images and assessment of liver sections stained with IHC of HBcAg. Lower limit of detection was shown as dotted lines. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; n.s., not statistically significant.

(Frelin et al., 2009), the marginal increase in replicative fitness of BCP + PC mutant observed in human hepatocyte chimeric mice might be magnified in presence of HBV-specific immune response. These findings indicated that cccDNA carrying mutations might possess greater replicative capacity and probably be benefit for the persistence of viral replication via intracellular replenishment.

Regarding the conflicting relationship of PC mutation with HBV DNA under different HBeAg status in meta-analysis, the following hypothesis was proposed to explain the contradictory phenomenon. The hallmark of transition from HBeAg-positive chronic infection to hepatitis is the activation of host immune response against HBV, which is partially characterized by occurrence of cytotoxic T cells (Iannacone and Guidotti, 2022; Frelin et al., 2009). Wildtype cccDNA pool would be decreased and further diluted during the long-term immune surveillance of HBeAg/HBcAg -specific cytotoxic T lymphocytes and compensatory proliferation of residual hepatocytes. Emergence of mutants generally implies the more powerful specific immune response and clearance of WT strain-infected hepatocytes (Chu et al., 2002). Therefore, it's conceivable that even hepatocytes carry replication-active mutants, HBV DNA levels will still be significantly decreased in HBeAg-positive patients. Along with disease progression, mutants, which could abolish HBeAg and evade host immune response, gradually accumulate. In HBeAg-negative stage, though there are a few infected hepatocytes due to immune selective pressure, cccDNA carrying mutations, which possesses a selective advantage and stronger replicative capacity, will be predominant. Thus, HBeAg-negative patients dominantly infected with PC and/or BCP mutants would have a relatively higher serum HBV DNA level (Fig. 8).

Interestingly, though liver injury was generally considered as a result of immune response against infected hepatocytes during chronic HBV infection, several studies indicated that BCP + PC mutants might exert direct cytopathic effects. Hayashi et al. (2019) showed BCP/PC mutations didn't significantly promote viral replication *in vitro*. And similar to human hepatocyte chimeric mice infected with WT strains, only mild inflammation was observed in mice infected with BCP/PC mutants, which was prepared from supernatants of 1.24-fold HBV construct

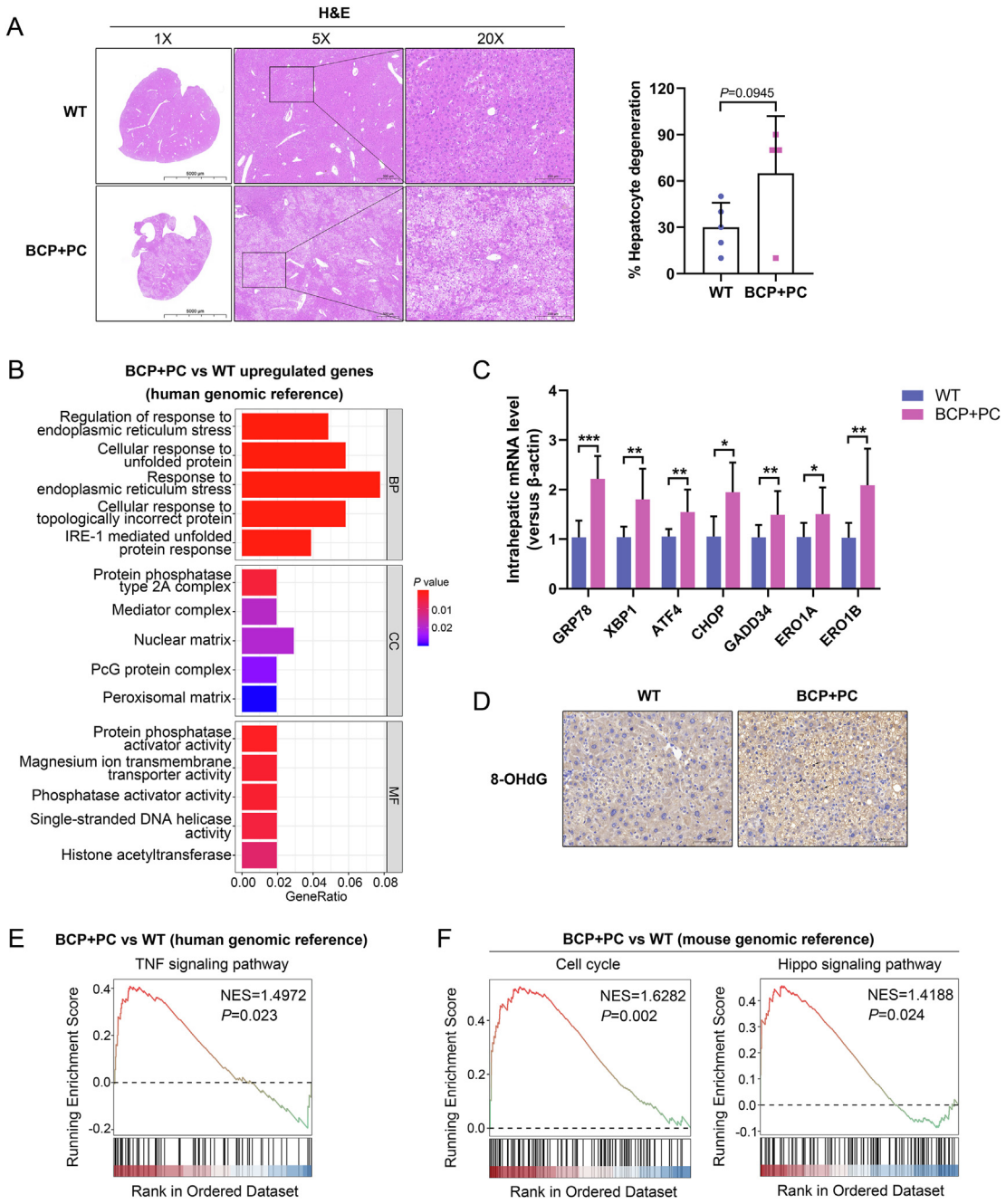

Fig. 7. BCP + PC mutations induce more severe liver injury in human hepatocyte chimeric mice. **A** Representative images and assessment of liver sections stained with H&E. RNA sequencing was conducted comparing the gene expression profiles of liver in WT strain- and BCP + PC mutant-infected chimeric mice. **B** The gene ontology enrichment analysis of upregulated genes aligned to human genomic reference. **C** The intrahepatic mRNA level of indicated genes by RT-qPCR. **D** Representative images of liver sections stained with IHC of 8-OHdG. Gene set enrichment analyses referring to Kyoto Encyclopedia of Genes and Genomes gene sets aligned to human genomic reference (**E**) or mouse genomic reference (**F**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(genotype F1b)-transfected Huh7 cells (Hayashi et al., 2019). However, a recent report demonstrated that high replication phenotype and accumulation of HBsAg led to ERS-mediated cell death in humanized mice infected with BCP/PC mutants, which was genotype C and derived from the sera of chronic HBV-infected patients (Uchida et al., 2023). Another study also suggested that HBV carrying PC mutation from sera of patients of genotype B1 caused a significant increase in viral replication, hepatocyte degeneration and fibrosis in humanized mouse model (Sugiyama et al., 2009). Noticeably, here we also observed more severe hepatocyte degeneration and cytoplasmic vacuolation in human hepatocyte chimeric mice infected with BCP + PC mutants (genotype D). Consistently, activation of cell cycle and hippo signaling by BCP + PC mutants aligned to mouse genomic reference suggested the compensatory proliferation of mouse hepatocytes.

Mechanistically, the enrichment analysis indicated the activation of ERS and TNF signaling pathways in human hepatocytes of the BCP + PC mutant-infected chimeric mice. Upon ERS, the UPR is activated to remedy the situation. Then the level of ER chaperone gene GRP78 and XBP1 mRNA, of which the active form could augment ER size and function, is enhanced (Lee et al., 2002). In addition, the downstream of PKR-like ER kinase pathway, ATF4 is selectively increased, transcriptionally inducing the expression of CHOP, XBP1 and chaperones to promote apoptosis or restore ER homeostasis (Malhi and Kaufman, 2011). CHOP upregulates the expression of GADD34, which reinitiates the translation. But if the

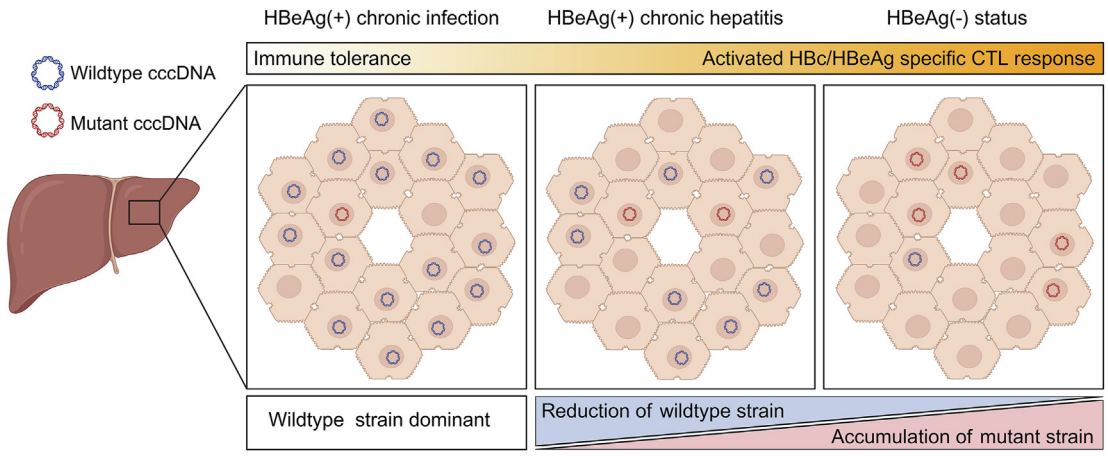

Fig. 8. A hypothetical model of wildtype and mutant virus dynamic over the course of chronic HBV infection (Created with BioRender.com).

ERS has not been completely resolved, the entry of nascent proteins into the ER can generate byproduct ROS via the ERO1A and ERO1B with deleterious consequences (Malhi and Kaufman, 2011). We also validated the occurrence of ERS and ROS formation by detecting the expression of indicated genes and products via RT-qPCR and IHC staining. Nevertheless, BCP + PC mutations enhanced intrahepatic HBcAg accumulation in current study, rather than HBsAg described in a previous report (Uchida et al., 2023). Moreover, we observed the correlation of severe hepatocyte damage with the enhanced intracellular distribution of HBcAg. The discrepancy between us and others might result from the HBV genotypes and source of inoculums. Additionally, as WT strains might coexist with mutants in sera of patients, the infectivity and the proportion of mutants in inoculums should be taken into full consideration. Another discovery in current study was that BCP + PC mutations inhibited IFNα/γ signaling pathway in hepatocytes, supporting the notion that cccDNA carrying BCP + PC mutations probably responded poorly to IFN treatment (Brunetto et al., 1993; Sonneveld et al., 2012). But the molecular mechanisms of HBV mutations in IFN response need to be further explored.

This study verified that PC alone and in combination with BCP mutations could promote HBV replication and elucidated the relevant mechanisms comprehensively in multiple systems *in vitro/vivo*. Meanwhile, our study gave hints for the pathogenic mechanisms of BCP + PC mutations in HBV genotype D by using human hepatocyte chimeric mouse model. However, the results of *in vivo* models should be cautiously interpreted when extrapolating to patients as HBV-specific immune response was not taken into account. And the effects of mutations in different HBV genotypes needed further investigation.

5. Conclusion

In conclusion, HBeAg-negative patients with PC mutation are likely to have higher HBV DNA level. PC and BCP mutations enhance HBV replication and have pathogenicity. HBeAg-negative patients should be monitored on HBV mutations regularly and are expected to receive early antiviral treatment to prevent disease progression.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

This study was approved by the Institutional Review Board of Peking University (No. LA2022396).

Author contributions

Guixin Li: conceptualization, investigation, writing-original draft. Danli Yang: investigation, writing-original draft. Xin Liu: validation, writing-original draft. Ting Zhang: investigation. Hui Liu: investigation. Jun Zou: investigation. Zimeng Xu: investigation. Xiangmei Chen: investigation. Lizhong Dai: resources, writing-review. Hongsong Chen: writing-review. Fengmin Lu: conceptualization, writing-review.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2024.03.003.

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