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Active HBV replication in hypoxic pericentral zone 3 is upregulated by multiple host factors including HIF-1α

To the Editor:

With great interest, we read the manuscript "Hypoxia inducible factors regulate hepatitis B virus replication by activating the basal core promoter" by Wing *et al.* published in the *Journal of Hepatology*.[1] The authors identified 2 conserved hypoxia response elements (HREs) within the HBV genome, through which stabilized hypoxia inducible factor-1α (HIF-1α) bound to HBV DNA, activated the basal core promoter, and prompted HBV replication under hypoxic conditions. Physically, cross sections of liver lobules from the portal vein (PV) to the central vein (CV) could be arbitrarily divided into 3 concentric zones, including periportal zone 1, midlobular zone 2 and hypoxic pericentral zone 3,[2] and it would be interesting to explore whether the physically formed lower oxygen tension in zone 3 would be more adaptive for HBV replication. Herein, we aimed to provide more evidence for the promotion of HBV replication by hypoxia and HIF-1α, and search for other factors involved in the upregulation of HBV replication under hypoxic conditions.

To provide extra experimental evidence supporting the role of HIF-1α in HBV replication, precursor recombinant covalently closed circular HBV DNA (prcccDNA)/pCMV-Cre plasmids were co-transfected with HIF-1α expression plasmid (or vector control) into HepG2 cells for 72 h, then levels of HBcAg, supernatant HBV DNA and HBV RNA were detected. The increased levels of HBcAg protein, supernatant HBV DNA and supernatant HBV RNA further confirmed that HIF-1α could indeed prompt HBV replication (Fig. 1A). It has been reported that HBx could also stabilize HIF-1α by preventing its ubiquitin-dependent degradation, independently of hypoxia.[3] We next constructed HBx-depleted prcccDNA plasmid (prcccDNAΔHBx) by nonsense mutation at the second ATG within the HBx open reading frame, and co-transfected prcccDNAΔHBx/pCMV-Cre with HBx expression plasmid (or vector control) into HepG2 cells for 72 h, before measuring the levels of HIF-1α protein and indicators of active HBV replication. It came out that HBx could stabilize HIF-1α at the protein level and synergistically increase the levels of HBcAg protein, supernatant HBV DNA and HBeAg, indicating the involvement of HIF-1α in the upregulation of HBV replication induced by HBx (Fig. 1B). Taken together, HIF-1α could upregulate HBV replication, which further supported Wing *et al.*'s research.

The HBeAg-positive chronic HBV infection (EPI) phase is characterized by higher HBV replication due to a lack of host immune responses,[4] and is a suitable stage at which to measure the real influence of hypoxia on HBV replication. Therefore, liver samples from 38 chronic HBV-infected individuals within the EPI phase were collected from Beijing 302 Hospital and stained for HBcAg by immunohistochemistry (Table S1). In total, 158 zonated structures were recognized and it was found that the percentage of HBcAg-positive hepatocytes was significantly zonated, and was higher in hypoxic pericentral zone 3 (16.5%) than in midlobular zone 2 (7.0%) or periportal zone 1 (5.1%) (Fig. 1C), which further verified the upregulation of HBV replication induced by hypoxia.

Since the cellular gene expression profile was zonated within 3 zones,[5] we wonder whether the expression pattern of other host factors could also contribute to zonated HBV replication seen above. The single cell RNA-sequencing dataset (GSE115469) was downloaded for further analysis. According to the expression profile of established cell-specific marker genes, there were 639 cells clustered into periportal zone 1, 103 cells for midlobular zone 2 and 2,570 cells for pericentral zone 3 (Fig. S1A). Given that HIF-1α was stabilized at the protein level rather than the transcriptional level, we further compared the expression pattern of *SLC10A1*, *CTNNB1* and *HNF4A* within 3 zones (Fig. S1B-D). The level of sodium taurocholate cotransporting polypeptide (NTCP) encoded by *SLC10A1* was higher in zone 3 than in zone 2 or zone 1 (Fig. 1D, left panel). NTCP is a multiple transmembrane transporter and participated in the enterohepatic circulation of bile salts.[6] Physically, bile salts were mainly removed by periportal zone 1 hepatocytes, and pericentral zone 3 hepatocytes were recruited at higher bile salt loads,[7] thus higher expression of NTCP in zone 3 may guarantee the complete absorption of bile salts. Meanwhile, NCTP was reported as the functional receptor of HBV,[8] therefore its zonated expression pattern may support HBV infection in hypoxic pericentral zone 3. Apart from *SLC10A1*, the expression levels of *CTNNB1* and *HNF4A* were also higher in zone 3 than in zone 1, showing a similar pattern to each other (Fig. 1D, middle and right panel). Since the Wnt/β-catenin pathway and HNF-4α were both reported to promote HBV replication,[9,10] their zonated expression pattern may also contribute to the upregulation of HBV replication in pericentral zone 3.

Keywords: HBV replication; hypoxia; HIF-1α; NTCP; β-catenin; HNF-4α; liver zonation.
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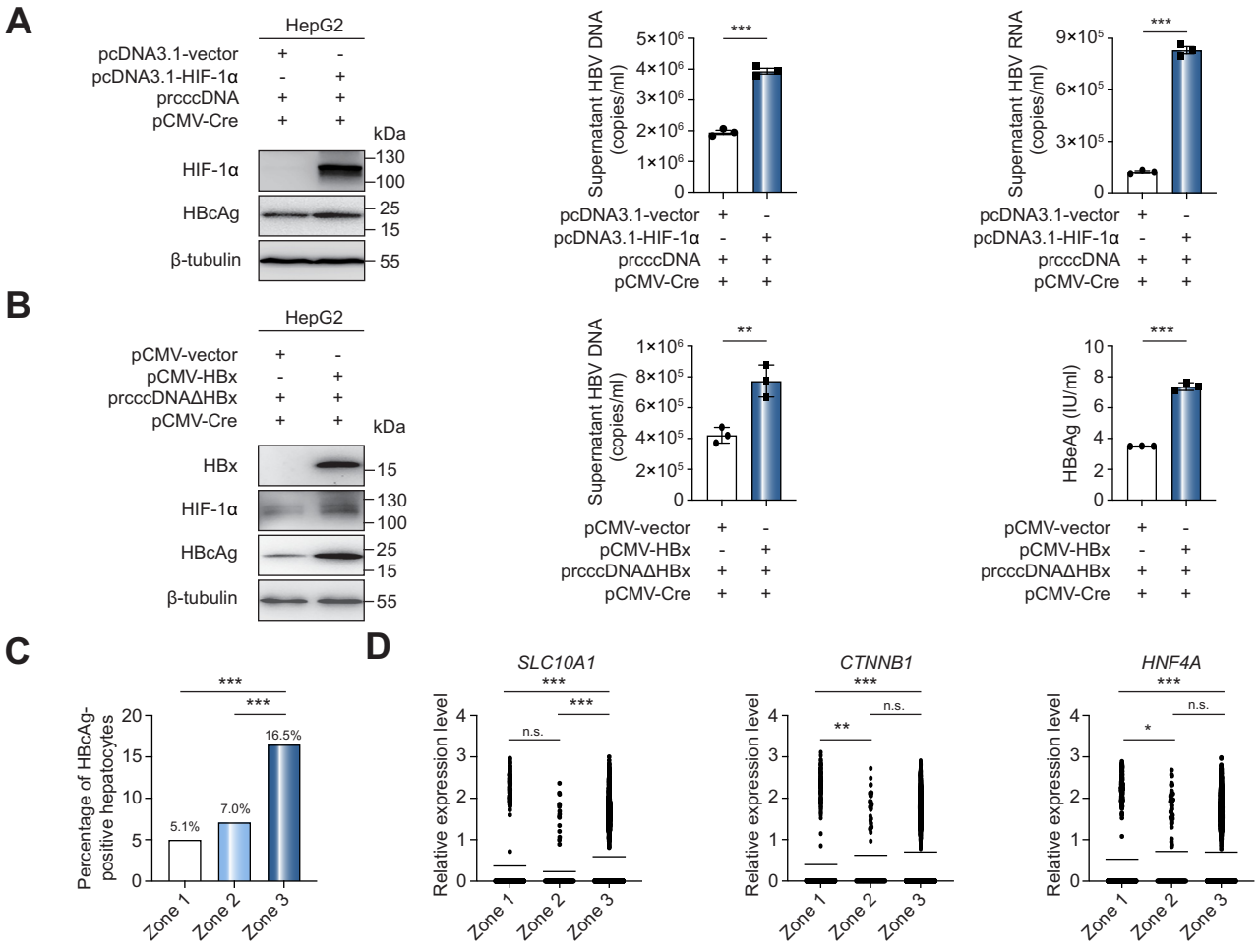

Fig. 1. Multiple factors involved in promoting HBV replication in hypoxic pericentral zone 3. (A) Levels of HIF-1α, HBcAg, supernatant HBV DNA and RNA detected in HepG2 cells ectopic expressed HIF-1α/prcccDNA/pCMV-Cre. (B) Levels of HBx, HIF-1α, HBcAg, supernatant HBV DNA and HBeAg detected in cultured HepG2 cells with ectopic expressed HBx/prcccDNAΔHBx/pCMV-Cre. Data are means±SEM. (C) Zonated expression of HBcAg in liver tissues of chronic HBV-infected individuals in EPI phase (Chi-square test). (D) Zonated expression of *SLC10A1*, *CTNNB1* and *HNF4A* analyzed from scRNA-seq dataset. (Two-tailed nonparametric tests for A, B and D, **p* <0.05, ***p* <0.01, ****p* <0.001, n.s., none-significant).

In conclusion, our study validated the role of hypoxia and HIF-1α in promoting HBV replication reported by Wing *et al.* Our results also suggested that the expression pattern of other host factors was involved in the upregulation of HBV replication according to liver zonation, which provided additional mechanistic explanations for the upregulation of HBV replication in hypoxic pericentral zone 3. In the future, it would be worthwhile to elucidate the HBV replication-promoting role of hypoxia and HIF-1α during chronic hepatitis B disease progression.

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Conflict of interest
The authors of this study declared that they do not have any conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions
Huang H and Yuan D contributed equally to this work; Lu F designed the research; Huang H, Yuan D, Li M and Abulaiti A performed the research; all authors analyzed the data; Huang H and Yuan D wrote the paper; Lu F revised the paper.

Data availability statement
The scRNA-seq dataset were downloaded from http://www.ncbi.nlm.nih.gov/geo with the accession number of GSE115469. Data related to this paper are available on reasonable request.

Ethical statement
The study was approved by the Ethics Committee of Peking University. Written informed consent was obtained from each patient.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.01.031.

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Author names in bold designate shared co-first authorship

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Reply to: 'Active HBV replication in hypoxic pericentral zone 3 is upregulated by multiple host factors including HIF-1α'

Hypoxia is more than HIFs

To the Editor:

The letter from Huang and colleagues[1] in response to our earlier publication[2] further highlights the importance of hypoxic signalling in viral hepatitis. The authors' data support our findings that hypoxia inducible factors (HIFs) promote HBV replication. The viral encoded protein HBx regulates virus transcription and mutant viruses lacking this protein are recognised to show an attenuated phenotype. Huang *et al.* show reduced replication of a HBx-deficient virus and exogenous expression of HBx stabilised HIF-1α, suggesting a mechanism for HBx to promote viral transcription. In contrast, we found limited evidence that HBV infection stabilised HIFs in various *in vitro* and murine models.[3] However, we observed a significant enrichment of hypoxic-regulated genes in the chronically infected liver. Importantly, this was not unique to HBV and was a hallmark of inflammatory chronic liver disease.[3]

Huang *et al.* show an increased frequency of HBcAg-expressing hepatocytes within the low oxygen pericentral area (zone 3) of the liver from 38 HBeAg-positive patients. Analysing published scRNA-seq human liver data[4] identified enrichment of *HIF-1α*, *HNF4α*, and *CTNNB1* transcripts in this zone, indicative of a pro-viral niche. In collaboration with Riedl and colleagues, we reported HIF-1α and HBcAg co-expression in the chronic hepatitis B liver.[5] One of the first publications reporting on a HBV transgenic mouse model identified a pericentral location of viral antigen-expressing cells.[6] To extend these observations we silenced HIF-1β in HBV

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