Original Article

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Hepatitis B virus dampens autophagy maturation *via* negative regulation of Rab7 expression

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Summary **Hepatitis B virus (HBV) infection brings a huge challenge for medical health practitioners. It has been reported that invaded HBV escapes autophagic degradation through inhibiting lysosome maturation following enhanced autophagy formation, which putatively contributes to HBV replication and infection. However, the underlying mechanism by which HBV escapes from autophagic degradation remains elusive. In this study, we monitored the autophagic process using HepG2 cells and mice without or with transient HBV DNA plasmid transfection (pHepG2) or stable HBV infection (HepG2.2.15 cells) *in vitro* and *in vivo*. The results of Western blot, transmission electron microscopy and confocal microscopy, confirmed that HBV induced autophagy, while the fusion of autophagosomes with lysosomes was arrested. Furthermore, Rab7, a small GTPase that functions as a molecular switch responsible for the autophagosome-lysosome fusion, was inhibited, suggesting a potential mechanism for HBV-induced inhibition of autophagic degradation. In conclusion, our study proposes a potential mechanism for how HBV escapes autophagic degradation, which might be a novel therapeutic target for controlling HBV infection.**

Keywords: HBV, Rab7, Autophagy, Autophagosome-lysosome fusion

1. Introduction

Hepatitis B virus (HBV) infection threats about 248 million individuals' health globally. It remains a dreadful public health problem worldwide although the pervasive application of effective vaccination limits the number of newly HBV-infected patients dramatically (*1*). The availability of potent antiviral drugs decreases the morbidity and mortality inflicted by HBV infection. Nevertheless, almost all the current therapeutic measures are unable to completely eradicate the HBV DNA even after long-term antiviral therapy (*2*), and HBV could be reactivated when host immunity is compromised. The persistence of HBV DNA in the nucleus of infected hepatocytes is one of the intractable enigmas in approaches for the cure of chronic hepatitis B (CHB) (*3*). A better understanding of this refractory challenge may help to develop reliable priorities against HBV infection.

Macroautophagy (hereafter autophagy), a highly conserved cellular self-digestive process, plays a pivotal role in the degradation of dysfunctional proteins and

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damaged organelles to maintains cellular homeostasis or responses to harmful stimuli in higher eukaryotes (*4*). Autophagy initiates with the formation of an autophagosome, following docking and fusion with a lysosome. Only after the fusion can the contents of the autophagosome be degraded (*5*). Moreover, accumulating evidence shows that autophagy is also involved in many pathophysiologic processes of human diseases including microbial infection (*6*). For HBV, Sir D *et al*. first reported that HBV could activate the early autophagic pathway (*7*). But it seems like autophagy causes no harm to HBV (*8*). The precise underlying mechanisms why the enhanced autophagosomes promote HBV DNA replication instead of engulfing it need to be elucidated.

Rab7, a small GTPase of Rab superfamily, functions as a key organizer of multiple membrane trafficking processes. It has been well established that Rab7 serves as a master regulatory component for the biogenesis of autophagosomes, lysosomes and other lysosome-related organelles (*9*). More recently, Rab7 has been implicated in the maturation of autophagosomes and their fusion with lysosomes. Hampered fusion of autophagosomes and lysosomes caused by deficiency of Rab7 has been covered in mycobacterium tuberculosis and human parainfluenza virus infection (*10,11*). Nevertheless, it is still uncertain whether Rab7 also plays an indispensable role in HBV-induced incomplete autophagy.

In the study, HBV infection induced autophagy was investigated, and the potential molecular mechanism that hindered autophagosome-lysosome fusion in HBV induced incomplete autophagic degradation was further explored.

2. Materials and Methods

2.1. *Cell culture*

HepG2 cells were obtained from the American Type Culture Collection (ATCC) and maintained according to ATCC instructions. HepG2.2.15 cells, which contained a stably integrated 1.3-copy HBV genome and could support persistent replication of HBV and produce intact HBV particles (*12*), was provided by the Department of Infectious Diseases of Changzheng Hospital. It was maintained in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen), 380 μg/mL G418 (Sigma), 125 μg/mL penicillin and 50 μg/mL streptomycin at 37°C in a humidified incubator containing 50 mL/L CO_2 (*13*).

2.2. *Plasmids and cell transfection*

HBV DNA plasmid (pHBV), which contains the 1.3-mer overlength HBV genome, and backbone plasmid pUC19 were a kind gift of professor James Ou from University of Southern California, Los Angeles, CA (*14*). Transient transfection into HepG2 of pHBV (pHepG2) or the backbone plasmid pUC19 (as the mock-transfected HepG2) were performed using Fugene 6 reagent (Roche) according to the manufacturer's instructions, respectively. HepG2 cells were lysed for both protein and RNA analysis after 48 h transfection. Meanwhile, level of HBsAg in the cell supernatant was determined using the ARCHITECT i2000SR HBsAg QT assay (Abbott). The cut-off value for determining if HBsAg is positive is S/N ratio ≥ 0.05, and the reporting units are S/N ratio. Level of HBV DNA in the cell supernatant was detected using the Roche COBAS HBV Amplicor MonitorTM assay. Tandem mRFP-GFP-LC3 expressing plasmid ptfLC3 (Addgene) was obtained from Addgene (*15*), and transfection was performed using Lipofectamine 2000 (Invitrogen).

2.3. *Animal analysis*

C57BL/6 mice (8 weeks old, male) were injected *via* the tail vein with phosphate buffer saline (PBS) in a volume that was equivalent to 8% of the body weight. The PBS with or without 10 μg of pHBV was delivered within 5 to 8 sec. Four days later, mice were sacrificed and liver tissues or serums were collected. Total liver protein was analyzed. Serum levels of HBsAg and HBV DNA of the mice were determined. The animal experiments were approved by the Ethics Committee of Ruijin Hospital.

2.4. *Immunoblotting*

Cells were directly lysed with RIPA containing protease and phosphatase inhibitors (Roche) and proteins were separated by SDS-PAGE after denaturation (*16*). Immunoblot analysis was performed by initial transfer of proteins onto polyvinylidenefluoride filters using a Mini Trans-Blot (Bio-Rad) followed by a blocking step with 5% nonfat dried milk plus 0.1% Tween 20 for 2 hours at room temperature and exposed to primary antibodies diluted 2,000-fold that recognized microtubule-associated protein light chain 3 (LC3, Cell Signal), autophagy-related protein 5 (Atg5, Cell Signal), Rab7 (Cell Signal), or 5000-fold for GAPDH (Santa Cruz) overnight at 4°C and subsequently washed. The blots were then incubated with a secondary antibody conjugated with Horse Radish Peroxidase diluted 5000-fold for 1 hour at room temperature. Signals were detected by a FluorChem E system (Alpha Innotech Corp). For some immunoblots, band densitormetry was quantified using ImageJ software (National Institutes of Health).

2.5. *RT-PCR*

RT-PCR for Rab7 was performed as previously described (*17*), the primer pairs used in the study were as follows: Forward: 5'-ATGACCTCTAGGAAGAAAGTGTTGC TG-3'; Reverse: 5'-TCAGCAACTGCAGCTTTCTGCC

GAG-3'. Total RNA was extracted using TRIzol reagent (Invitrogen), and RNA was reverse transcribed using Superscript II (Invitrogen) and a random hexamer primer. The cDNA was used as template for PCR with Rab7-specific primer pairs, and PCR products were analyzed by agarose gel electrophoresis and visualized under UV light with ethidium bromide.

2.6. *Transmission electron microscopy*

For ultrastructure analysis, cells were trypsinized, centrifuged, and fixed with 2.0% glutaraldehyde in 0.2M PBS (pH 7.2) for 12 h at 4°C, and washed with PBS, then post-fixed with 1% OsO_4 in 0.2M PBS (pH 7.2) for 2 h at 4°C. After a further wash with PBS, cells were dehydrated *via* a graded ethanol series and then embedded before trimming and sectioning. Embedded cells were sectioned at a thickness of 75 nm using a Reichert Ultramicrotome and mounted onto 200-mesh copper grids. After counterstaining with uranyl acetate and lead citrate for 5 min, the grids were visualized using a CM-120 transmission electron microscope (Philip) with an operating voltage of 80kV (*18*).

2.7. *Fluorescence confocal microscopy*

Fluorescence confocal microscopy was performed as previously described (*16*). Cells grown on coverslips were fixed in BD Cytofix/Cytoperm solution (BD Biosciences) at room temperature for 15 min. These coverslips were visualized with single-line excitation at 488 or 594 nm for Alexa Fluor on an Olympus Fluoview confocal microscope with appropriate emission filters (Olympus).

2.8. *Autophagy analyses*

For starvation, the cells were incubated in serum-free Earle's balanced salt solution (Invitrogen) for 6 hours. Autophagy was analyzed by immunoblotting or fluorescence microscopy, as described previously (*19*). In the immunoblotting analysis, cells were treated as indicated, and cell lysates were immunoblotted with anti-LC3 antibody to monitor the LC3-II generated during the formation of autophagosomes. In the fluorescence confocal microscopy analysis, cells were transfected with a mRFP-GFP-LC3 expressing plasmid ptfLC3 construct and treated as indicated. These cells were imaged by fluorescence confocal microscopy and autophagy cell numbers were recorded. Total LC3-positive dots (GFP^+RFP^+ and GFP^-RFP^+ dots), early autophagosomes (GFP^+RFP^+ dots) and autolysosomes (GFP^- RFP^+ dots) were analyzed.

2.9. *Statistical analysis*

The 2-tailed Student t test or one-way analysis of variance followed by the Turkey post-hoc test was used for all statistical analyses in the study using SPSS 18.0. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. *HBV DNA stimulates the formation of autophagosomes*

In order to study whether HBV DNA induces autophagy, first we established an HBV infection cellular system. In our system, the levels of HBsAg and HBV DNA in cell supernatant were monitored. Compared to the HepG2 control and the mock-transfected HepG2, HepG2.2.15 and pHepG2 showed significantly higher levels of both HBsAg and HBV DNA (Figure 1A, B). Atg5 and LC3, two pivotal ubiquitin-like proteins that are involved in the initiation of autophagy, serve as hallmarks of the formation of autophagosomes (*5*). In the current study, markedly elevated LC3-II and Atg5 were detected in pHepG2, and HepG2.2.15 cells, similar to that in starved HepG2 cells (Figure 1C, D). Additionally, autophagy-featured intracellular double membrane structures were readily observed in pHepG2 under transmission electron microscopy, whereas there were barely double membrane vacuoles in HepG2 (Figure 1E). Collectively, it suggests that HBV infection induces autophagosome formation.

3.2. *HBV infection induces autophagosome accumulation in vivo*

To decipher whether HBV infection could induce autophagosome formation *in vivo*, pHBV was injected into C57BL/6 mice to simulate infection of HBV. Compared with those in the control, serum HBsAg and HBV DNA levels significantly increased in the pHBV injection group (Figure 2A and B). Histology showed disorganized hepatic lobules and lymphocytic infiltration in portal tracts, which indicated obvious inflammation in liver tissue (Figure 2C). Higher expression of LC3-II and Atg5 protein levels were observed in liver tissue of pHBV transfected mice than that of control mice (Figure 2D). Thus, our data indicates that HBV infection induces the formation of autophagosomes *in vivo*.

3.3. *Autophagy maturation is hampered in pHepG2 cells*

Autophagy maturation (docking and fusion of lysosome) is a key step for microbial degradation and is susceptible to pathogen hijacking. To uncover the underlying mechanism by which HBV induced autophagy promoted HBV replication instead of engulfing it, HepG2 cells were transfected with pHBV, followed by transfection with mRFP-GFP-LC3 plasmid. GFP is susceptible to

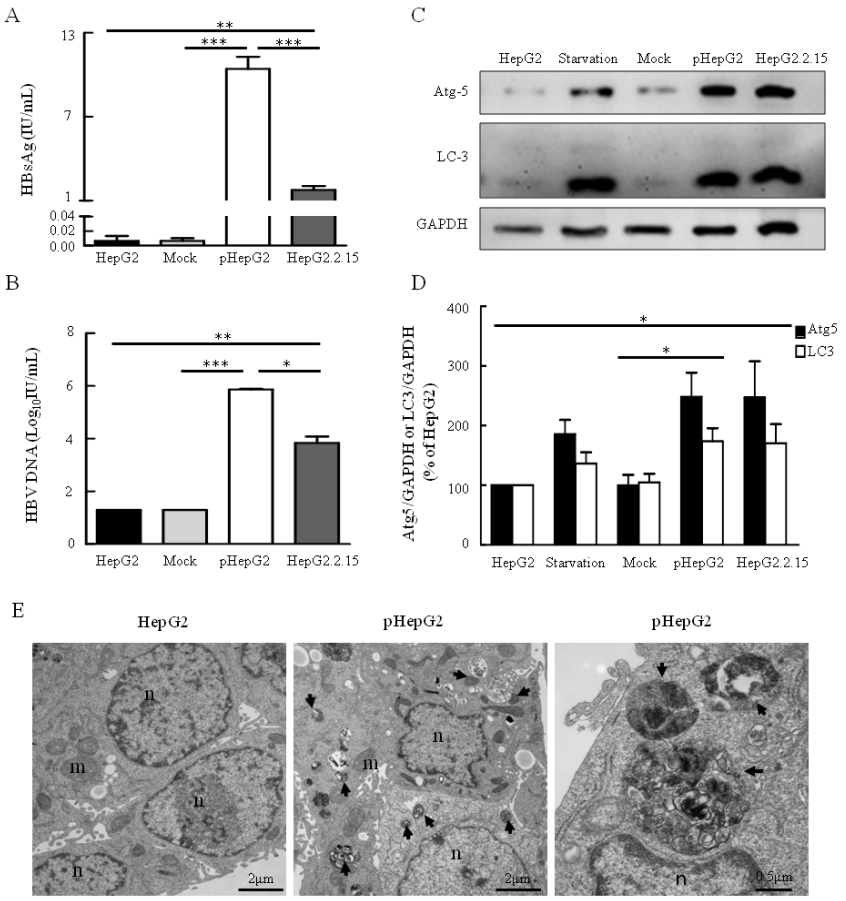

Figure 1. HBV DNA induces autophagosome accumulation. HepG2 cells were starved, transfected with pHBV or pUC19 for 48h, respectively. **(A, B)** Levels of HBsAg and HBV DNA in supernatant of HepG2, mock-transfected HepG2, pHepG2 and HepG2.2.15 were measured. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **(C)** The Atg5 and LC3 levels in HepG2, starvation treated HepG2, mock-transfected HepG2, pHepG2 and HepG2.2.15 were assessed by Western blot. **(D)** The bands density ratios of Atg5/GAPDH or LC3-II/GAPDH were determined by Western blot, and the results of control HepG2 was taken to be 100%. It was calculated as the average value of the data obtained from 3 independent experiments. *, $p < 0.05$. **(E)** Autophagic vacuoles in HepG2 and pHepG2 were observed by electron microscopy. The arrow indicates autophagosome, "m" represents mitochondria, and "n" represents nuclear.

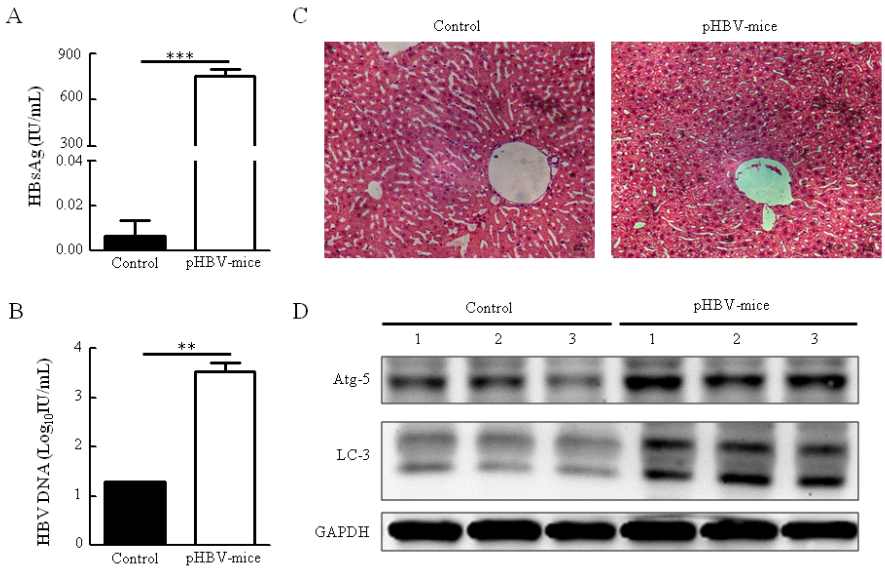

Figure 2. pHBV increases autophagosome formation in mice. Male C57BL/6 mice (8 weeks old) were randomly injected with (pHBV, $n = 3$) or without (control, $n = 3$) 10 μg of pHBV. Ninety-six hours later, mice were sacrificed and liver tissues or sera were collected. **(A and B)** Mice serum HBsAg and HBV DNA levels were measured. **, $p < 0.01$; ***, $p < 0.001$. **(C)** Liver tissues of mice in control and pHBV-treated mice were stained with hematoxylin-eosin staining. **(D)** Atg5 and LC3 levels in mice liver tissue were assessed by Western blot.

acidified conditions, while mRFP signals are relatively stable, so the autophagosome is displayed as yellow punctate dots (both red and green) before fusion with lysosome, and red for mature autolysosomes. As shown in Figure3A, yellow punctate dots were more obvious in pHepG2 cells than HepG2, while only sporadic red punctate dots were observed, confirming dominant presence of autophagosomes (yellow punctate dots), with rather few autolysosomes (red punctate dots). Cell numbers of HepG2 with autophagy were significantly increased after transfection with pHBV (Figure 3B). Above all, our data indicates inhibitory effects on autopahgy maturation.

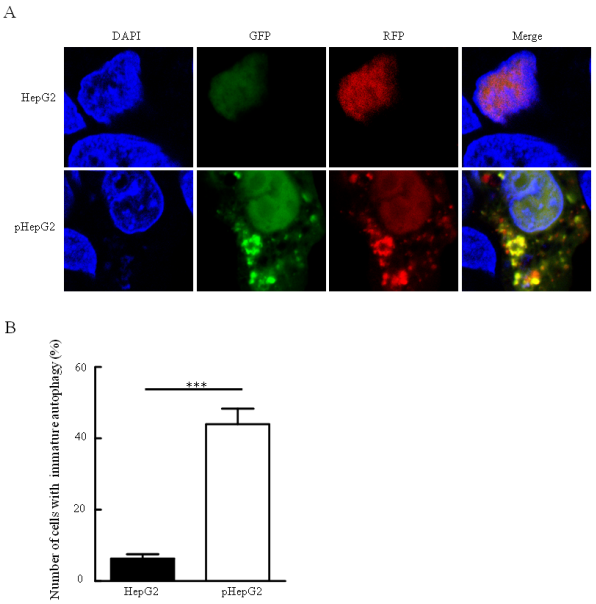

Figure 3. Autophagosome-lysosome fusion-block by pHBV transfection. (A) HepG2 cells transfected without or with HBV DNA plasmid (pHepG2) were grown on coverslips, then transiently transfected with mRFP-GFP-LC3 expressing plasmid ptfLC3. Digital images were captured with confocal microscopy. **(B)** Under confocal microscopy, cells with yellow punctate dots was identified as cells with autophagy. The number of autophagic cells was recorded in every 100 cells counted. The results were calculated as the average value of the data obtained from 3 independent experiments. ***, $p < 0.001$.

3.4. *HBV infection induces reduction of Rab7*

Small GTPase Rab7 has been considered as a master molecular switch during the formation of autolysosomes (*20*). Consequently, the expression of Rab7 was investigated to determine the role of Rab7 in HBV induced autophagy. Our results showed significant drops in Rab7 protein expression in pHepG2 cells and HepG2.2.15 cells (Figure 4A, B), as well as in Rab7 mRNA level (Figure 4C, D). Thus, it shows that HBV infection inhibits Rab7 expression and dampens autophagy maturation.

4. Discussion

Autophagy, a conserved cellular protective mechanism, has been regarded as a key component of the innate immune system (*21*). Induction of autophagy results in the elimination of invading pathogens, whereas some microbes have evolved strategies to manipulate autophagy for their own benefit. Several reports have proclaimed that HBV infection is able to induce autophagy (*7,22,23*), and transfection of pHBV in hepatic and hepatoma cell lines both promote formation of autophagosomes (*23*). Consistently, our results also discovered increased autophagy in pHepG2 cells and HBV-expressing HepG2.2.15 cells. Furthermore, the *in vivo* results support our conclusion when transfecting C57BL/6 mice with pHBV.

HBV-induced autophagy have been extensively investigated, and the views of its effect on HBV and chronic infection were still elucidated. Several studies suggested that HBV could manipulate autophagy to affect virus production and chronic infection

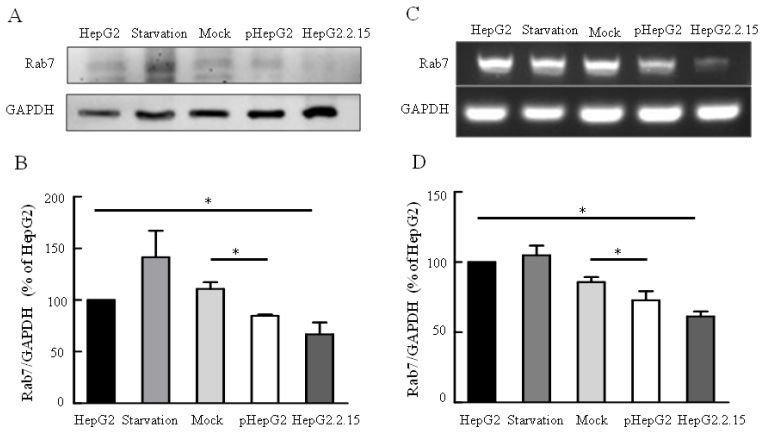

Figure 4. HBV DNA downregulates Rab7 expression. HepG2 cells were starved, transfected with pHBV or pUC19 for 48h, respectively. **(A)** The cellular lysis was subjected to Western blot for Rab7 protein expression analysis. **(C)** The cellular Rab7 mRNA levels in HepG2, starvation treated HepG2, mock-transfected HepG2, pHepG2 and HepG2.2.15 were detected by RT-PCR. **(B, D)** The bands density ratios of Rab7/GAPDH were determined by Western blot and RT-PCR, and the results of control HepG2 was taken to be 100%. It was calculated as the average value of the data obtained from 3 independent experiments. *, $p < 0.05$.

establishment (*24,25*). On the contrary, Tian *et al*. indicated that incomplete autophagy could regulate HBV DNA replication instead of HBV virions *in vivo* (*26*). However, how autophagy might implement regulation and help pathogen escape from the following autophagic degradation still remained elusive. A recent study demonstrated that hepatitis B virus x protein impaired lysosomal degradative capacity by disturbing its acidification without influencing the fusion of autophagosomes and lysosomes (*27*). Intriguingly, one of the most important findings from our current study turned out that the fusion of autophagosomes and lysosomes was hampered in pHepG2 cells. This discrepancy might be due to transfection of a different pHBV in our experiment system. Besides, the effects of different cell lines also should be considered. Some intracellular pathogens, mycobacterium tuberculosis and human parainfluenza virus (*10,11*), hijack autophagy and inhibit its maturation for their replication, prompting us to speculate that HBV might induce autophagosome formation as its replication site to facilitate the viral life cycle but block the following autophagosomes-lysosomes fusion to evade autophagic degradation.

Fusion proteins, such as VAMP1, 2, 7 and 8, Vti1b, syntaxin1, and 8, VPS28, LAMP2 and Rab7, play a key role in the fusion of autophagosomes and lysosomes. Our data suggested that a difference merely existed in Rab7 mRNA levels between HepG2 and pHepG2. Rab7, a late endosome-/lysosome-related small GTPase, may be the only lysosomal Rab protein identified so far (*9*), which functions as a crucial molecular switch in autophagy. Rab7 can act as a master factor to regulate the biogenesis and maintenance of lysosomes (*28*). Furthermore, blocking Rab7-mediated trafficking machineries downregulate autophagy-mediated clearance (*29*). Extensive research delineated that Rab7 could promote maturation of autophagosomes and regulate their fusion with lysosomes (*30*). However, abnormal expression of Rab7 has been implicated in invasion of many other pathogens. For example, Hu D *et al*. reported that Rab7 could be blocked to prevent autophagosome-lysosome fusion in mycobacterium tuberculosis infection (*10*). Hampered fusion of autophagsomes and lysosomes caused by deficiency of Rab7 has also been covered in human parainfluenza virus infection (*11*). Moreover, Inoue J *et al*. has reported that HBV could hijack Rab7 for its replication, as siRNA-mediated depletion of Rab7 dramatically augmented the secretion of virions (*31*). Accordingly, our data showed that HBV infection inhibited Rab7 expression in pHepG2 cells and HepG2.2.15 cells. Whereas, starvation treatment, which induced autophagy in the whole process, increased expression of Rab7 in HepG2 cells. Combined with these findings, our study made it clear that Rab7 might be responsible for the HBV-induced incomplete autophagic progress, which could convincingly show how HBV could enhance the formation of autophagosomes and escape subsequent autophagic degradation.

In conclusion, this study confirms that HBV induces autophagosome formation but blocks autophagy maturation. Moreover, it supports the idea that HBV dampens Rab7 expression, which is responsible for the autophagosome-lysosome fusion. Our findings help to clarify the role of Rab7 in HBV-induced incomplete autophagic degradation and provide a potential target for further manipulation of autophagy in the treatment of HBV infection.

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References

1. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. The Lancet. 2015; 386:1546-1555.
2. Liang TJ, Block TM, McMahon BJ, Ghany MG, Urban S, Guo JT, Locarnini S, Zoulim F, Chang KM, Lok AS. Present and future therapies of hepatitis B: From discovery to cure. Hepatology. 2015; 62:1893-908.
3. Thomas D, Zoulim F. New challenges in viral hepatitis. Gut. 2012; 61 Suppl 1:i1-5.
4. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. Science. 2004; 306:990-995.
5. Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. Nat Cell Biol. 2010; 12:814-822.
6. Kudchodkar SB, Levine B. Viruses and autophagy. Rev Med Virol. 2009; 19:359-378.
7. Sir D, Tian Y, Chen Wl, Ann DK, Yen TSB, Ou JhJ. The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. Proceedings of the National Academy of Sciences. 2010; 107:4383-4388.
8. Zhong L, Hu J, Shu W, Gao B, Xiong S. Epigallocatechin-3-gallate opposes HBV-induced incomplete autophagy by enhancing lysosomal acidification, which is unfavorable for HBV replication. Cell Death Dis. 2015; 6:e1770.
9. Zhang M, Chen L, Wang S, Wang T. Rab7: roles in membrane trafficking and disease. Biosci Rep. 2009; 29:193-209.
10. Hu D, Wu J, Wang W, Mu M, Zhao R, Xu X, Chen Z, Xiao J, Hu F, Yang Y, Zhang R. Autophagy regulation revealed by SapM-induced block of autophagosome-lysosome fusion *via* binding RAB7. Biochem Biophys Res Commun. 2015; 461:401-407.
11. Ding B, Zhang G, Yang X, Zhang S, Chen L, Yan Q, Xu M, Banerjee AK, Chen M. Phosphoprotein of human parainfluenza virus type 3 blocks autophagosome-

lysosome fusion to increase virus production. Cell Host Microbe. 2014; 15:564-577.
12. Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci U S A. 1987; 84:1005-1009.
13. Xu WS, Zhao KK, Miao XH, Ni W, Cai X, Zhang RQ, Wang JX. Effect of oxymatrine on the replication cycle of hepatitis B virus *in vitro*. World J Gastroenterol. 2010; 16:2028-2037.
14. Zheng Y, Li J, Johnson DL, Ou JH. Regulation of hepatitis B virus replication by the ras-mitogen-activated protein kinase signaling pathway. J Virol. 2003; 77:7707-7712.
15. Kimura S, Fujita N, Noda T, Yoshimori T. Monitoring autophagy in mammalian cultured cells through the dynamics of LC3. Methods Enzymol. 2009; 452:1-12.
16. Xu C, Feng K, Zhao X, Huang S, Cheng Y, Qian L, Wang Y, Sun H, Jin M, Chuang TH, Zhang Y. Regulation of autophagy by E3 ubiquitin ligase RNF216 through BECN1 ubiquitination. Autophagy. 2014; 10:2239-2250.
17. Cai W, Du A, Feng K, Zhao X, Qian L, Ostrom RS, Xu C. Adenylyl cyclase 6 activation negatively regulates TLR4 signaling through lipid raft-mediated endocytosis. J Immunol. 2013; 191:6093-6100.
18. Jiang Y, Tong Y, Lu S. Visualizing the three-dimensional mesoscopic structure of dermal tissues. J Tissue Eng Regen Med. 2014; 8:794-800.
19. Xu C, Liu J, Hsu LC, Luo Y, Xiang R, Chuang TH. Functional interaction of heat shock protein 90 and Beclin 1 modulates Toll-like receptor-mediated autophagy. FASEB J. 2011; 25:2700-2710.
20. Ng EL, Gan BQ, Ng F, Tang BL. Rab GTPases regulating receptor trafficking at the late endosome-lysosome membranes. Cell Biochem Funct. 2012; 30:515-523.
21. Devenish RJ, Lai SC. Autophagy and burkholderia. Immunol Cell Biol. 2015; 93:18-24.
22. Sir D, Ann DK, Ou JH. Autophagy by hepatitis B virus and for hepatitis B virus. Autophagy. 2010; 6:548-549.
23. Tang H, Da L, Mao Y, Li Y, Li D, Xu Z, Li F, Wang Y, Tiollais P, Li T, Zhao M. Hepatitis B virus X protein sensitizes cells to starvation-induced autophagy *via* up-regulation of beclin 1 expression. Hepatology. 2009; 49:60-71.
24. Li J, Liu Y, Wang Z, Liu K, Wang Y, Liu J, Ding H, Yuan Z. Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. J Virol. 2011; 85:6319-6333.
25. Yang H, Fu Q, Liu C, Li T, Wang Y, Zhang H, Lu X, Sang X, Zhong S, Huang J, Mao Y. Hepatitis B virus promotes autophagic degradation but not replication in autophagosome. Biosci Trends. 2015; 9:111-116.
26. Tian Y, Sir D, Kuo CF, Ann DK, Ou JH. Autophagy required for hepatitis B virus replication in transgenic mice. J Virol. 2011; 85:13453-13456.
27. Liu B, Fang M, Hu Y, Huang B, Li N, Chang C, Huang R, Xu X, Yang Z, Chen Z, Liu W. Hepatitis B virus X protein inhibits autophagic degradation by impairing lysosomal maturation. Autophagy. 2014; 10:416-430.
28. Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B. Rab7: a key to lysosome biogenesis. Mol Biol Cell. 2000; 11:467-480.
29. Rawet-Slobodkin M, Elazar Z. PLEKHM1: a multiprotein adaptor for the endolysosomal system. Mol Cell. 2015; 57:1-3.
30. Ganley IG, Wong PM, Jiang X. Thapsigargin distinguishes membrane fusion in the late stages of endocytosis and autophagy. Autophagy. 2011; 7:1397-1399.
31. Inoue J, Krueger EW, Chen J, Cao H, Ninomiya M, McNiven MA. HBV secretion is regulated through the activation of endocytic and autophagic compartments mediated by Rab7 stimulation. J Cell Sci. 2015; 128:1696-1706.

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