Original Article

Paclitaxel-induced Immune Dysfunction and Activation of Transcription Factor AP-1 Facilitate Hepatitis B Virus Replication

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Abstract

Background and Aims: Hepatitis B virus (HBV) reactivation is commonly observed in individuals with chronic HBV infection undergoing antineoplastic drug therapy. Paclitaxel (PTX) treatment has been identified as a potential trigger for HBV reactivation. This study aimed to uncover the mechanisms of PTX-induced HBV reactivation *in vitro* and *in vivo*, which may inform new strategies for HBV antiviral treatment. ***Methods:*** The impact of PTX on HBV replication was assessed through various methods including enzyme-linked immunosorbent assay, dual-luciferase reporter assay, quantitative real-time PCR, chromatin immunoprecipitation, and immunohistochemical staining. Transcriptome sequencing and 16S rRNA sequencing were employed to assess alterations in the transcriptome and microbial diversity in PTX-treated HBV transgenic mice. ***Results:*** PTX enhanced the levels of HBV 3.5-kb mRNA, HBV DNA, HBeAg, and HBsAg both *in vitro* and *in vivo*. PTX also promoted the activity of the HBV core promoter and transcription factor AP-1. Inhibition of AP-1 gene expression markedly suppressed PTX-induced HBV reactivation. Transcriptome sequencing revealed that PTX activated the immune-related signaling networks such as IL-17, NF-κB, and MAPK signaling pathways, with the pivotal common key molecule being AP-1. The 16S rRNA sequencing revealed that PTX induced dysbiosis of gut microbiota. ***Conclusions:*** PTX-induced HBV reactivation was likely a synergistic outcome of immune suppression and direct stimulation of HBV replication through the enhancement of HBV core promoter activity mediated by the transcription factor AP-1. These findings propose a novel molecular mechanism, underscoring the critical role of AP-1 in PTX-induced HBV reactivation.

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Introduction

The continuing high prevalence of chronic hepatitis B virus (CHB) infection is a global public health concern, particularly in China and other countries in the Asia-Pacific region. Approximately 296 million people globally are believed to have CHB, and hepatitis B virus (HBV) infection-related illnesses led to approximately 820,000 fatalities in 2019.[1,2] HBV infection can cause varying degrees of liver inflammation and fibrosis. In the absence of antiviral treatment, persistent CHB infection can lead to cirrhosis and hepatocellular carcinoma. HBV infection is amenable to early diagnosis and effective treatment, but the current diagnosis and treatment rates of HBV in China are only 22% and 15%, respectively.[3] Despite the widespread implementation of hepatitis B vaccination and other interventions, the prevention and treatment of HBV still pose significant challenges.

HBV is transported to the liver through bloodstream transmission and infects hepatocytes. After attaching to its functional receptor, sodium taurocholate cotransporting polypep-

tide (NTCP), the virus enters the host cell cytoplasm through endocytosis. Covalently closed circular DNA (cccDNA) is formed in the nucleoplasm by the release of relaxed circular DNA (rcDNA) from the nuclear capsid.[4] HBV cccDNA is the primary source of sustained viral replication and transcription. It exists in a microchromosomal form for an extended period and is highly stable, acting as the persistent reservoir of the virus and a significant cause of HBV reactivation during immunosuppression.[5] Even after HBV infection clearance and serologic conversion of HBsAg, the presence of HBV cccDNA can still be observed within the liver.[6]

The definition of HBV reactivation may vary slightly among different guidelines, but the underlying concept remains the same. It refers to the activation of HBV in individuals with quiescent infection or those having low levels of viral replication. HBV reactivation is a frequent complication of immunosuppressive or antineoplastic therapy in individuals with chronic HBV infection leading to liver damage.[7] The main underlying mechanism of HBV reactivation is the disruption of the balance between the virus and the host's immune function. Treatment with chemotherapeutic drugs or immunosuppressants can compromise the immune function of patients, leading to a significant increase in viral replication within the host. Subsequently, when these drugs are discontinued and the immune function is restored, an overly strong immune response can occur, resulting in hepatocyte injury.[8] Of note, the likelihood of HBV reactivation is particularly high among individuals treated with B-cell depleting agents such as rituximab.[9] However, in more than half of all cases, HBV reactivation occurs early in the course of chemotherapy, rather than during the interval after the completion of chemotherapy.[10] This suggests that immune dysfunction alone cannot explain all the clinical phenomena, and it is possible that certain chemotherapeutic agents directly stimulate HBV replication.

Paclitaxel (PTX) is a widely utilized first-line therapeutic agent for various types of cancers. Its primary mechanism of action is to stabilize and enhance the polymerization of microtubule proteins, leading to the prevention of microtubule depolymerization and the inhibition of cell mitosis. However, clinical trials have reported cases of severe HBV reactivation leading to liver damage in breast cancer patients following PTX treatment.[11]

While previous clinical studies have documented the reactivation of HBV in individuals concurrently diagnosed with HBV infection and tumors following PTX treatment, the direct impact of PTX on HBV itself remains a subject of controversy. Furthermore, there is a paucity of comprehensive investigations elucidating the molecular mechanisms underlying chemotherapeutic drug-induced HBV reactivation. Therefore, in this study, we conducted a series of *in vitro* and *in vivo* experiments to examine the relationship between PTX and HBV replication. The objective was to shed light on the potential mechanisms of PTX-induced HBV reactivation.

Methods

Cell culture

HepAD38 (HB-8065, ATCC, Manassas, VA, USA) cells were obtained from the American Type Culture Collection, while HepG2-NTCP cells were generously provided by Prof. Ningshao Xia (Xiamen University, Fujian, China). HepG2.2.15 cells were stored in our laboratory. HepAD38 cells were cultured in a growth medium comprising Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT, USA) with the addition of 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 1 µg/mL tetracycline, 500 µg/mL G418, 100

units/mL penicillin (HyClone, Logan, UT, USA), and 100 µg/mL streptomycin (HyClone, Logan, UT, USA) as previously described.[12] To boost NTCP receptor expression in HepG2-NTCP, an additional 2 µg/mL of doxycycline was added to the growth medium.[13]

Antibodies and reagents

The following antibodies were used for immunoblot analysis: anti-hepatitis B core antigen (HBcAg) (B0586) from Dako (Glostrup, Denmark) and anti-GAPDH (Cat. no. 60004-1) from Proteintech (Rosemont, IL, USA). Anti-Rabbit-HRP (ab6721) from Abcam (Cambridge, UK) and anti-c-Jun (Cat. no. 9165S) from Cell Signaling Technology (Danvers, MA, USA). Paclitaxel (PTX, Cat#HY-B0015) was obtained from MedChemExpress (Monmouth Junction, USA). Sangon Biotech (Shanghai, China) provided G418, tetracycline (A100422), and doxycycline (DOX).

Animal studies

HBV transgenic mice (HBV-Tg, C57BL/6), a gift from Xiamen University, were housed in the SPF of the Animal Centre of Chongqing Medical University. A total of 8 HBV-Tg mice, aged 6–8 weeks, were included in each group. Animal administration used in this study has been described in the previous study.[12] The mice were administered intraperitoneal injections of phosphate-buffered saline (PBS, control) or PTX (10 mg/kg body weight) every other day for a total of seven injections. Half of the mice were sacrificed after the injection on the 14th day, and the remaining mice were kept under observation until one week after drug withdrawal. The liver tissue samples were harvested and quickly frozen in liquid nitrogen and stored at −80°C, together with the colon contents samples for sequencing purposes.

Western blot analysis

Total proteins were extracted from the cells using RIPA lysis buffer (Cat. no. C0005; Beyotime Biotechnology, Shanghai, China) with 1 mM phenylmethanesulfonyl fluoride (Beyotime Biotechnology, Shanghai, China). The protein samples were subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then electrically transferred onto a PVDF membrane (IPVH00010, Merck Millipore, Billerica, MA, USA). The protein bands were detected using an enhanced chemiluminescence (ECL) detection kit (Beyotime Biotechnology, Shanghai, China).

RNA isolation and RT-qPCR

Total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. All operations were carried out under enzyme-free conditions. For RT-qPCR detection, cDNA synthesis was performed using modified Moloney murine leukemia virus reverse transcriptase (RR047A, TaKaRa, Tokyo, Japan). The SYBR Green qPCR Master Mix with special primers was utilized for RT-qPCR analysis on a Bio-Rad CFX Connect Real-time PCR Detection System (Bio-Rad). The fold change was examined using the $2^{-\Delta\Delta Ct}$ method with β-actin serving as the internal control (Supplementary File 1). All primers used in the study are listed in Supplementary Table 1.

Immunohistochemistry

Immunohistochemistry was performed as described.[14] After fixation of liver tissues with 4% paraformaldehyde, paraffin-embedded sections were prepared according to the standard procedure and stored at room temperature. The tissue sections were incubated overnight with primary antibodies

at 4 °C, followed by coating with biotin-labelled sheep anti-mouse/-rabbit IgG polymer (ZSGB-BIO, Beijing, China) at room temperature for 30 minutes. Diaminobenzidine solution (ZLI-9019, ZSGB-BIO, Beijing, China) was applied for colour development at the end. The Pannoramic Scan 250 Flash or MIDI system was used to scan the stained slides, and the Pannoramic Viewer 1.15.2 (3DHistech, Budapest, Hungary) was employed for image acquisition.

Dual-Luciferase reporter assay

Briefly, dual-Luciferase reporter assay was performed as previously described.[15] A luciferase reporter plasmid containing the HBV core promoter Cp, X gene promoter Xp, and surface antigen promoters Sp1 and Sp2 was constructed using pGL3-Basic as a vector (constructed and preserved by the laboratory). Cells were co-transfected with plasmids pcDNA3.1, TK-RL, and the corresponding HBV promoter plasmids (pGL3-Cp, pGL3-Xp, pGL3-Sp1, pGL3-Sp2). After 48 hours of incubation, the luciferase activity was quantified using the Dual-Luciferase Reporter Gene Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's guidelines.

RNA sequencing

Mouse liver tissues harvested from PTX and CON groups were snap-frozen in liquid nitrogen and used for transcriptome sequencing. RNA sequencing analysis was conducted at Shanghai Majorbio Bio-pharm Technology Co. Ltd. (Shanghai, China) following the manufacturer's descriptions. Total RNA was extracted using TRIzol® Reagent and the mRNA was purified using oligo (dT) beads for library construction. The paired-end RNA sequencing library was subjected to sequencing using the Illumina NovaSeq 6000 sequencer, with a read length of 2×150 bp, to generate the sequencing data.

16S rRNA sequencing

Samples of intestinal contents were collected and total DNA was extracted. PCR was employed to amplify the 16S rRNA of bacteria present in the samples. The resulting PCR products were detected by agarose gel electrophoresis. Based on the initial quantitative analysis of the electrophoresis results, the PCR products were measured using the fluorescence-based quantification method. Subsequently, high-throughput sequencing was conducted using the Illumina platform. The sequence of 338F was 5'-ACTC CTACGGGAGGCAGCAG-3', and the sequence of 806R was 5'-GGACTACHVGGGTWTCT AAT-3'. The primer sequences have been previously reported.[16]

Statistical analysis

GraphPad Prism 8.0 was used for statistical analyses. For continuous variables, mean (±standard deviation) values from three independent experiments are presented. Inter-group comparisons were conducted using the *t*-test, while one-way ANOVA was used for multi-group comparisons. P values <0.05 were considered indicative of statistical significance.

Results

Paclitaxel directly stimulates HBV replication in vitro

We utilized the stable HBV-expression hepatocellular carcinoma (HCC) cell line (HepAD38, HepG2.2.15) and the HBV naturally infected cell model (HepG2-NTCP) to investigate whether PTX has a direct effect on HBV replication *in vitro*. Initially, we assessed the cytotoxicity of PTX within a specific concentration range (0–4 μM) and found that it had a low cytotoxic effect, with cell viability >80% (Supplementary Fig. 1A). Furthermore, the IC50 value of PTX was determined using the CCK8 assay kit. The IC50 value of PTX in HepAD38, HepG2-NTCP, and HepG2.2.15 was 4.468 μM, 5.562 μM, and 6.489 μM, respectively (Supplementary Fig. 1B). To assess the impact of PTX on HBV replication within a secure range, we incubated HepAD38 and HepG2-NTCP cells with PTX at concentrations ranging from 0 to 4 μM.

Following treatment of cells with PTX at various concentrations (0–4 μM) for 120 hours, the intracellular HBV 3.5-kb mRNA levels (Fig. 1A), HBV DNA levels (Fig. 1B), and HBV cccDNA levels (Fig. 1C) were significantly increased in a concentration-dependent manner. The intracellular HBcAg expression was similarly increased (Fig. 1D–E). Enzyme-linked immunosorbent assay (ELISA) of cell culture supernatants showed that PTX treatment enhanced HBeAg and HBsAg secretion levels (Fig. 1F and Supplementary Fig. 2A) in a concentration-dependent manner. Similar findings were observed in HepG2.2.15 cells (Supplementary Fig. 2B–F). These findings indicated that PTX may potentially enhance HBV replication *in vitro*.

Paclitaxel can promote HBV replication in vivo

The impact of PTX on HBV replication *in vivo* was investigated using HBV-Tg mice. HBV-Tg mice were divided into 2 groups (n=8). Intraperitoneal injection of PTX (10 mg/kg) was administered in the PTX group while PBS was administered in the control group (Con). The injections were administered every other day for a total of seven injections (Fig. 2A). The observation time was extended to one week after the withdrawal of the drug. Serum samples of the mice were collected on the 7th, 14th, and 21st day, respectively. PTX administration resulted in a significant increase in serum HBV DNA levels, along with increased levels of HBeAg and HBsAg (Figure. 2B–D), but their secretion levels showed a slight decrease one week after drug withdrawal. HBV 3.5-kb mRNA, HBV DNA, and HBV cccDNA levels in the liver were also significantly increased (Fig. 2E–G). Immunohistochemical staining showed a significant increase in HBcAg and HBsAg protein expression in hepatocytes (Fig. 2H), which was correlated with the duration of drug action, and the replication levels were slightly decreased after drug withdrawal. In conclusion, PTX treatment significantly enhanced HBV replication levels in HBV-Tg mice. We also detected serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels to explore whether PTX-mediated HBV reactivation would induce liver injury. Our findings suggested no significant damaging effect of PTX on the liver of HBV transgenic mice (Supplementary Fig. 2G–H). We speculated that the time point of our observations may be in the active phase of HBV replication, which has not yet progressed to the phase of hepatic impairment.

Paclitaxel treatment induces altered gene expression in HBV-Tg mice

We performed liver transcriptome analysis to further explore the molecular mechanism of PTX-induced HBV reactivation. The PCA plot (Fig. 3A) showed clustering of samples in the Con and PTX groups with a clear separation of samples in each group. This indicated the repeatability of samples in each group. Using the criteria of adjusted P value <0.05 and Fold change ≥2, we found significant up-regulation of 453 genes and significant down-regulation of 259 genes in the PTX group compared to the Con group (Fig. 3B). KEGG enrichment analysis of differentially expressed genes (DEGs) revealed notable enrichment of various biological processes, including cancer:overview,
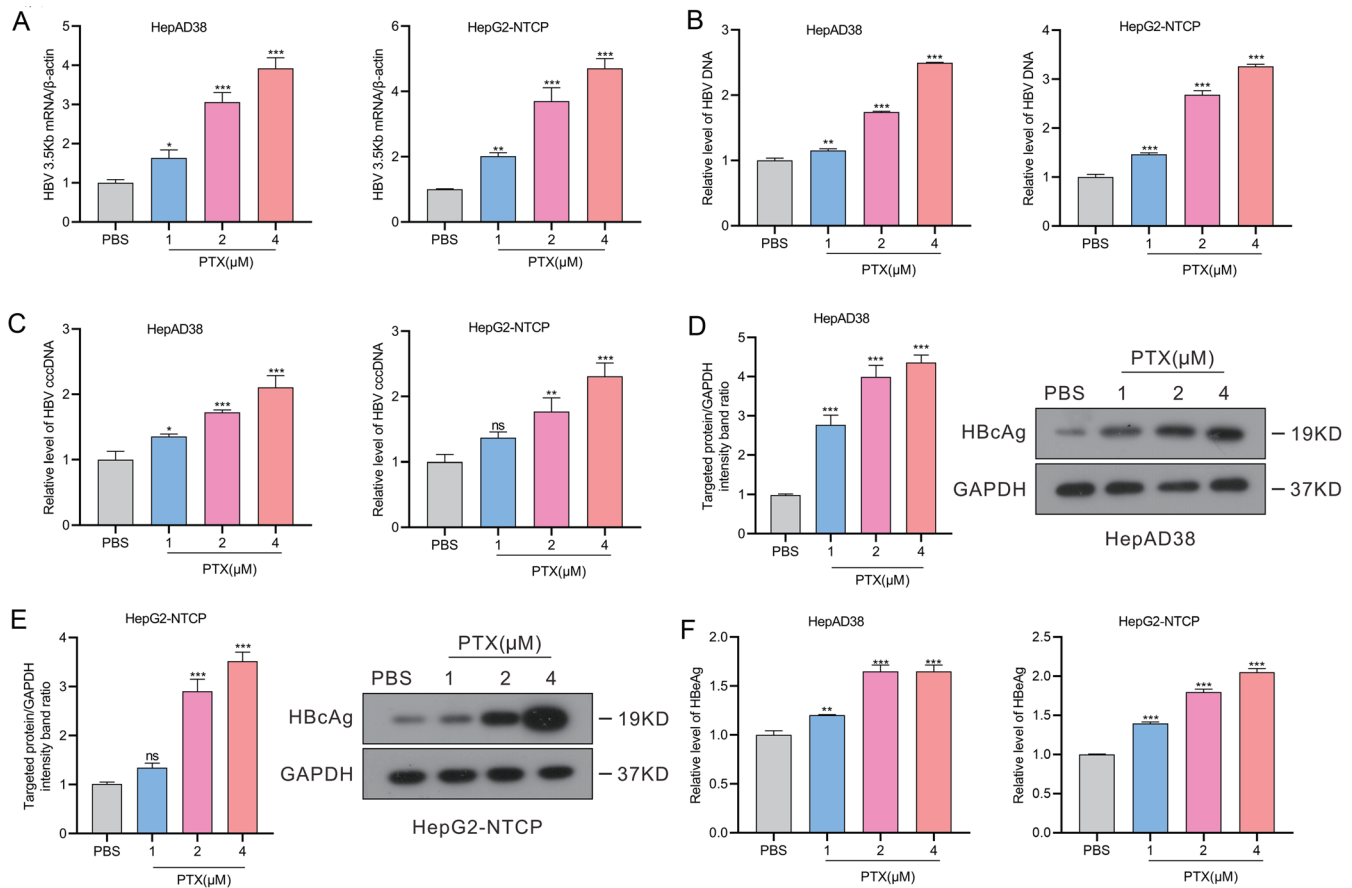
Fig. 1. PTX enhances HBV replication *in vitro*. (A–C) qPCR assay was employed to quantify the levels of HBV 3.5-Kb mRNA, HBV DNA, and HBV cccDNA in both HepAD38 cells and HepG2-NTCP cells following PTX treatment. (D–E) Immunoblot analysis of HBcAg expression levels in HepAD38 cells and HepG2-NTCP cells. Densitometry analysis was used to measure the relative levels of HBcAg. (F) Results of ELISA showing HBeAg levels in the culture medium supernatants of HepAD38 cells and HepG2-NTCP cells. Mean±SD values from three independent experiments are presented. Statistical significance was assessed using the *t*-test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. HBV, hepatitis B virus; PTX, paclitaxel; HBV cccDNA, HBV covalently closed circular DNA; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

infectious disease:viral, immune system and signal transduction (Fig. 3C–D). Further analysis revealed that PTX upregulates immune response-related pathways such as the IL-17 signaling pathway, NF-Kappa B signaling pathway, and MAPK signaling pathway (Fig. 3E). The heatmaps depicting the enrichment of the DEGs in the three immune response pathways are presented in Figure 3F–H. Transcription factor family statistics were also performed on the DEGs. The results showed that they could be clustered in the Fos related Jun related transcription factor family (Fig. 3I), including Jun, Fosl2, and Jund. Jun is a member of the AP-1 family, and AP-1 has been reported to have a significant impact on HBV replication.[17]

AP-1 activation can stimulate HBV replication

HBV replication is known to be finely regulated by a wide range of liver-enriched transcription factors. Therefore, we screened the transcription factors involved in the regulation of HBV expression and analyzed their expression levels after PTX treatment. We observed a remarkable increase in the mRNA levels of transcription factors AP-1 and CREB1 (Fig. 4A, Fig. 3A). Notably, AP-1 exhibited a greater increase, which was consistent with the results of transcriptome sequencing (Fig. 3I). Additionally, the protein expression of

the c-Jun subunit of AP-1 was also significantly increased (Fig. 4B, Supplementary Fig. 3B). Previous *in vitro* investigations have shown that PTX induces upregulation of AP-1 mRNA and protein levels, and we verified whether PTX promotes the enhancement of AP-1 activity in HBV-Tg mice. Immunohistochemistry showed a significant increase in AP-1 protein levels in the liver tissues of mice on the 14th day of PTX administration and one week after withdrawal of the drug (day 21) as compared to the PBS control (Fig. 4C). Based on these findings, we selected AP-1 as the target for further investigation in subsequent experiments. To validate the role of AP-1 in HBV replication, we employed siRNA targeting to silence AP-1 expression (Fig. 4D–E, Supplementary Fig. 3C–D). Silencing of AP-1 led to downregulation of the expression levels of HBV 3.5-kb mRNA, HBV DNA, and HBV cccDNA (Fig. 4F–H). The protein expression of HBcAg was decreased, while the levels of HBeAg and HBsAg in the supernatant were reduced (Fig. 4I–K), suggesting that silencing AP-1 significantly inhibited the replication levels of HBV-expressing cells.

PTX enhances viral HBV replication by activating the binding of AP-1 to HBV core promoter

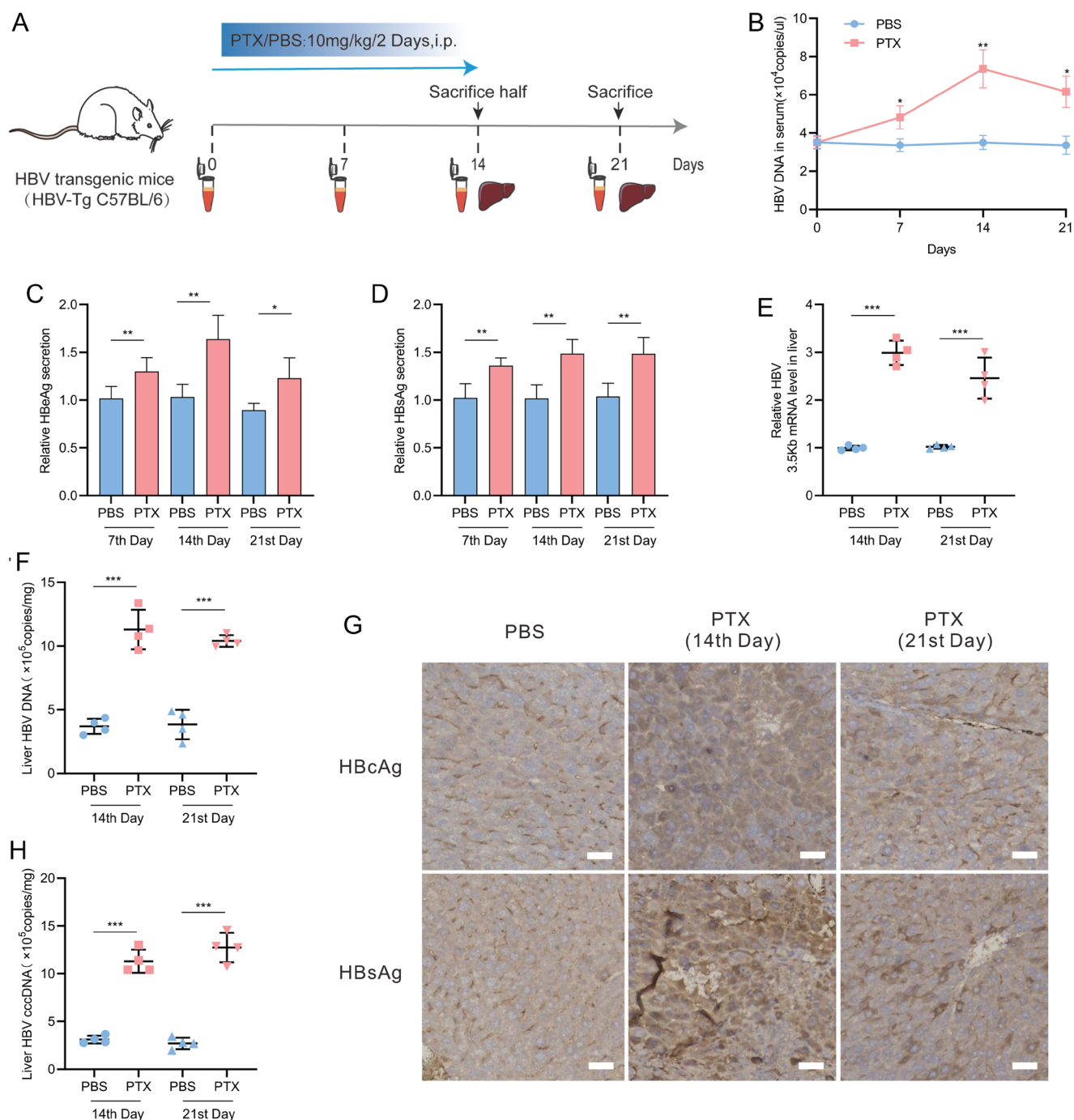Numerous transcription factors can bind to HBV promoter/

Fig. 2. PTX administration promotes viral biosynthesis *in vivo*. (A) Schematic illustration of the PTX treatment regimen in HBV transgenic mice. (B) HBV DNA levels in mouse serum were quantified using qPCR. (C–D) HBeAg and HBsAg levels in mouse serum were measured using ELISA. (E–G) The levels of HBV 3.5-Kb mRNA, HBV DNA, and HBV cccDNA in liver tissues were determined using qPCR. (H) Immunohistochemistry analysis of HBcAg and HBsAg expression in liver tissues (scale bar: 60 μm). Mean±SD values from three independent experiments are presented. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs. PBS control. HBV, hepatitis B virus; HBV-Tg mice, HBV-transgenic mice; PTX, paclitaxel; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV cccDNA, HBV covalently closed circular DNA.

enhancer elements to regulate HBV transcription and replication. We found that PTX induced AP-1 activation to promote HBV transcription and replication, but the specific mechanism by which AP-1 regulates HBV transcription and replication is not clear. We initially assessed the impact of PTX on the activity of four HBV promoters: HBV Cp, HBV Xp, HBV Sp1, and HBV Sp2. The findings indicated a notable enhancement in the activity of the core promoter (Fig. 5A, Supplementary Fig. 3E). Silencing of AP-1 affected the activity of the HBV core promoter (Fig. 5B, Supplementary Fig. 3F), while CHIP assays suggested an interaction between AP-1 and the HBV core promoter (Fig. 5C, Supplementary Fig. 3G). Because si-
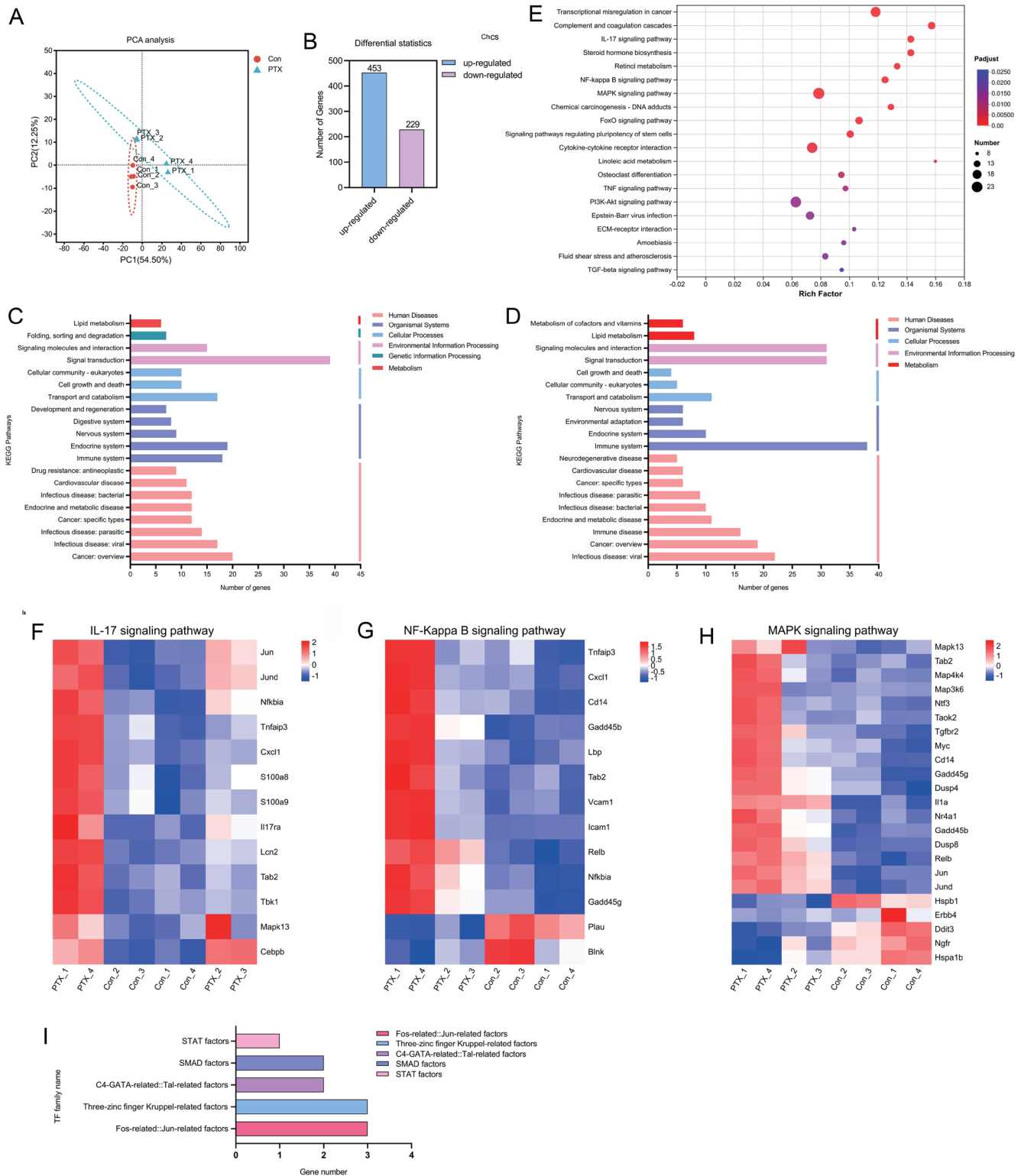
Fig. 3. Differentially expressed genes (DEGs) in the liver tissues of PTX-treated (PTX) mice and control (Con) mice. (A) Principal component analysis (PCA) of transcriptional analysis of liver tissue of mice in Con and PTX groups. (B) Statistical chart of significantly up- and down-regulated genes (P-value < 0.05 and at least twofold change) in Con vs PTX. (C–D) The distribution of Kyoto Encyclopedia of Genes and Genomes (KEGG) terms for different pathways assigned to significantly up-regulated and down-regulated genes. (E) The top 20 significantly changed pathways analyzed by KEGG pathway enrichment. (F–H) Heatmap of three immune-related pathways enriched in the DEGs. (I) Statistical chart of transcription factor family among the Con and PTX groups. PCA, Principal component analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.
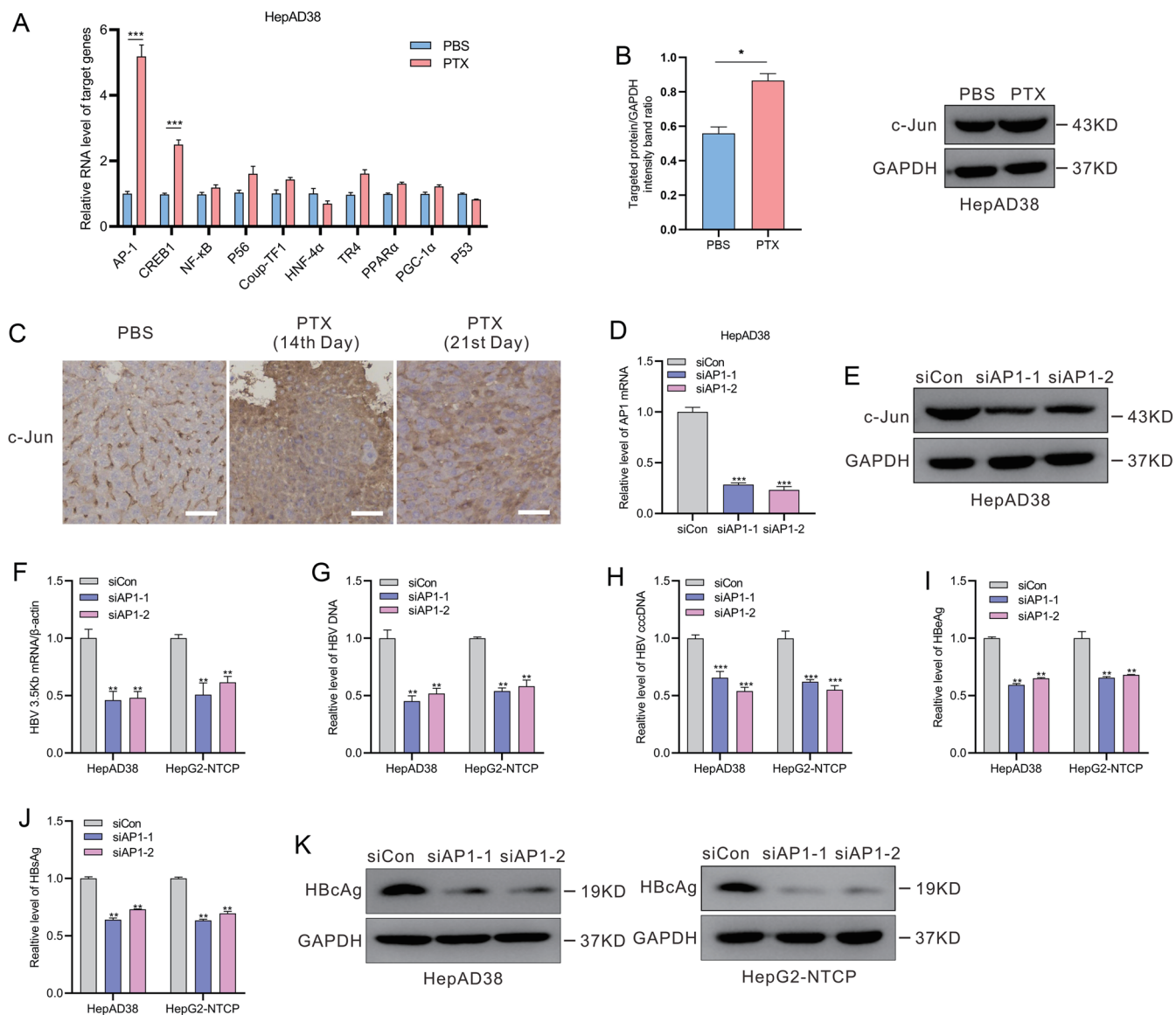
Fig. 4. Effect of AP-1 on HBV replication. (A) Gene expression of HBV replication-associated transcription factors in HepAD38 cells analyzed by qPCR. (B) Immunoblot analysis of c-Jun expression levels in HepAD38 cells after PTX treatment. Relative levels of c-Jun were measured by densitometry. (C) Immunohistochemical staining of c-Jun in the liver tissue (scale bar: 60 μm). (D–E) Transcription and protein expression of AP-1 in HepAD38 cells following AP-1 silencing. (F–H) qPCR analysis of 3.5-kb mRNA, HBV DNA, and HBV cccDNA expression levels following AP-1 silencing. (I–J) Quantitative analysis of ELISA for HBeAg and HBsAg levels in HepAD38 cells following AP-1 silencing. (K) Immunoblot analysis of HBcAg expression levels in HepAD38 and HepG2-NTCP following AP-1 silencing. Mean±SD values from 3 independent experiments are presented. *$p<0.05$, **$p<0.01$, ***$p<0.001$. PTX, paclitaxel; AP-1, activator protein 1; HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV cccDNA, HBV covalently closed circular DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

lencing of AP-1 inhibited HBV replication, we further explored whether PTX promotes HBV replication by modulating AP-1 activity and thereby promoting HBV replication. The promotion of HBV 3.5-kb mRNA, HBV DNA, and HBV cccDNA was significantly attenuated after targeted silencing of AP-1 (Fig. 5D–F, Supplementary Fig. 3H–J). The increased HBeAg and HBsAg secretion levels and HBcAg protein expression levels were also significantly ameliorated (Fig. 5G–I, Supplementary Fig. 3K–M). Moreover, we used pGEM-HBV1.3MUT plasmid containing mutated AP-1 binding site and found that mutation of the AP-1 binding site in the HBV core promoter abrogated PTX-induced elevation of HBV replication level (Fig. 5J–O). These findings suggested that PTX may enhance viral

replication by activating the binding of AP-1 to the HBV core promoter.

Dysbiosis of gut microbiota induced by PTX treatment

To assess the effects of PTX administration on the gut microbiota, we performed 16S rRNA sequencing. The results of the rarefaction curve of the Sobs index at the ASV level showed a leveling-off of the rarefaction curves of both the Con and PTX groups, with sufficient sequencing volume to cover the microbial communities in all samples (Fig. 6A). The analysis of α-diversity, as measured by the Chao1 index and Shannon index, showed no significant differences between the groups (Fig. 6B–C). The results of the hierarchical clustering tree
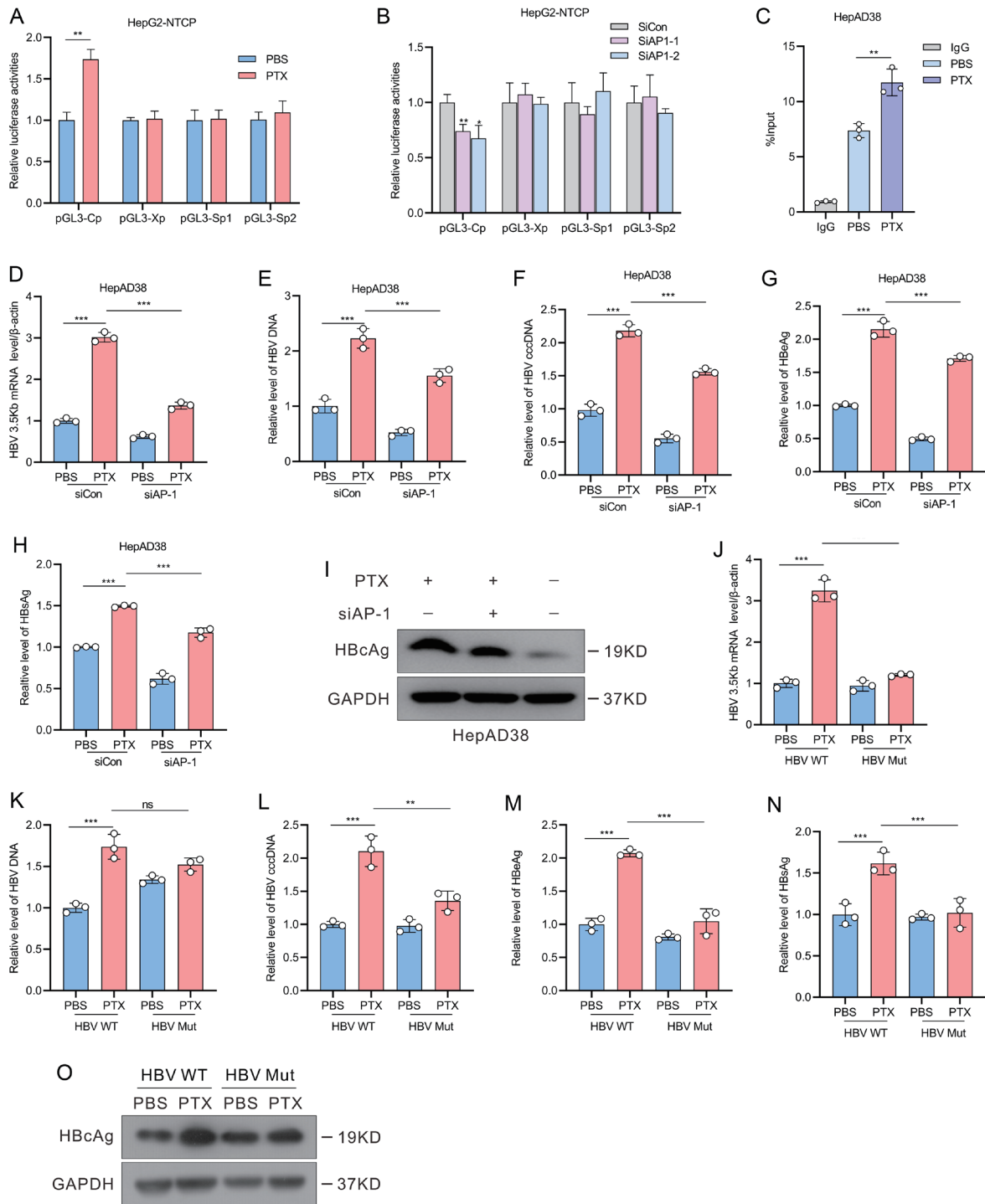
Fig. 5. PTX enhances viral HBV replication by activating the binding of AP-1 to HBV core promoter. (A) HepG2-NTCP cells treated with 4 µM PTX for 48 h after 12 h of transfection with HBV core, PreS1, PreS2, or X promoter luciferase reporter vectors. (B) Dual-luciferase reporter assays for detecting the effect of silencing AP-1 on HBV promoter activity. (C) CHIP-qPCR assay was used to detect the interaction between AP-1 and HBV core promoter. (D–F) Quantitative analysis of qPCR results showing the effect of silencing AP-1 on the PTX regulation of 3.5-kb mRNA, HBV DNA, and HBV cccDNA levels in HepAD38 cells. (G–H) ELISA analysis of the effect of silencing AP-1 in HepAD38 cells on PTX regulation of HBeAg and HBsAg levels. (I) Immunoblot analysis of HBcAg expression levels in AP-1 silenced HepAD38 cells. +, siAP-1 or PTX; − , siControl or DMSO. (J–L) HepG2 cells were transfected with pGEM-HBV1.3 or pGEM-HBV1.3MUT, and then treated with 4 µM PTX. qPCR assay was employed to quantify the levels of HBV 3.5-Kb mRNA, HBV DNA, and HBV cccDNA. (M–O) ELISA assays were used to detect HBeAg and HBsAg levels in the culture medium supernatant. (O) Immunoblot analysis of HBcAg expression levels in HepG2 cells. Mean±SD values from three independent experiments are presented. *$p<0.05$, **$p<0.01$, ***$p<0.001$. PTX, paclitaxel; HBV Cp, HBV core promoter; HBV Xp, HBV X promoter; HBV Sp1, HBV pre S1 promoter; HBV Sp2, HBV pre S2 promoter; AP-1, activator protein 1; HBV, hepatitis B virus; WT, wild type; Mut, mutant; HBcAg, hepatitis B core antigen; HbeAg, hepatitis B e-antigen; HbsAg, hepatitis B surface antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
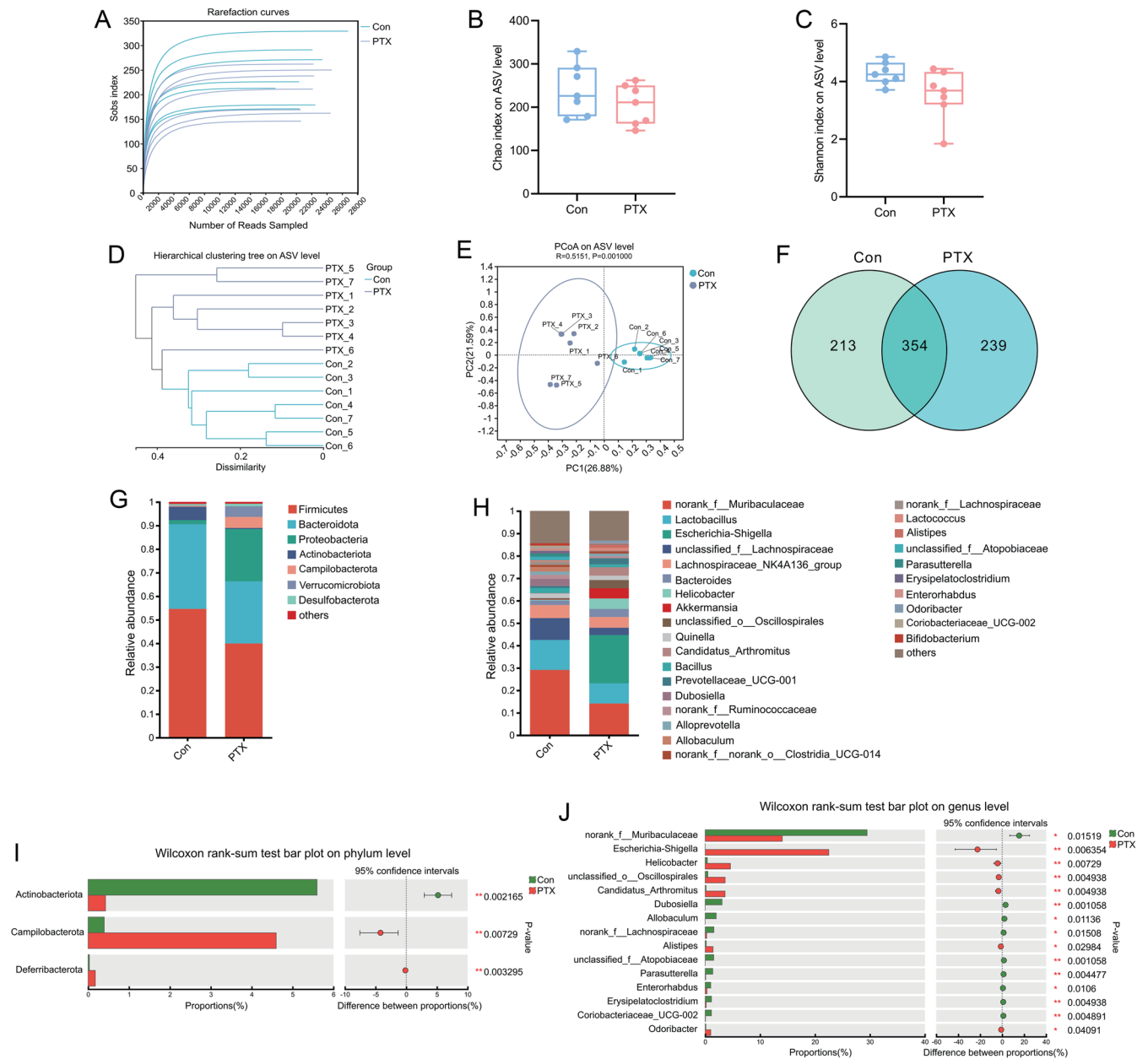
Fig. 6. Changes in the gut microbiota of HBV-Tg mice following treatment with PTX. (A) Rarefaction curve at the ASV level; (B–C) Chao index and Shannon index calculated at the ASV level; (D–E) The β-diversity of ASV-level microbial community was evaluated using hierarchical clustering (D) and principal coordinate analysis (PcoA) I. (F) Venn diagram of common and peculiar ASVs in the two groups. (G–H) The relative occurrence of bacteria at the phylum (G) and genus (H) levels. (I–J) Comparative analysis at the phylum level (I) and genus level (J). *p<0.05, **p<0.01. PCoA, principal coordinate analysis; Con, control; PTX, paclitaxel.

at the ASV level showed significant clustering of the colony structure in both Con and PTX groups (Fig. 6D). The PCoA plot (Fig. 6E), also built from the heterogeneity matrix using the Bray-Curtis index to explain the β-diversity, showed individual clustering between the groups, showing significant differences at the ASV level. These results indicated that PTX treatment altered the composition of the gut microbiota in HBV-Tg mice. A total of 354 ASVs were shared in both groups, while 213 and 239 ASVs were exclusively found in Con and PTX groups, respectively (Fig. 6F). The relative abundance of bacteria was assessed at both the phylum and genus levels (Fig. 6G–H). Using the Kruskal-Wallis rank-sum test, we

observed a significant increase in the relative abundance of *Campilobacterota* and *Deferribacterota* at the phylum level in the PTX group compared to the Con group. Conversely, the relative abundance of *Actinobacteriota* was significantly lower in the PTX group (Fig. 6I). At the genus level, the proportional representation of detrimental bacteria such as *Escherichia-Shigella*, *Helicobacter,* and *unclassified_o__Oscillospirales* was remarkably elevated in the PTX group, while the proportional representation of advantageous bacteria such as *norank_f__Muribaculaceae*, *Dubosiella*, *Allobaculum*, *norank_f__Lachnospiraceae*, and *Enterorhabdus* were considerably reduced.

Discussion

The persistence of HBV cccDNA within the nucleus of infected hepatocytes provides the virological foundation for HBV reactivation. Disruptions in the balance between viral replication and host immune control, resulting from alterations in either the virus or the immune system, can lead to HBV reactivation. Immunosuppression induced by antineoplastic drugs may impair control of HBV replication. It may also directly promote HBV replication, leading to HBV reactivation.[18] Immunomodulators, such as corticosteroids, not only inhibit the function of cytotoxic T-cells but also activate regulatory elements within the HBV gene in cultured human HCC cells, thereby promoting HBV replication.[19] Cisplatin-induced autophagy was found to enhance HBV replication by triggering the ROS/JNK pathway and inhibiting the Akt/mTOR signaling pathway.[20] The present study demonstrates that PTX may activate HBV core promoter activity, thereby enhancing HBV replication through the activation of the transcription factor AP-1. Furthermore, we conducted transcriptome sequencing and 16S rRNA sequencing to perform a comprehensive quantitative analysis of the changes in the transcriptome and microbial diversity of HBV-Tg mice following PTX treatment, which may reduce host resistance to HBV infection.

PTX is a class of cytotoxic drugs commonly used in the management of diverse cancer types, including advanced ovarian cancer, metastatic breast cancer, and non-small cell lung cancer.[21] It is often used in combination with other chemotherapeutic agents or hormones. PTX has been reported as one of the chemotherapeutic agents associated with HBV reactivation.[22] Trastuzumab in combination with PTX was found to increase the risk of HBV reactivation.[23] A case report documented PTX-induced HBV reactivation in a breast cancer patient who eventually died due to severe liver and kidney failure.[11] However, there is no clear consensus on whether HBV reactivation occurs as a result of the underlying disease itself, the PTX treatment, or the interaction between the two factors.[4] In the present study, PTX was found to directly promote HBV replication *in vitro* in a concentration-dependent manner. The *in vivo* study using HBV-Tg mice also suggested that PTX promotes HBV reactivation.

In the present study, PTX treatment increased the activity of the HBV core promoter. Further investigation identified AP-1 as a potential regulator of the HBV core promoter in response to PTX. The AP-1 transcription factor is composed of Jun proteins (c-Jun, JunB, and JunD), Fos proteins (c-Fos, FosB, Fra-1, and Fra-2), as well as ATF and MAF proteins, forming a dimeric complex.[24] Activation of AP-1 has been linked to different aspects of cancer biology, such as apoptosis, metastasis, cell growth, angiogenesis, invasion, and drug resistance.[25] The transcription factor AP-1 plays a crucial role in many aspects of liver biology. For instance, treatment with nitidine chloride was shown to induce mitochondrial damage and apoptosis in hepatocytes through the activation of the JNK/c-Jun signaling pathway.[26] Additionally, the histone methyltransferase SETD2 was found to induce the activation of c-Jun/AP-1 in the liver by promoting the accumulation of lipids, thereby contributing to hepatocellular carcinogenesis.[27] AP-1 has also been implicated in the pathogenesis of viral hepatitis,[28] as HBX interacts with Jab1 to enhance AP-1 activation.[29] AP-1 has been shown to bind to the Enh II/Xp region of the HBV genome.[17] Additionally, AP-1 binding motifs have been identified in the Enh II/Cp region of HBV.[30] In the present study, PTX-mediated activation of AP-1 had no significant effect on HBV X promoter activity. PTX-mediated HBV reactivation is a complex process, and the activity of the HBV X promoter may also be regulated by multiple factors, possibly by transcription factors other than AP-1. Our results offer additional support for the role of AP-1 in controlling HBV replication and its involvement in modulating the activity of the HBV core promoter upon PTX treatment.

For an in-depth characterization of the mechanism underlying the regulation of HBV by PTX, we performed additional investigations by integrating transcriptome and gut microbiota sequencing. Transcriptome sequencing results revealed significant changes in biological processes such as viral infectious diseases, immune system, and signal transduction after PTX treatment. We detected enrichment of immune response-related DEGs in the liver, such as the IL-17 signaling pathway, NF-KappaB signaling pathway, and MAPK signaling pathway,[31–33] which are critical modulators of the immune response. Previous studies have indicated that immunosuppressive agents or chemotherapy drugs can induce immune system dysregulation, leading to HBV reactivation. Transcriptome sequencing suggested that PTX not only directly promotes HBV replication but also enhances HBV replication by inducing immune dysfunction and inflammatory responses. PTX-induced HBV reactivation may be a combined result of immunosuppression and direct enhancement of HBV replication. Additionally, our analysis of transcription factor families in liver DEGs revealed a concentration of Fos related::Jun related transcription factor families, which were consistent with our *in vitro* results. The gut microbiota has a pivotal influence on the maintenance of intestinal homeostasis and liver health via the intricate interplay of the gut–liver axis,[34] and dysbiosis of the gut microbiota is associated with immune imbalance.[35] Emerging research has revealed a correlation between dyshomeostasis of gut microbiota and the onset and progression of viral hepatitis.[36] We observed significant differences between the PTX and Con groups with respect to the microbial structure and composition within the colon. The relative occurrence of harmful bacteria was significantly increased in the PTX group, while the relative occurrence of beneficial bacteria was significantly decreased. This suggested that PTX-induced dysbiosis of the gut microbiota may be one of the reasons for immune imbalance, ultimately leading to enhanced HBV replication.

Conclusion

In this study, PTX treatment was found to directly promote HBV replication and transcription, leading to HBV reactivation in HBV stable expression cell models, HBV natural infection cell models, and HBV transgenic mouse models. Further molecular analysis indicated that PTX may enhance HBV replication and transcription through the promotion of HBV core promoter activity mediated by the transcription factor AP-1. Furthermore, PTX treatment was found to induce immune system dysregulation and dysbiosis of the gut microbiota, which may potentially contribute to the induction of HBV reactivation. However, it is important to acknowledge that our experimental models may not fully replicate the complex dynamics of HBV infection and reactivation observed in clinical patients. Therefore, more comprehensive and in-depth research is required to provide references for the development of prevention and treatment strategies for clinical HBV reactivation.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceptualization and study design were conducted by JG, LC, and XL. AR was responsible for developing the methodology structure. Data acquisition was performed by SC, BL, and QY. SC, BL, and WL were also involved in data processing and interpretation. The manuscript was drafted by SC, MH, and SW. JG, LC, and XL provided materials and technical support. SC, WL, and QY critically revised the manuscript for important intellectual content. SC and SW supervised the study. All authors have made significant contributions to the article and have provided their consent for the publication of the final version.

Ethical statement

The animal study conducted in this research was subjected to a thorough review and approval process by the Laboratory Animal Center of Chongqing Medical University (IACUC-CQMU-2023-0342). The study followed the prescribed national protocols and regulations in China regarding the utilization of experimental animals and their well-being.

Data sharing statement

The raw data presented in the study have been deposited in the NCBI Sequence Read Archive (Bioproject ID:PRJNA1081780 and PRJNA1081628). All other data used in the study are available from the corresponding author upon reasonable request.

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