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Letter

Sphingomyelin phosphodiesterase 3 as a novel host factor inhibiting hepatitis B virus transcription

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Dear Editor,

Hepatitis B virus (HBV) is a small, enveloped DNA virus and a member of the *Hepadnaviridae* family (Zhao et al., 2020). It is a major human pathogen causing chronic liver disease, leading to significant morbidity and mortality worldwide (Xia and Liang, 2019). According to the World Health Organization (WHO), an estimated 296 million people live with chronic HBV infection, contributing to around 820,000 deaths annually due to complications such as liver cirrhosis and hepatocellular carcinoma (HCC) (Easterbrook et al., 2021). Despite the availability of effective vaccines, HBV remains a global health challenge. Current antiviral therapies, including nucleos(t)ide analogs and interferons, can suppress HBV replication but do not eradicate the virus due to the persistence of covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes (Zhao et al., 2025). The presence of cccDNA, a stable episomal form of the viral genome, serves as a transcriptional template for viral mRNA and is the main obstacle to achieving a cure (Xia and Guo, 2020).

Recently, we discovered that sphingomyelin phosphodiesterase 3 (SMPD3) plays a critical role in regulating sphingolipid metabolism during liver development, with Mettl3-mediated m6A modification being key to controlling Smpd3 levels and ensuring normal liver maturation and growth (Wang et al., 2023). This highlights the importance of epitranscriptomic regulation in coordinating organ development and functional maturation. As the development of liver is crucial for HBV infection, the potential role of SMPD3 in HBV infection remains to be determined.

To examine the effect of SMPD3 on HBV replication, we first assessed its impact in Huh7-NTCP cells. We transfected these cells with small interfering RNA (siRNA) targeting SMPD3, followed by transfection with the pCMV-HBV plasmid. As shown in Fig. 1A, both siRNAs achieved significant knockdown at the mRNA and protein levels. SMPD3 downregulation was associated with increased levels of HBsAg, HBeAg, and HBV DNA in the cell culture supernatants (Fig. 1A). Additionally, intracellular HBV replication markers, including HBV RNA, and HBc, were elevated following SMPD3 knockdown (Fig. 1A). To further validate SMPD3's role in HBV replication, we co-transfected Huh7-NTCP cells with both the pCMV-HBV and pXJ40-HA-SMPD3 plasmids. As expected, SMPD3 overexpression significantly inhibited HBV replication markers, including HBeAg, HBsAg, HBV DNA, as well as intracellular HBc and HBV RNA (Supplementary Fig. S1). To further investigate the role of SMPD3, we transfected cells with varying concentrations of pXJ40-HA-SMPD3. The results showed that SMPD3 inhibits HBV replication in a dose-dependent manner (Fig. 1B) and that different concentrations of SMPD3 had no impact on cell viability (Supplementary Fig. S2). To validate these findings, we knocked down SMPD3 in HBV-replicating HepAD38 cells and observed that SMPD3 depletion promoted HBV replication (Fig. 1C). To test the effect in HBV infection model, we verified SMPD3's function using HBV-infected HepG2-NTCP cells. As expected, SMPD3 knockdown enhanced HBV RNA, antigen production, and progeny virus levels, though no changes were detected in HBV cccDNA by qPCR (Fig. 1D). We then established SMPD3-stably knocked-down HepG2-NTCP cells using lentiviral shRNAs, both of which significantly reduced SMPD3 mRNA levels (Fig. 1E). Consistent with siRNA-mediated knockdown, we observed significantly elevated levels of HBeAg, HBsAg, HBV DNA and intracellular HBV RNA. However, the levels of HBV cccDNA remained unchanged (Fig. 1E). Our data indicate that SMPD3 exerts an inhibitory effect on HBV at RNA level. To explore the mechanism, we first examined the effect on RNA stability. As shown in Fig. 1F, SMPD3 overexpression did not affect

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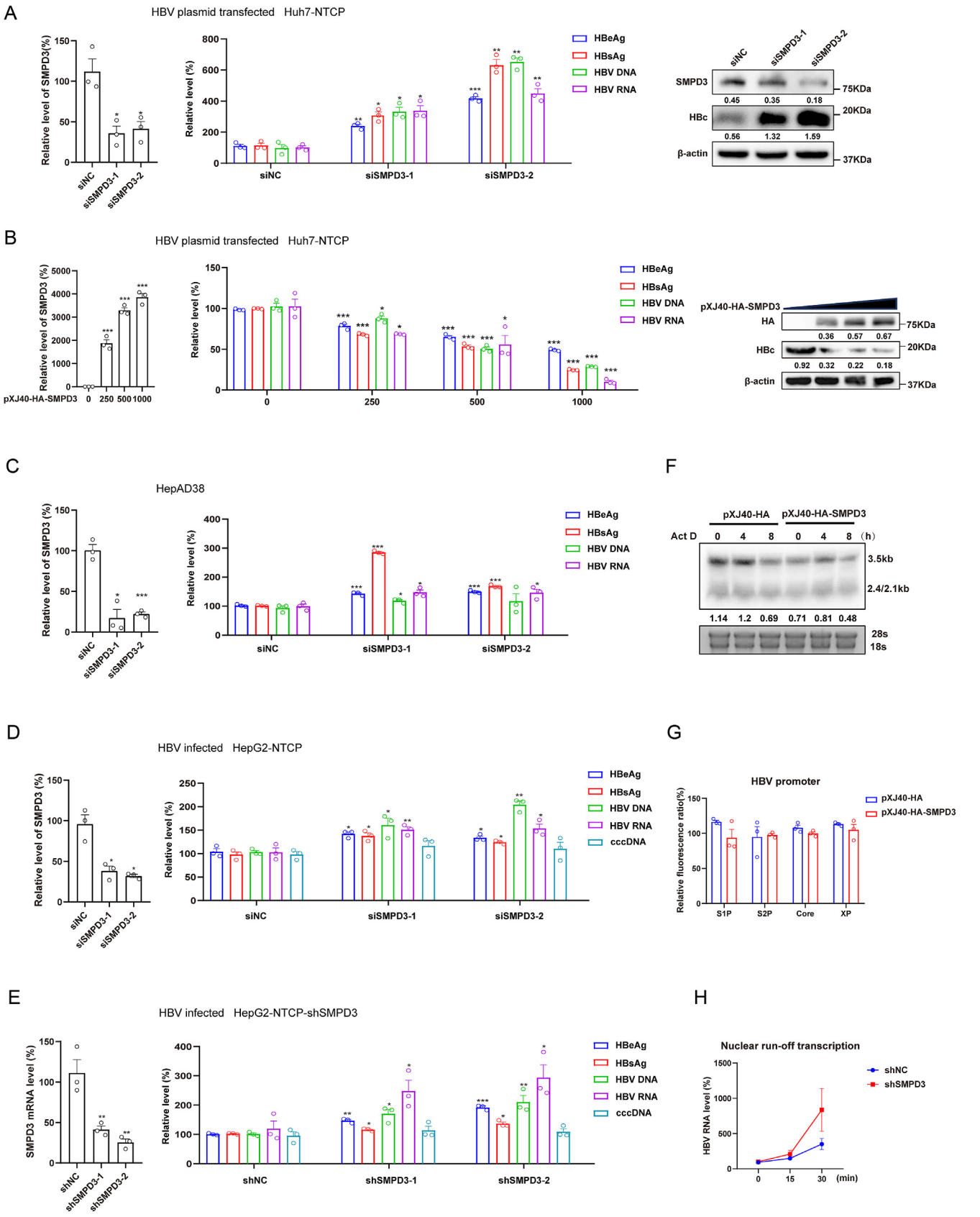

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Fig. 1. The effect of SMPD3 on HBV replication in cell cultures. **A** Huh7-NTCP cells were transfected with siRNA targeting SMPD3 in 24-well plates, and 12 hours post-transfection, transfected with pCMV-HBV plasmid, and then cells were harvested 7 days post-transfection. The expression of HBV RNA, SMPD3 was detected by qRT-PCR and Western blot, and the expression of HBc was detected. ELISA and qPCR were used to detect HBeAg, HBsAg, and HBV DNA secreted in culture supernatants. **B** Huh7-NTCP cells were transfected with different concentrations of pXJ40-HA-SMPD3 and pCMV-HBV plasmids in 24-well plates, and then the cells were harvested 7 days after transfection. The qRT-PCR and Western blot were used to detect the expression of SMPD3, HBV RNA and to detect the expression of HBc. ELISA and qPCR were used to detect the secreted HBeAg, HBsAg and HBV DNA in the culture supernatant. **C** HepAD38 cells were transfected with siRNA targeting SMPD3 in 24-well plates, and then the cells were harvested 7 days after transfection. HBeAg, HBsAg and HBV DNA secreted in culture supernatants were detected by ELISA and qPCR, and intracellular HBV RNA was detected by qRT-PCR. **D** HepG2-NTCP cells were transfected with siRNA targeting SMPD3 in 24-well plates, infected with HBV at a multiplicity of infection (MOI) of 100, 48 hours post-transfection, and then harvested seven days post-infection. Secreted viral antigens, HBV DNA in culture supernatants, intracellular HBV RNA, and HBV cccDNA were detected by ELISA, qPCR, and qRT-PCR. **E** HepG2-NTCP cells stably knocked down for SMPD3 were infected with HBV at an MOI of 100, and then harvested 7 days after infection. HBeAg, HBsAg and HBV DNA in supernatants and intracellular HBV RNA and HBV cccDNA were detected by ELISA and qRT-PCR. **F** Huh7-NTCP cells were co-transfected with SMPD3 expression plasmid and the pCMV-HBV plasmids. After plasmids were transfected for 72 h, cell was treated with Actinomycin D. Cells were collected at 0, 4, and 8 hours after add Actinomycin D, HBV RNA was detected by Northern blot. **G** Huh7-NTCP cells were co-transfected with SMPD3 expression plasmid and the HBV promoter luciferase reporter plasmids. After promoter plasmids were transfected for 48 h, cells were harvested. Firefly luciferase activity was measured. **H** A nuclear run-off transcription assay was used to detect the effect of SMPD3 on transcriptional elongation of HBV circle. HBV RNA was detected by qRT-PCR. Densitometry was performed with ImageJ software, expressed as the ratio of 3.5 kb (pgRNA) to 28 s/18 s. The protein levels were performed with ImageJ software and standardized against β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. cccDNA, covalently closed circular DNA; HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; MOI, multiple infection; pgRNA, pregenomic RNA; qPCR, quantitative PCR; qRT-PCR, the quantitative reverse transcription PCR.

HBV RNA stability. Furthermore, promoter assay demonstrated that HBV promoter activities were not affected (Fig. 1G, Supplementary Fig. S3). To further confirm the role of SMPD3 in HBV transcription, we conducted a nuclear run-on transcription assay, which revealed that SMPD3 knockdown increased HBV transcript levels *in vitro* (Fig. 1H). In conclusion, these findings suggest that SMPD3 functions as an antiviral host factor, inhibiting HBV transcription.

Our results suggest a mechanism that SMPD3 inhibits HBV transcription in a promoter independent manner. HBV transcription can be affected by mechanisms beyond the promoter, such as changes in enhancers, transcription factor binding sites, chromatin structure, RNA polymerase pausing, gene body methylation, regulatory non-coding RNAs, structural DNA variants, and splicing regulatory elements (Xia and Guo, 2020; Teng et al., 2021; Xia et al., 2023). These elements influence transcription efficiency, elongation, or gene accessibility, highlighting the complexity of gene regulation that extends far beyond the promoter region itself.

The SMPD3 gene encodes neutral sphingomyelinase 2, a member of the sphingomyelinase family that hydrolyzes sphingolipids to produce ceramides (Kim et al., 2008; Matsuzaka et al., 2020). Studies indicate that ceramide restricts viral replication in human lung epithelial cells, where influenza A virus induces ceramide biosynthesis *de novo* (Soudani et al., 2019). Overall elevation of ceramide by bacterial sphingomyelinase interferes with hepatitis C virus uptake at the level of receptor isolation (Voisset et al., 2008). Current findings suggest that the ceramide signaling pathway regulated by the SMPD3 gene may significantly influence viral replication and transmission (Stoffel et al., 2016; Zhou et al., 2019). Revill et al. (2013) demonstrated that SMPD3 acts as a potential suppressor of hepatocellular carcinoma through a genome-wide study, revealing that knockdown of SMPD3 promotes cell invasion and migration *in vitro* and enhances tumor formation *in vivo*. Furthermore, SMPD3 has been associated with various cancers, including leukemia, adenocarcinomas, and hepatocellular carcinomas.

Our study suggests that SMPD3 acts as a suppressor of HBV transcription, providing a novel layer of viral-host interaction. HBV transcription is tightly regulated by host cellular factors, and the involvement of lipid signaling pathways adds a new dimension to understanding viral persistence and replication. By generating ceramide, a bioactive lipid molecule known to participate in various signaling pathways, SMPD3 may create a cellular environment less favorable for HBV RNA synthesis. The suppression of HBV transcription by SMPD3 could be attributed to ceramide's role in modulating nuclear receptors, transcription factors, or chromatin structure that are crucial for HBV cccDNA activity. This opens up potential therapeutic avenues targeting SMPD3 or its downstream lipid mediators to control HBV infection, particularly in the context of persistent HBV, where transcriptional control is vital for disease management and reducing viral replication. Future research focusing on the specific molecular mechanisms by which SMPD3 influences HBV transcription could unveil new strategies for antiviral therapies.

FOOTNOTES

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