Autophagy machinery impaired interferon signalling pathways to benefit hepatitis B virus replication

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Summary

Background: **Autophagy-related genes ATG4B, ATG7, and ATG12 have been identified to play a critical role in viral replication. However, these genes have yet to be identified in hepatitis B virus (HBV).**

Objective: **To characterise the role of ATG4B, ATG7, and ATG12 genes in HBV infection.**

Methods: **The mRNA expression was examined by quantitative real-time RT-PCR and Western blotting. Short hairpin RNA (shRNA) of the target gene was used to examine the function of the gene in HBV replication. Evaluation of HBV DNA level was performed by real-time PCR.**

Results: **Our findings revealed that ATG12 gene expression was significantly up-regulated (p < 0.005), whereas ATG7 gene expression was down-regulated (p < 0.0001) in HepG2.2.15 cells when compared to HepG2 cells. However, no significant difference in mRNA level of ATG4B was observed. These results were consistent with protein level findings. Moreover, we analysed the function of ATG12 in HBV replication by using ATG12 shRNA and evaluated HBV DNA level. We found that the amount of HBV was decreased in ATG12-knockdown HepG2.2.15 cells when compared to control HepG2.2.15 cells (P <0.05). The mRNA expression of interferon-alpha (IFN-α), interferon-beta (IFN-β), and interferon-inducible genes (IFI) was also investigated. Our results showed that the expression of IFN-α, IFN-β, and IFI27 genes were increased in ATG12-knockdown cells but not in Mx1 gene when compared to control cells (p < 0.005, p < 0.0001 and p <0.005, respectively).**

Conclusion: **These autophagy-related genes, ATG12 may play a role in HBV replication via impairing IFN pathway. However, the biological significance of other autophagic genes such as ATG7 warrants further study.** ***(Asian Pac J Allergy Immunol 2016;34:77-85)***

Keywords: *autophagy, hepatitis B virus, ATG4B, ATG7, ATG12, interferon*

Introduction

Hepatitis B virus (HBV) infection is a serious and life-threatening health problem. Approximately 350 million people worldwide are chronically infected with HBV. If left untreated, this deadly virus leads to chronic diseases including liver cirrhosis and hepatocellular carcinoma (HCC) which claims the lives of approximately 1 million people each year.[1] Autophagy is a catabolic pathway for recycling nutrients to maintain cellular homeostasis. It has also been implicated in innate and adaptive immunity to eliminate intracellular pathogens.[2] However, recent studies have demonstrated that some viruses defeat the autophagic pathway for their own benefit. A recent report showed that HBV induces autophagic process for its replication without increasing autophagic protein degradation.[3-5]

Numerous studies have been conducted to identify molecular pathways that regulate autophagy. A class III phosphatidylinositol 3-kinase (PIK3C3) has been reported as an important regulator in the autophagic process. PIK3C3 consists of a catalytic subunit hVps34 and a regulatory subunit p150. It

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has been shown to complex with Beclin-1/ATG6, UVRAG, and Bif-1, which is necessary for the initiation of autophagy.[6] A recent study showed that HBV induces autophagy for its DNA replication through the X protein (HBx) which binds to PIK3C3.[3] Moreover, a function of HBx in increasing autophagy through the up-regulation of beclin 1 expression was observed in the study of Tang et al.[7] In addition to the regulators of initiation of autophagy, the conjugation systems for the formation of autophagosomes are also crucial. There are two ubiquitin-like conjugation systems. The first is the coupling of autophagy-related genes (ATG) 12 to ATG5 to form a covalently linked heterodimer, which then recruits ATG16 to form ATG12-ATG5-ATG16 complex. The second system is the ATG8/LC3 conjugated to the phospholipid phosphatidylethanolamine. Several kinds of enzymes composed of ATG4B, ATG7, and ATG3 are required for lipidation of LC3, which is important for the formation of autophagosomes.[8,9] Some genes and/or proteins in the ubiquitin-like conjugation systems have been reported to play a role in hepatitis C virus (HCV) infection such as ATG12, ATG4B, and ATG7.[10,11] To clarify these autophagy-related genes in HBV disease, the ATG4B, ATG7, and ATG12 were characterised under starvation conditions.

Methods

Cell lines

The human hepatoma cell line (HepG2) and the HBV-transfected HepG2.2.15 cell line were kindly provided from Professor Antonio Bertoletti (Singapore Institute for Clinical Sciences (A*Star). Both cell lines were cultured in Dulbecco's modified Eagles' medium supplemented with 10% foetal bovine serum and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) (Invitrogen). In addition, the HepG2.2.15 cells were supplemented with 150 μg/ml Geneticin® (G418 Sulphate; Gibco) for maintaining HBV plasmid. For autophagy induction, HepG2 and HepG2.2.15 cells were incubated in starvation medium (Earle's Balanced Salt Solution: EBSS; Gibco) at 37ºC in 5% CO_2 for 4 and 8 h. All cells were collected by trypsinisation with 0.25% trypsin/EDTA (Gibco) and washed with phosphate-buffered saline (PBS). The cell pellet was re-suspended in RNAlater® Solution (Ambion) and stored at -20ºC until used.

Table 1. Primer sequences used in this study.

Gene names	Primer sequences (5'–>3')	
ATG12	ATG12-F	TTGTGGCCTCAGAACAGTTG
	ATG12-R	GAGAGTTCCAACTTCTTGGTCTG
ATG7	ATG7-F	CCTCCTCTTGACATTTGC
	ATG7-R	CTTCGTCCTTTGACCTTG
ATG4B	ATG4B-F	TCCATAGGCCAGTGGTACG
	ATG4B-R	TGCACAACCTTCTGATTTCC
Beclin1	BECN1F	GGATCAGGAGGAAGC
	BECN1R	GATGTGGAAGGTTGC
PIK3C3	PIK3C3F	CCTGTAGGAGGAACAAC
	PIK3C3R	GCAAGACGGCTCATCTG
B-actin	B-actinF	ACCAACTGGGACGACATGGAGAA
	B-actinR	GTGGTGGTGAAGCTGTAGCC
IFNA1	IFNA1-F	GCTTTACTGATGGTCCTGGTGGTG
	IFNA1-R	GAGATTCTGCTCATTTGTGCCAG
IFNB1	IFNB1-F	GAATGGGAGGCTTGAATACTGCCT
	IFNB1-R	TAGCAAAGATGTTCTGGAGCATCTC
Mx1	Mx1-F	CCTTGCATGAGAGCAGTGATG
	Mx1-R	AGCCTCATCCGCCTAGTCAA
IFI27	IFI27-F	GCAGTCACTGGGAGCAACTG
	IFI27-R	CAATGGAGCCCAGGATGAAC
PreS1	PreS1-F	GGGTCACCATATTCTTGGGAAC
	PreS1-R	CCTGAGCCTGAGGGCTCCAC

RNA isolation and quantitative real-time RT–PCR

Total RNA was extracted from HepG2 and HepG2.2.15 cells using RNA extraction kit (RBC Bioscience). One microgram of total RNA were converted to cDNA by random primers using MultiScribe™ Reverse Transcriptase (High Capacity cDNA Reverse Transcription Kits; Applied Biosystems®). A 2 μl of cDNA sample was added to 20 μl of master mix containing (1X) Power SYBR Green PCR Master Mix (Applied Biosystems®), 1.25 μM forward and reverse primers of specific genes (Table 1). The PCR amplification was performed on the Applied Biosystems 7500 Real-Time PCR System using thermal cycling conditions: 10 min at 95ºC, followed by 40 cycles of 15 sec at 95ºC, 1 min at 60ºC. The derivation of the 2^{-ddCT} method was applied for relative quantification of mRNA expression.[12] Beta-actin was used as an endogenous reference control. The expression of a target gene in HepG2 or HepG2.2.15 cells was subtracted by the reference gene. The ddC_T of HepG2.2.15 gene expression were subtracted with HepG2 expression. Data were calculated with the following equation,

where $ddC_T = (C_{T, target} - C_{T, actin})_{HepG2.2.15} - (C_{T, target} - C_{T, actin})_{HepG2}$ and analysed with the fold change of 2^{-ddCT}.

Western blot analysis

Non-starved and starved HepG2 and HepG2.2.15 cells were collected and lysed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). After sonication, the protein concentration of cell lysates was measured using the BCA assay (Pierce™). A total of 20 µg of proteins from each cell line was loaded onto 12% polyacrylamide gel and run at 130 V for 75 min. The proteins were transferred onto nitrocellulose membrane and were immunoblotted with the primary monoclonal antibodies (mAb) against Atg7, Atg12, Beta actin (dilution, 1:1000; Rabbit mAb; Cell Signaling Technologies) and Atg4B (dilution, 1:1000; Mouse mAb; Abcam). After washing, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (dilution 1:2000) and were then analysed with enhanced chemiluminescence (ECL) substrate (SuperSignal West Femto Chemiluminescent Substrate; Pierce ™). Image was quantified using Licor-odyssey image system (LI-COR®).

RNA interference

HepG2.2.15 cells were transiently transfected with 500 ng of short hairpin RNA (shRNA) plasmid specific to ATG12 and negative control (SureSilencing shRNA Plasmid; QIAGEN). The shATG12 plasmid contained following sequences: 5'-CGAACCATCCAAGGACTCATT-3'. Negative control inserted with a mock target sequence as follows: 5'-GGAATCTCATTCGATGCATAC-3'. Transfection of shRNA plasmids were carried out using Lipofectamine®2000 (Invitrogen) according to the manufacturer's instructions. At 72 h post-transfection, cells were collected and the silencing efficiency of the shRNA was detected by Western blotting.

Quantification of intracellular HBV replication

Total DNA from silencing ATG12-HepG2.2.15 cells and mock-treatment HepG2.2.15 cells were isolated using the QIAamp DNA Blood Mini Kit (Qiagen). For viral DNA quantification, a standard copy number of HBV was used. Specific primers for PreS1 HBV gene were newly designed, as shown in Table 1. The PCR amplification was performed with SYBR green on the Applied Biosystems 7500 Real-Time PCR System.

Apoptosis assay

Apoptotic cells were detected by using APC-labelled Annexin V binding (Invitrogen, CA, USA) and Fixable Viability Dye eFluor® 780 staining (eBioscience). Total apoptotic cells are early apoptotic cells defined as annexin V-positive and Fixable Viability Dye eFluor® 780-negative combined with late apoptotic cells defined as both annexin V-positive and Fixable Viability Dye eFluor® 780-positive. Cells were acquired on a FACScan (BD Biosciences) and analysed with FlowJo 10.0 software (FlowJo LLC., USA).

Statistical analysis

Significant differences were determined by unpaired t test with GraphPad Prism, version 5.0 software (San Diego, California, USA). The statistical significance was set at P-value of $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0001$ (***).

Result

ATG12 is up-regulated in HBV-transfected cells under starvation condition.

More than 30 autophagy-related genes are involved in autophagic processes.[13] Beclin1 is an essential autophagy regulator binding to PIK3C3 (Vps34) which is a critical enzyme for the initiation of autophagy.[14] Previous studies have shown that autophagy induction is mediated by the HBx protein, which binds to PIK3C3 for viral replication.[3] Moreover, the viral regulatory protein HBx was involved in starvation-induced autophagy via the up-regulation of Beclin1 expression.[7] Therefore, we chose Beclin1 and PIK3C3 as a marker for HBV-induced autophagy in starvation condition. We analysed the Beclin1 and PIK3C3 mRNA expression in HBV-transfected HepG2.2.15 and HepG2 cells. At 4 h post-starvation, Beclin1 and PIK3C3 were up-regulated in both of cell lines and were then down-regulated at 8 h (data not shown). Afterwards, we selected the optimal condition of autophagy induction for both cell lines with starvation in EBSS for 4 h. The candidate of autophagy related genes including ATG4B, ATG7, and ATG12 were determined the mRNA expression level at 4 h post-culture in EBSS. In the present study, we found that the mRNA expression of ATG12 was significantly increased in HBV-transfected HepG2.2.15 cells when compared to HepG2 cells ($p = 0.0039$). In the case of ATG7, the mRNA expression of this gene was significantly down-regulated in HBV-transfected HepG2.2.15

cells ($p < 0.0001$). However, no significant difference was observed with ATG4B gene between HBV-transfected HepG2.2.15 cells and HepG2 cells (Figure 1). Furthermore, we confirmed the results of mRNA level with Western blot analysis. We consistently found that ATG12 protein was up-regulated in HBV-transfected HepG2.2.15, whereas ATG7 was down-regulated compared to HepG2 cells. As for ATG4B protein expression, there was no difference between these two cells (Figure 2). The ATG12 protein expression was also up-regulated in non-starved HepG2.2.15 cells compared to the HepG2 cells (Figure 3).

Decreased ATG12 affects HBV replication.

ATG12 is required for ubiquitin-like conjugation systems in the early steps of autophagosome formation.[8] We hypothesised that HBV induces the up-regulation of ATG12 to benefit its replication. To test this hypothesis, we knocked-down the ATG12 gene in HepG2.2.15 cells with ATG12 shRNA. Our results showed that the protein level of ATG12 in the cells treated with ATG12 shRNA was decreased when compared to the mock control (Figure 4A). To determine the effect of ATG12 on HBV replication, HBV DNA was quantified from shATG12-transfected cells compared to mock cells. Our findings showed that HBV DNA was diminished in shATG12-transfected HepG2.2.15 cells in comparison to the mock control ($p < 0.05$) (Figure 4B). Therefore, the impairment of ATG12 reduces HBV replication in HepG2.2.15 cells. In addition, we performed an apoptosis assay to confirm that the role of ATG12 in HBV replication in HepG2.2.15 cells was not due to cell death. In this study, we found an increase of apoptotic cells in shATG12-transfected HepG2 cells as compared to the mock control cells, but not different in shATG12-transfected HepG2.2.15 cells (Figure 5).

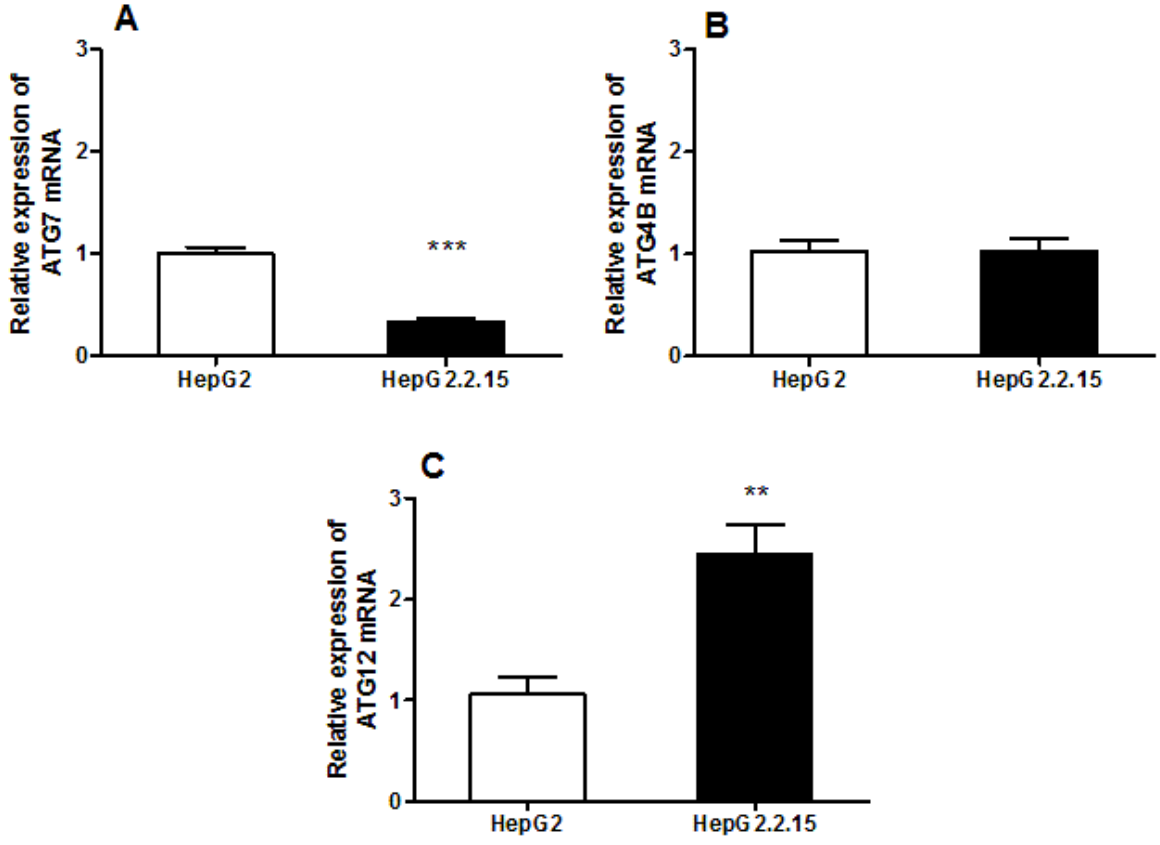

Figure 1. Quantitative real-time RT-PCR analysis of ATG7 (A), ATG4B (B), and ATG12 (C) mRNA in HepG2 and HepG2.2.15 cell lines at 4 h post-cultured in EBSS. Beta actin was used as an internal control. Data is shown as mean with SEM of five independent experiments.

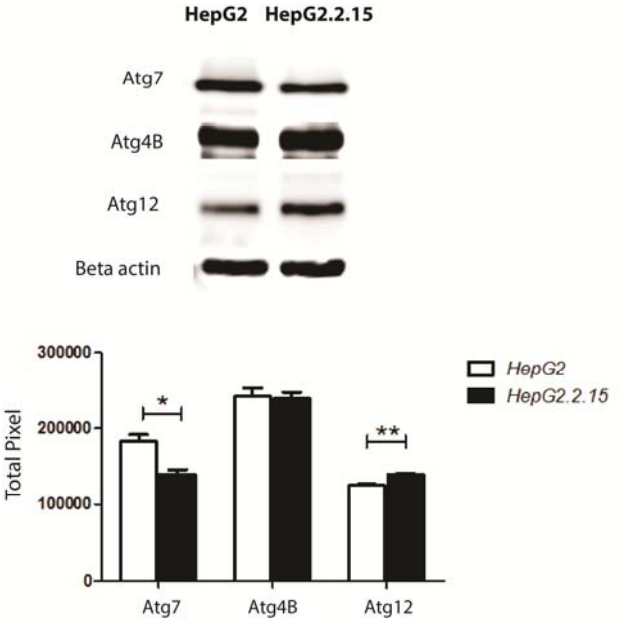

Figure 2. Western blotting with specific antibodies was used to analyze the ATG7, ATG4B, and ATG12 protein expression in HepG2 and HepG2.2.15 cells at 4 h post-cultured in EBSS. Beta-actin was used as a protein loading control. The quantification of band intensity is shown in the panel below. Data is shown as mean with SEM of three independent experiments.

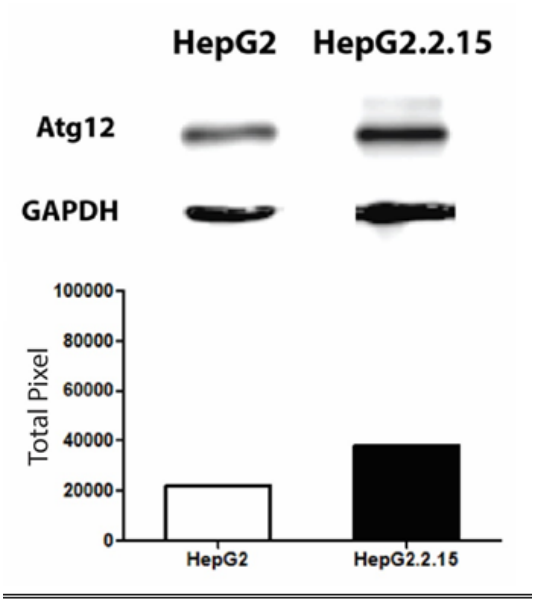

Figure 3. Western blotting analysis of ATG12 protein expression in HepG2 and HepG2.2.15 cells without starvation. GAPDH was used as a protein loading control. The quantification of band intensity is shown in the panel below.

Silencing of ATG12 in HBV-transfected cells induces interferon (IFN) signalling

It has been reported that HCV infection enhances IFN signalling in autophagy-knockdown immortalised human hepatocytes (IHHs).[15] Thus, we determined whether or not the knockdown of the ATG12 gene after HBV infection affects the IFN signalling pathway. To verify this, the mRNA expression of IFN-α, IFN-β and two interferon-inducible genes (IFI27 and Mx1) was analysed. Our results show that IFN-α, IFN-β and IFI27 were significantly up-regulated in shATG12 HepG2.2.15 cells when compared to mock treatment cells. However, no significant difference in the Mx1 gene was found in this study (Figure 4C). Therefore, these results suggest that inhibition of ATG12 enhances IFN signalling in HBV-transfected HepG2.2.15 cells.

Discussion

In this study, we analysed the role of autophagy-related genes (ATG4B, ATG7, and ATG12) on HBV infection. We determined the expression profile of these autophagic genes in HBV-transfected HepG.2.15 cells and parental HepG2 cells and found that ATG12 was significantly up-regulated in HepG2.2.15 cells when compared with HepG2 cells, which was consistent with our protein expression results. ATG12 is an ubiquitin-like protein that is important in the expansion and completion of the early steps of autophagosome formation.[16] Our functional study of ATG12 in HepG2.2.15 cells transfected with shATG12 showed that viral DNA was decreased. Moreover, we demonstrated that IFN-α and IFN-β as well as IFI27 mRNA expressions were up-regulated in shATG12-transfected HepG2.2.15 cells. This is consistent with the study of Shrivastava et al., which demonstrated that even in different families of viruses, after silencing of some autophagy genes in HCV-infected IHHs, an increased expression of IFN-α, IFN-β, and IFI27 was found.[15] Moreover, a previous report showed that ATG12-ATG5 conjugate inhibits type I IFN production through the caspase recruitment domains (CARDs) by binding with the retinoic acid-inducible gene I (RIG-I) and IFN-β promoter stimulator 1 (IPS-1) in mouse embryonic fibroblasts (MEFs).[17] Our result confirmed the role of ATG12 in IFN production, although we did not directly show the effect of ATG12-ATG5 conjugate.

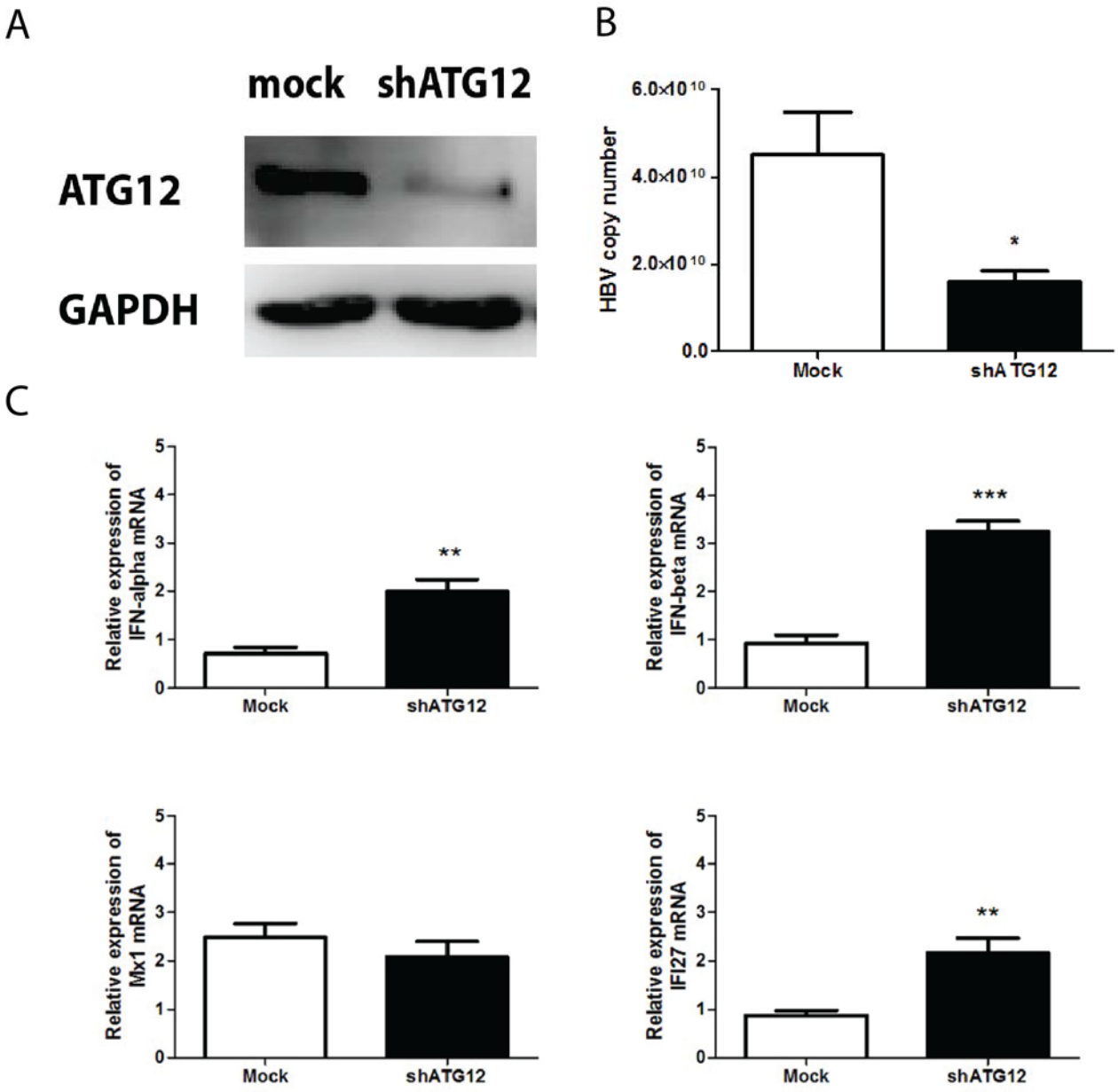

Figure 4. Silencing of ATG12 affects on HBV DNA and IFN expression. (A) Suppression of ATG12 expression by RNA interference. Western blotting method was applied to analyze ATG12 protein from mock treatment (control) and ATG12 knockdown (shATG12) HepG2.2.15 cells. (B) Quantitation of HBV DNA by real-time PCR. Total purified DNA from mock treatment (mock) and ATG12 knockdown (shATG12) in HepG2.2.15 cells were amplified using preS1 specific primers. HBV preS1 plasmid was used as standard copy number. Data is shown as mean with SEM of four independent experiments. (C) Quantitative real-time RT-PCR analysis of IFN-α, IFN-β, Mx1, and IFI27 mRNA in shATG12 and mock - HepG2.2.15 cells. Data is shown as mean with SEM of four independent experiments.

Therefore, we suggest that ATG12 is required for HBV replication and impediment of the IFN signalling pathway. These findings raise the possibility of targeting the autophagic pathway for the treatment of HBV patients.

Furthermore, our study showed that ATG7 was significantly down-regulated in HepG2.2.15 cells when compared with HepG2 cells under induction of autophagy. ATG7 is homologous to the E1 ubiquitin-activating enzyme, which is an essential enzyme in two ubiquitin-like protein conjugation

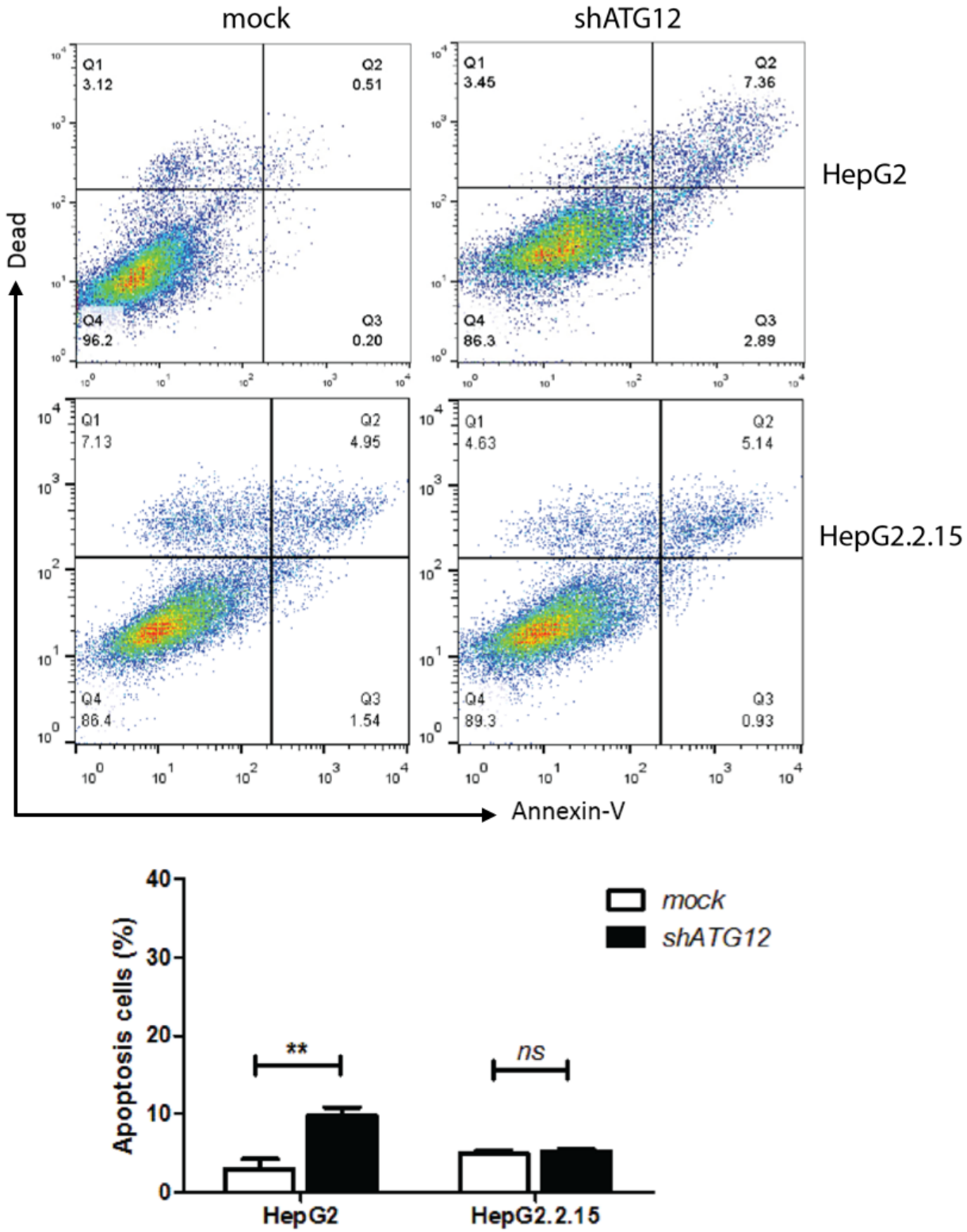

Figure 5. Apoptosis assays in HepG2 and HepG2.2.15 transfected with shRNA specific for *ATG12* or mock control (shNeg). Cells were transiently transfected with shRNA for 72h. Total apoptotic cells were detected by Annexin V binding. Values are mean with SEM of at least three independent experiments.

systems. It is required for the conjugation of ATG12 to ATG5 and the activation of ATG8 to form autophagosomes.[18,19] Several studies reported that autophagy was inhibited after siAtg7 transfection.[20,21] A study by Tanida et al. showed that the level of HCV particles in the medium was decreased by the ATG7-knockdown.[11] For HBV, however, a study by Sir et al. found that knocking down ATG7 with siRNA led to the suppression of HBV DNA replication, which was similar to the PI3KC3 results.[3] In order to verify our findings, further study of the overexpression of ATG7 is needed to clarify its biological function in HBV infection.

In terms of ATG4B, our results demonstrated that ATG4B was not significantly different between HepG2.2.15 cells and HepG2 cells. ATG4B is a dominant protease enzyme that cleaves the ATG8/LC3 for the conjugation process of ATG8/LC3 and phosphatidylethanolamine.[22] A previous study showed that ATG4B is required for HCV replication.[10] Nevertheless, our negative findings suggest that ATG4B might not participate in HBV infection.

In conclusion, our study demonstrated the role of autophagy machinery in HBV replication. We found that ATG12-knockdown reduces HBV DNA level in HepG2.2.15 cells and induces the IFN signalling pathway. Thus, this ATG12 machinery of autophagy may aid HBV survival by reducing antiviral innate immunity. However, there might be other autophagy components or other mechanisms involved in HBV persistence; therefore, clarification of the interaction between autophagy and other pathways in hepatitis B disease warrants future study.

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