Article

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# HBx Regulation on HBV Pregenome Promoter in the Episomal Form Versus the Integrated Form

Hui Ding[1,†], Shuang Hu[1,†], Huihui Zhu[1], Ziyang Liu[1], Yuan Li[2], Jianping Liu[2], Xiaofang Li[1], Shuangyin Han[1], Suofeng Sun[1,*]

[1]Department of Gastroenterology, People's Hospital of Henan University, Henan Provincial People's Hospital, 450003 Zhengzhou, Henan, China
[2]Department of Traditional Chinese Medicine, The Third Affiliated Hospital Affiliated of Henan University of Traditional Chinese Medicine, 450003 Zhengzhou, Henan, China
*Correspondence: sunsuofeng1982@yeah.net (Suofeng Sun)
[†]These authors contributed equally.
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**Background: Hepatitis B virus (HBV) genome structure is an incomplete closed double stranded circular DNA and it uses covalently closed circular DNA (cccDNA) as template for replication. To study the antiviral effect on different HBV replication forms, a stable cell line expressing HBV using Huh7 cells with shuttle plasmid to imitate the real HBV replication form was stablished. Unlike the HepG2.2.15 cells, the replication of HBV-expressing Huh7 cells present significant decrease after 9 days of interferon-$\alpha$ (IFN-$\alpha$) treatment. This study aimed to verify whether hepatitis B virus X (HBx) epigenetic regulation by HBV promoter is affected by the DNA form and discuss the differences between the episomal form and the integrated form.**
**Material and Methods: Huh7 cells were used with two different plasmids containing HBV genome to imitate HBV-expressing cells with the episomal form and the integrated form. Luciferase reporting system was used to determine the activation of the promoter after treatment with IFN-$\alpha$ with different concentrations and promoter regulation factor HBx. HBx-expressing plasmid was transfected to evaluate its effect on HBV replication in the episomal form. HBV DNA and pregenomic RNA (pgRNA) in HBx knockdown cell line was determined and HBx-expressing plasmid was transfected to evaluate its effect on HBx in the episomal form.**
**Results: The two cell lines were established successfully and used for further experiments after selection. IFN-$\alpha$ showed significant inhibition effect on HBV pregenome promoter in the episomal form DNA while was not observed in the integrated form. After HBx-expressing plasmid was transfected, HBV pregenome promoter activity was higher in the episomal form rather than the integrated form. HBx showed a concentration-dependant activation on HBV replication in the episomal form. HBx knockdown reduced HBV production and HBV concentration significantly increased after transfection by HBx-expressing plasmid.**
**Conclusions: HBx regulation effect on HBV pregenome promoter is influenced by the HBV genome form. The epigenetic regulation effect on HBV pregenome promoter is more active in the episomal form rather than the integrated form.**

**Keywords:** HBV; HBx; pregenome promoter; episomal form; integrate form

## Introduction

Chronic hepatitis B caused by the persistent presence of the hepatitis B virus (HBV) infection is seriously harmful. Every year, about 400 million people are chronically infected worldwide, from which 600000 people die of HBV infection related liver cirrhosis, liver failure and primary liver cancer among other diseases [1,2]. At present, clinical therapeutic drugs including interferon (IFN) and nucleoside (acid) analogue (NAs), can effectively inhibit HBV transcription or replication, but cannot eliminate HBV. In addition, IFN has some problems, such as low treatment response rate or low antigen negative conversion rate. IFN is one of the most used drugs in clinical treatment for chronic HBV infection. It can inhibit the HBV reproduction, promote the Hepatitis B virus e antigen (HBeAg) negative conversion and serum conversion, reduce alanine aminotransferase (ALT), and even decrease liver cancer risk. However, IFN overall clinical efficacy to treat hepatitis B is not very satisfactory. The sustained and effective treatment effect is only found in about 1/3 of the patients with hepatitis B and the side effects associated with this treatment limits its clinical application [3].

HBV viral envelope wraps up the nucleocapsid, which contains part of the double-stranded relaxed circular DNA (rcDNA) genome [4]. In the HBV-infected hepatocytes cytoplasm, nucleocapsids are transported via microtubule-mediated nuclear pore complexes. After capsid breakdown, rcDNA is released into the nucleus and converted by host factors into covalently closed circular DNA (cccDNA), forming a stable minichromosomes [5,6]. In HBV-infected liver cells, the uncoated viral genome is transported

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to the nucleus and converted into covalently closed circular DNA, which is a stable form of the viral genome and serves as the template for viral transcripts synthesis. cccDNA is transcribed into pregenomic RNA (pgRNA). And pgRNA is translated into HBV DNA polymerase and nucleocapsid protein, and then used as template for rcDNA synthesis. HBV pgRNA can be used not only as messenger RNA (mRNA) encoding core protein and polymerase protein, but also be packaged into nucleocapsids as an intermediate of pregenomic RNA [7]. The nucleocapsid with partially double-stranded HBV DNA is enveloped and the virion secreted. A part of the nucleocapsid is recycled into the nucleus and the rcDNA is converted to cccDNA. Although the viral episomal genomic cccDNA serves as a template for transcription, it cannot be integrated into its formation [8,9]. HBV cccDNA is the template for transcription of all viral mRNAs and accumulates as a stable episome in the nucleus of infected cells. It is organized into minichromosomes by histone and nonhistone viral and cellular proteins. HBV-encoded regulatory protein hepatitis B virus X protein (HBx) behaves as a promiscuous transactivator of viral and cellular promoters. The HBx regulatory proteins produced in the HBV replicating cells are recruited by the cccDNA mini-chromosome. HBx recruiting kinetics by cccDNA are similar to HBV replication, suggesting that HBx may interact with cccDNA to regulate its transcription. Collectively, these results indicate that HBx may promote HBV oncogenicity by regulating cccDNA pgRNA transcription [10,11]. Hence, a new HBV-related hepatocellular carcinoma (HCC) model was established by transfecting the hepatoma cell line Huh-7 with lentivirus expressing HBx [11–15].

The HepG2.2.15 cells containing two integrated head-to-tail copies of the genotype D and stably replicating the HBV genome [16] are currently used to study HB replication cycle and evaluate the effect of antiviral compounds [17,18]. HBV genome structure is an incomplete closed double stranded circular DNA. Thus, in a previous study, we established a stable cell line according to shuttle plasmid pBE2-Multi, which can stabilize and replicate HBV genome [19]. In this study, IFN-$\alpha$ treatment for 9 days on pEB-HBCe cells resulted in a significant HBV inhibition of HBV DNA supernatants. In contrast, same treatment on Hep2.2.15 cells showed no anti-HBV effect. We presumed that the regulation of HBV promoter activities may be influenced by the DNA forms. Furthermore, IFN's effects on epigenetic regulation of HBV promoter may be preferentially involved in the episomal form but not in the integrated form of the viral DNA. The above discussion shows that HepG2.2.15 is different from our new cell line. In our new pEB cell line, the HBV DNA is transcribed independently of the chromosome by using the HBV self promoter, but in HepG2.2.15 cell line, the HBV gene chimerism in the chromosome is transcribed by using the chromosome promoter.

## Material and Methods

### *Plasmid Construction*

To verify whether the effect of HBx on HBV pregenome promoter show differences between the episomal form and the integrated form, we constructed a plasmid using two different vectors. The pEB-multi-puro plasmid was used to simulate episomal form promoter and the pFC-MCS-pGK-RFP-puro plasmid was used to simulate the integrated form promoter. Both plasmids can produce HBV because of the HBV pregenome promoter sequence for HBV replication promoter. Renilla sequence on the plasmid used to evaluate plasmid transcription by luciferase reporter system was used. We also constructed a plasmid express HBx. This plasmid was used to assess HBx effect on HBV pregenome promoter among the episomal form and the integrated form.

### *Cell Culture, Transfection and Stable Cell Lines Stablishment*

Huh7 liver cancer cells of human origin were purchased from ATCC (American type culture collection) and cultured by Dulbecco's Modified Eagle medium (11965092, DMEM, Gibico, NY, USA). They were supplemented with 10% fetal bovine serum (16140071, FBS, Gibico, NY, USA). Two 10 cm dishes of Huh7 cells were transient transfected by pEB-HBV-promoter plasmid and pFC-HBV-promoter plasmid respectively using lipo3000 (L3000075, invitrogen, Carlsbad, CA, USA). The medium was changed and treated with 5 $\mu$g/mL puromycin after 2 days of transfection. After 3 weeks of selection using puromycin, transfected Huh7 cells showed a stable expression and were ready for next experiment. Mycoplasma testing was performed on the cell line used.

### *Western Blot*

Cells were lysed, gels were prepared using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After 1 h, the membrane transfer stopped, and anti-HBc antibody was prepared using Hepatitis B core (HBc) rabbit protein, Hepatitis B surface (HBs) antibody (2AHB16, Immunology Institute, Tokyo, Japan), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-47724, Santa Cruz Biotechnology, Carlsbad, CA, USA), it was subjected to immunization and biological expression. After washing by tris buffered saline with tween (TBST), they were incubated with horseradish peroxidase (HRP)-labeled second antibody (7076P2, Cell Signaling Technology, Danvers, MA, USA) for 1 h. The dilution ratio of the first antibody was 1:1000, and the dilution ratio of the second antibody was 1:1000 at 5% nonfat milk. The antigen antibody complex was determined by chemidoc imaging system (Doc$^{TM}$ XR+ 1708195, bio rad laboratory, Tokyo, Japan). Image J v1.8.0.112 (National

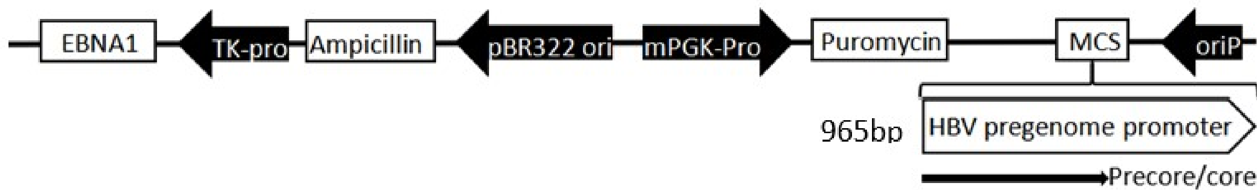


**Fig. 1. pEB-HBV-expressing plasmids construction.** The master vector contained the oriP-EBNA1 system and a puromycin resistance gene. HBV pregenomic promote genome insertion into multiple cloning sites.

Institutes of Health, Bethesda, MD, USA) was used to measure western blot bands grayscale.

### *DNA and RNA Quantification*

HBV DNA in transfected cells culture supernatant was treated with PNE solution (8.45% polyethylene glycol (PEG), 0.445 mol NaCl, 13 mmol EDTA) in ice for 1 h, and incubated with Dnase I (2270A, TAKARA, Shiga, Japan) and Rnase (2158, TAKARA , Shiga, Japan) at 37 °C for 1 h. Microspheres were treated with proteinase K at 56 °C for 12 h, and HBV DNA was isolated by phenol/chloroform extraction-ethanol precipitation method. HBV DNA copy number was determined by quantitative polymerase chain reaction (qPCR). To quantify HBV 3.5 kb pgRNA, total RNA was extracted from HBV transfected cells using total RNA extractor (TRI) reagent (TR 118, Molecular Research Center, Cincinnati, OH, USA). After treatment, using Dnase I and Rnase inhibitor, cDNA template was synthesized. Unspliced 3.5 kb RNA was quantified by qPCR using Synergy BrandsSynergy Brands (SYBR) qPCR Mix kit (QKD-201T, Toyobo, Osaka, Japan). The thermal cycling conditions comprised 1 min at 95 °C, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 30 sec. Primer sequences used in this study were: 5′-TCCCTCGCCTCGCAGACG-3′ and 5′-GTTTCCCACCTTATGAGTC-3′ for unspliced 3.5 kb RNA, and 5′-TTCTACAATGAGCTGCGTGTG-3′ and 5′-GGGGTGTTGAAGGTCTCAAA-3′ for $\beta$-actin mRNA.

### *Statistical Analysis*

SPSS 22.0 (IBM SPSS statistics, Chicago, IL, USA) statistical software was used for statistical analysis. Independent sample $T$-test was used to assess differences between two groups and One-Way ANOVA (analysis of variance) was used to assess differences between multiple groups. $p < 0.05$ was considered statistically significant difference.

## Results

### *Construction of HBV Promoter Plasmids Includes the Episomal Form and the Integrated Form*

Firstly, plasmids with two different vectors were constructed. One of the plasmids used pEB-multi-puro vector as pEB-HBV-promoter plasmid to establish episomal HBV pregenome promoter cell line and the other one used pFC-MCS-pGK-RFP-puro vector as pFC-HBV-promoter plasmid to establish the integrated HBV pregenome promoter cell line. Both plasmids contained HBV pregenome promoter sequence and renilla sequence. By sequencing, it was confirmed that the plasmids were successfully constructed (Fig. 1).

### *pEB-HBV-Promoter Plasmid-Based Cell Line and pFC-HBV-Promoter Plasmid-Based Cell Line Establishment*

The previously constructed plasmids were transfected to Huh7 cells. Then, they were selected by puromycin to establish stable cell lines for next experiments. Renilla luciferase activity was determined using a luciferase reporter system to verify pEB-HBV-promoter plasmid-based cell line establishment and pFC-HBV-promoter plamid based cell line. After being treated with increasing concentrations of 0, 2.5, 5 $\mu$m interferon-$\alpha$, the luciferase reporter system demonstrated that renilla luciferase activity presented no significant difference between the interferon treatment group and the control group in the pFC-HBV-promoter plamid based cell line (Fig. 2a). In addition, renilla luciferase activity and interferon-$\alpha$ concentration had a significant negative correlation ($p < 0.05$) in pEB-HBV-promoter plasmid-based cell line (Fig. 2b). The results showed that IFN-$\alpha$ present can significantly inhibit HBV pregenome promoter in the episomal form rather than the integrated form.

### *HBx Effect on HBV Pregenome Promoter in Different Cell Lines*

To evaluate the activation effect on pEB-pregenome promoter and pFC-pregenome promoter of different proteins, plasmids expressing HBx, HNF4a, CEBP-b were separately transfected to two previously established cell lines. The empty plasmid was transfected as negative control. Then, pEB-pregenome promoter and pFC-pregenome promoter activation effect was evaluated detecting the promoter relative protein expression. Protein presented a notable higher expression ($p < 0.05$) after HBx plasmid transfection in the cell line expressing pEB-pregenome promoter (Fig. 3a). However, there was no significant expression

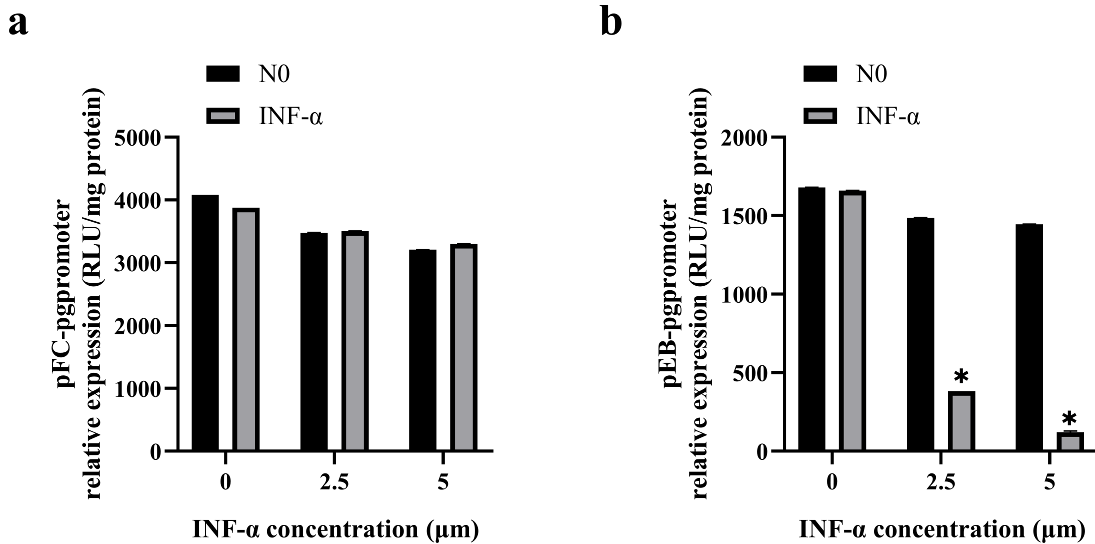


**Fig. 2. Effects of IFN-$\alpha$ on HBV pregenomic promoter in fragment DNA and episomal DNA form.** After being treated with increasing concentration of 0, 2.5, 5 $\mu$m interferon-$\alpha$, (a) there is no significant difference in the integrate DNA form expression depending on interferon-$\alpha$ concentration. (b) HBV pregenome promoter expression decreased significantly in the episomal DNA form in 2.5 and 5 $\mu$m interferon-$\alpha$ concentration. $^{*}p < 0.05$.

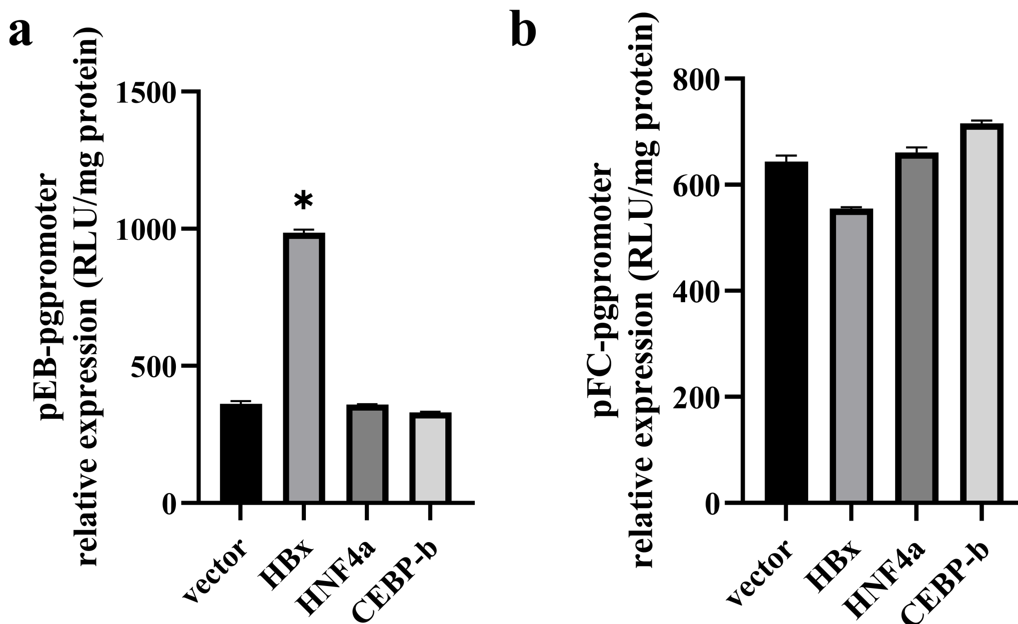


**Fig. 3. Effects of HBx on HBV pregenomic promoter in fragment DNA and episomal DNA form.** After transfection by different plasmids, separately, into previously established cell lines, (a) HBV pregenome promoter expression after transfection by HBx-expressing plasmid is significantly higher compared to the others in the episomal form (b) and there is no significant expression difference in the integrate form. $^{*}p < 0.05$.

difference in the cell line expressing pFC-pregenome promoter (Fig. 3b). HBx presented a promotion effect on HBV pregenome promoter. HBx activation was observed in the episomal form rather than the integrated form.

### *HBx Effect on HBV Replication Verification*

To verify HBx effect on HBV replication, pEB-HBCe cells were transfected with 0.3 $\mu$g, 0.6 $\mu$g, 0.9 $\mu$g HBx-expression plasmid, these were considered as experimental groups and non-HBx-expression plasmid was considered as negative control. Then HBx protein expression was confirmed by western blot. HBV DNA concentrations in su-

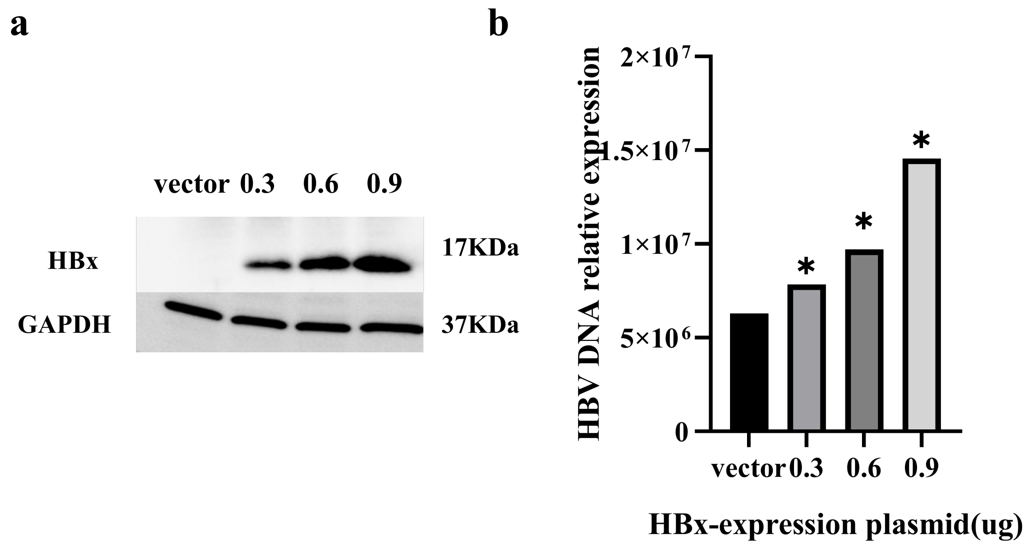


**Fig. 4. The vector plasmid and 0.3 $\mu$g, 0.6 $\mu$g, 0.9 $\mu$g HBx-expressing plasmid were transfected to pEB-HBCe cells respectively.** (a) HBx protein expression was confirmed by western blot. (b) HBV replication was determined by qPCR. HBV expression is positively correlated with HBx expression in the episomal form. $^{*}p < 0.05$.

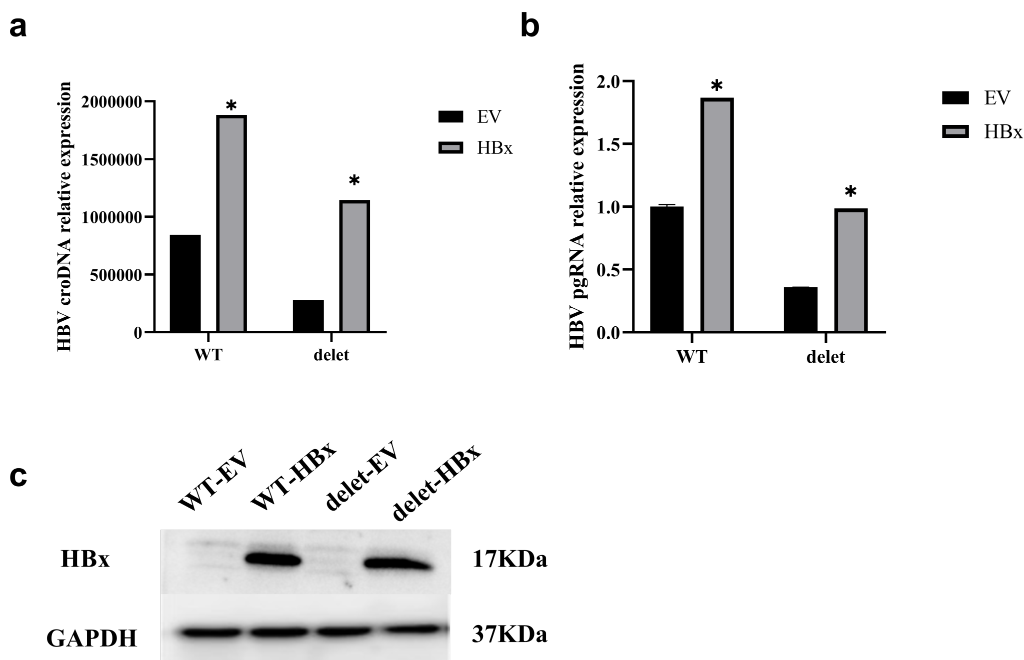


**Fig. 5. HBx-expressing plasmid and vector plasmid were transfected to HBx wild type cells and HBx delete cells.** HBV DNA (a) and viral pgRNA (b) levels in supernatant and cells were determined by real-time PCR. It was found that HBx presented significantly activated HBV replication in the episomal form. (c) HBx protein expression was confirmed by western blot. $^{*}p < 0.05$.

pernatant were tested. HBV replication was positively correlated with HBx expression ($p < 0.05$) (Fig. 4).

To further confirm that HBx showed a promotion effect on HBV replication in the episomal form, HBx was knocked down in HBV expressed Huh7 cell. Real-time PCR was used to determine HBV DNA and pgRNA in supernatant and cells. As expected, a decrease in HBx expression can effectively reduce HBV replication by detecting HBV core DNA and RNA concentration in supernatant compared with the negative control. Then, HBx plasmid and empty plasmid were respectively transfected in WT Huh7 cell and HBx-deleted Huh7 cell. By measuring the concentration of HBV core DNA and pgRNA in supernatant, the results showed that HBx significantly activated HBV replication in the episomal form again (Fig. 5a,b). HBx expression was confirmed by western blot (Fig. 5c).

## Discussion

HepG2.2.15 cells are stable and contain two complete HBV genome head-to-tail copies. Although there are obvious limitations, such as virus replication does not use cccDNA as template, but it is still widely used in cell HBV life cycle analysis and antiviral research. However, in hepatocytes infected with HBV, viral genomic cccDNA is used as a transcriptional template, but it is not integrated. Thus, to mimic HBV infected hepatocytes and study the antiviral effect, in the present study, a stable cell line based on episomal pEB-Multi vector was established to generate HBV genome. This study provides the basis for antiviral drugs evaluation and the study of their mechanism of action. The results indicated that the stable cell line based on episomal pEB-Multi vector is resistant to lamivudine and sensitive to interferon or entecavir. Furthermore, viral promoter activities regulation may be influenced by DNA forms. It is hypothesized that the effect of IFN on HBV epigenetic pregenome promoter regulation may be preferentially involved in HBV DNA in the episomal form rather than the integrated form.

In this study, two cell lines were established successfully and used for further experiments. IFN-$\alpha$ showed a significant inhibition effect on HBV pregenome promoter in the episomal form DNA while this was not observed in the integrated form. After HBx-expressing plasmid was transfected, HBV pregenome promoter activity was higher in the episomal form rather than the integrated form. HBx presented a concentration-dependant activation on HBV replication in the episomal form. HBx knockdown reduced HBV production and HBV concentration significantly increased HBx-expressing plasmid was transfected. HBx regulation effect on HBV pregenome promoter is influenced by HBV genome form. Epigenetic regulation effect on HBV pregenome promoter is more active in the episomal form rather than the integrated form. We used the pEB-multi-puro vector plasmid transfected into Huh7 to establish episomal replication form HBV cell line and the pFC-MCS-pGK-RFP-puro vector plasmid transfected into Huh7 to establish integrated replication form HBV cell line. Then, these two types of cell lines were treated with increasingly IFN-$\alpha$ concentration levels. Similar to previous studies, Huh7 cells expressing HBV were extremely sensitive to IFN-$\alpha$ when the HBV pregenome promoter was in the episomal form. Moreover, the inhibition effect to HBV pregenome promoter showed an IFN-$\alpha$ concentration dependent correlation. Same as HepG2.2.15 cells, Huh7 cells transfected with pFC-MCS-pGK plasmid were not sensitive due to the replication of the integrated form. The results showed that IFN-$\alpha$ had a significant influence on HBV pregenome promoter epigenetics regulation when the HBV genome is in the episomal form and this influence is dose-dependent inhibition.

Hepatitis B virus X (HBx) protein encoded by HBV gene plays an important role in chronic hepatitis B occurrence and development of, liver cirrhosis and hepatocellular carcinoma. In this study, we established a novel HBV-related HCC model by transfecting the hepatoma cell line Huh-7 with lentivirus expressing HBx. Then, IFN-$\alpha$ effect on the growth of cancer cells was evaluated to determine the potential of drugs to treat HBV-related HCC. HBx viral proteins were recruited onto cccDNA *in vivo*. HBx is required for HBV pregenomic RNA transcription. It enhances HBV replication [20]. As a result of HBx is crucial for HBV expression. HBx effect on HBV pregenome promoter in the episomal form and the integrated form was assessed to test and verify whether the regulation of HBV promoter activities may be influenced by DNA forms. The two previously established Huh7 cell expressed HBV cell lines were separately transfected into the HBx-expressing plasmid, HNF4a-expressing plasmid, CEBP-b-expressing plasmid and empty plasmid. HNF4a and CEBP-b are liver-enriched or widespread transcription factors that upregulate core promoter activity. Empty plasmids were used for negative controls. The results show that HBV pregenome promoter activity increase significantly after transfection by HBx-expressing plasmid in the episomal HBV-expressing cell line. There was not obvious HBx effect on HBV pregenome promoter activity in the integrated HBV-expressing cell line. To further verify HBx activation effect on HBV pregenome promoter, HBx-expressing plasmid was transfected with different expression levels into HBV-expressing Huh7 cell in the episomal form. This demonstrated that HBV expression is increased with increasing levels of HBx. Furthermore, HBV core DNA and pgRNA concentration was significantly reduced in HBx knockdown supernatant cell line. The concentration was significantly increased by HBx-expressing plasmid transfection in the episomal form. In a word, HBx presents a significant activation effect on HBV pregenome promoter when HBV genome is in the episomal form rather than the integrate form.

## Conclusions

In conclusion, HBx regulation effect on HBV pregenome promoter is influenced by HBV genome form. Moreover, HBV pregenome promoter effect on epigenetic regulation is more sensitive in the episomal form rather than the integrated form.

## Abbreviations

HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; IFN-$\alpha$, interferon-$\alpha$; HBx, hepatitis B virus X; pgRNA, pregenomic RNA; HBeAg, Hepatitis B virus e antigen; ALT, alanine aminotransferase; rcDNA, relaxed circular DNA; HBc, Hepatitis B virus core; HBs, Hepatitis B virus surface.

## Availability of Data and Materials

The dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Author Contributions

SFS and SYH—designed the research study; HD, SH, HHZ, ZYL, YL, JPL and XFL—performed the research; HD and SH—collected and analyzed the data; HD, SH and HHZ—has been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors give final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

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