Hepatitis B Virus Induces Autophagy to Promote its Replication by the Axis of miR-192-3p-XIAP Through NF kappa B Signaling

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Hepatitis B virus (HBV) is a major risk factor for the development and progression of hepatocellular carcinoma. It has been reported that viral infection can interfere with cellular microRNA (miRNA) expression and participate in the pathogenesis of oncogenicity. Here, we report that decreasing levels of the expression of the miRNA miR-192-3p is associated with rising levels of HBV DNA in the serum of HBV patients. We revealed that HBV infection repressed the expression of miR-192-3p through hepatitis B x protein interaction with c-myc. We further showed that miR-192-3p was repressed by HBV transfection *in vitro* and in a mouse model, leading to cellular autophagy. Using an miRNA target prediction database miRBase, we identified X-linked inhibitor of apoptosis protein (XIAP) as a target gene of miR-192-3p and demonstrated that miR-192-3p directly targeted the XIAP 3′-untranslated region of XIAP messenger RNA. Importantly, we discovered that HBV promoted autophagy through miR-192-3p-XIAP axis and that this process was important for HBV replication *in vitro* and *in vivo*. We demonstrated that miR-192-3p functioned through the nuclear factor kappa B signaling pathway to inhibit autophagy, thereby reducing HBV replication. *Conclusions:* Our findings indicate that miR-192-3p is a regulator of HBV infection and may play a potential role in hepatocellular carcinoma. It may also serve as a biomarker or therapeutic target for HBV patients. (HEPATOLOGY 2019;69:974-992).

Three hundred fifty million people worldwide are infected with hepatitis B virus (HBV), despite the availability of a vaccine that prevents its infection. Chronic infection of HBV is a major risk factor of hepatocellular carcinogenesis.

However, how HBV contributes to the development of hepatocellular carcinoma is still unclear.

Emerging evidence indicates that both autophagy and microRNAs (miRNAs) are involved in HBV replication and HBV-related hepatocarcinogenesis.[1,2]

miRNAs are a class of short, endogenous, noncoding RNAs that can regulate gene expression post-transcriptionally through binding to complementary sequences in the 3′-untranslated regions (3′-UTR) of the target transcripts.[3] Recently, miRNAs are thought to play an important role in HBV infection. It has been well documented that HBV infection can either activate or repress the expression of different cellular miRNAs.[2] Cellular miRNAs may participate in the elimination of viral infections in host cells by affecting different processes such as those important for viral replication.

It has been shown that HBV infection can cause autophagy in the host cells and that autophagy is important for HBV amplification in host cells.[4,5] Autophagy is a catabolic process by which long-lived proteins and damaged organelles are sequestered in the cytoplasm and removed for recycling. It is important for maintaining cellular homeostasis. Autophagy is also known as one of the host defense responses against infections.[6,7] Thus, some bacteria and viruses developed strategies to suppress or bypass cellular autophagy to ensure their survival. For example, herpes simplex virus-1[8] and Kaposi's sarcoma herpes virus[9] have evolved mechanisms to suppress autophagy for their survival. In contrast, other viruses have been shown to induce autophagy and often use it for their replication. These viruses include poliovirus,[10] hepatitis C,[11] and HBV.[4,5,12]

HBV makes use of autophagy during either its productive cycles or nonproductive infections or both.[13] HBV can affect autophagy through different pathways, such as hepatitis B x protein (HBx) binding to phosphatidylinositol-3-kinase (PI3K) C3 to enhance autophagy,[4] or activation of death-associated protein kinase in a pathway related to Beclin-1 by HBx to induce autophagy,[14] or direct up-regulation of Beclin-1 expression by HBx to increase autophagy,[15] suggesting that the HBx protein induces autophagy at the initiation stage of autophagic progression. Second, there are different reported effects of autophagy on HBV. Li et al. reported that HBV small-surface protein (SHBs)-induced autophagy does not affect the expression level of Beclin-1, and plays a major role during viral envelopment.[12] However, Tian et al. showed that autophagy had only a small effect on HBV RNA transcription and pregenomic RNA packaging, but was required for efficient HBV DNA replication.[4,5] Thus, further study on the role of autophagy on HBV is required for understanding the pathogenesis and biology of HBV.

The Atg family of proteins is critical for autophagy. Several of them, Atg4, Beclin-1 and LC3, have been shown to be the targets of miRNAs such as miR-30a and miR-204,[16,17] suggesting that miRNA can regulate autophagy. Here, we show that HBV levels are inversely correlated with the levels of cellular miRNA miR-192-3p in HBV patients as well as in cultured cells. We demonstrate that HBV induces autophagy through HBx interaction with c-myc to directly inhibit miR-192-3p expression. We further reveal that X-linked inhibitor of apoptosis protein (XIAP) is a direct target of miR-192-3p and that the miR-192-3p-XIAP axis promotes autophagy by increasing p-IκB (inhibitor of κB)-α to activate the nuclear factor kappa B (NF-κB) pathway, and eventually increases HBV replication. Our studies revealed a miRNA-mediated pathway by which HBV induces

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autophagy to promote its own replication, suggesting that targeting this pathway may offer an avenue for preventing and treating HBV pathogenesis.

Materials and Methods

PLASMIDS

The pHBV1.3 plasmid was a generous gift from Guangxia Gao (Chinese Academy of Sciences, China). pcDNA3.1-XIAP was generated. The human XIAP 3′-UTR of 717-bp bearing two predicted miR-192-3p target sites were cloned in the pMIR-REPORT (Applied Biosystems, Foster City, CA). The mutant 3′-UTR reporters were generated by using the mutated primers (Supporting Table S1). All constructs were confirmed by DNA sequencing.

CHEMICALS, ANTIBODIES, AND OTHER REAGENTS

Rapamycin, 3-methyladenine (3-MA), anti-glyceraldehyde 3-phosphate dehydrogenase, and anti-β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Anti-LC3I/II, anti-phospho-IκBα, and anti-total-IκBα antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-p62, anti-XIAP, anti-c-myc, anti-hemagglutinin (anti-HA), and anti-Beclin-1 antibodies were obtained from ABclonal (Woburn, MA). Small interfering RNAs (siRNAs) and the miR-192-3p mimics (or agomir)/inhibitor (or antagomir) and the respective negative controls were purchased from RiboBio (Guangzhou, China).

CELL CULTURE AND TRANSFECTION

HepG2, HepG2.2.15, and Huh7 cells were cultured as described.[18] Primary human hepatocytes (PHHs) were purchased from XenoTech LLC (Lenexa, KS; Shanghai, China) and were thawed and cultured with InVitroGRO CP complete medium for 24 hours. The cells were then infected with a multiplicity of 100 genome equivalents of HBV in the presence of 1% dimethyl sulfoxide (DMSO) and 4% PEG8000.[19] The InVitroGRO HI complete medium was changed every 2 days. After 4 days, the cells were transfected with miR-192-3p-agomir/antagomir or their negative control for 48 hours, and then both the PHHs and culture medium were harvested for analyses.

HBV VIRUS ISOLATION

Hep2.2.15 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% inactivated fetal bovine serum. After 4 days, HBV virus was concentrated from the medium culture.[20]

BLOOD SAMPLE PREPARATION AND MICRO-RNA ISOLATION

All patient serum samples were obtained from Zhongnan Hospital of Wuhan University. Written informed consent was obtained from each patient. miRNA was isolated from frozen serum samples using a serum/plasma miRNA Kit (Aidlab Biotechnology, Beijing, China) following the manufacturer's instructions.

REAL-TIME QUANTITATIVE PCR AND WESTERN BLOT ANALYSES

Total RNA, including miRNAs, and protein were prepared from cells or tissues. RNA was reverse-transcribed using the M-MLV Reverse Transcriptase Kit (Invitrogen; Thermo Fisher Scientific, Waltham, MA). Real-time quantitative PCR was performed using the SYBR Select Master Mix (Life Technologies, Carlsbad, CA). Protein concentration was determined using the bicinchoninic acid protein kit (Thermo Fisher Scientific).

LUCIFERASE ACTIVITY ASSAY

A recombind pMIR-Report luciferase vector, miR-192-3p or miR-NC, and pMIR-Report control vector were co-transfected into cells for 36 hours. The cells were lysed and subjected to luciferase activity assays by using the Dual-Glo system (Promega, WI).

AUTOPHAGOSOME FORMATION ASSAY BY CONFOCAL MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPY

Cells were co-transfected with HBV plasmid and/or miR-192-3p, XIAP plasmid, siXIAP as indicated. The cells were fixed and the nuclei were stained with 4-6-diamidino-2-phenylindole (DAPI, Promoter

Biotechnology). The fluorescence of green fluorescent protein (GFP)-LC3 was observed under a confocal fluorescence microscope (Leica LCS SP8 STED, German). Cells were fixed with 2.5% glutaraldehyde overnight and subjected to preparation for transmission electron microscopy (HT770; Hitachi, Tokyo, Japan) observation.

ENZYME-LINKED IMMUNOSORBENT ASSAYS

The hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) viral-secreted protein levels in the supernatant were determined using enzyme-linked immunosorbent assay (ELISA) kits (Kehua Bio-engineering, Shanghai, China).

SOUTHERN BLOT AND NORTHERN BLOT ANALYSIS

Extraction of HBV replicative intermediates was performed as described.[18] Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions, which were detected using the DIG Northern starter kit (Roche Diagnostics, Indianapolis, IN) for northern blot corresponding to nucleotides 1072 to 2171 of the HBV genome and for southern blot corresponding to nucleotides 157 to 1068 of the HBV genome as previously described.[18]

CO-IMMUNOPRECIPITATION

Cells were lysed in Lysis Buffer (20 mM Tris-Cl [pH 8.0], 150 mM NaCl, 2 mM ethylene diamine tetraacetic acid, 1% NP-40). After centrifugation, the supernatant was incubated with antibodies, and then protein A/G beads were added and the mixture was incubated overnight at 4°C. The beads were washed 3 times with washing buffer (20 mM Tris-Cl [pH 7.5], 300 mM NaCl). The proteins bound to the beads were analyzed by western blot.

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation (ChIP) assay was performed with Huh7 cells co-transfected with HBx-HA and c-myc-FLAG expression plasmids.

Briefly, cells were prepared after 4% formaldehyde (FA) or sequential disuccinimidyl glutarate–FA cross-linking, and ChIP assay was then performed using anti-HA, anti-FLAG, and anti-immunoglobulin G. The cross-links were reversed and the DNA was purified. The enriched DNA was detected by real-time quantitative PCR with a pair of primers.

HYDRODYNAMICS-BASED TRANSFECTION IN MICE

A mouse model of acute HBV infection was used in this study as described.[18] Briefly, pUC-HBV1.3 (10 μg) and 15-nmol agomir negative control or miR-192-3p agomir were injected into the tail veins together with XIAP expression construct or empty vector pcDNA3.1 (30 μg) within 6-8 seconds in a volume of saline equivalent to 10% of the mouse body weight. Sera were taken for analysis of HBsAg and HBeAg. Liver tissue was taken for southern blot analysis. The tissue proteins were visualized by immunohistochemical staining and checked by western blot. All mice were housed in a pathogen-free mouse colony, and the animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, People's Republic of China, 1998).

STATISTICAL ANALYSIS

All experiments were performed at least 3 times. The statistical significance was calculated using unpaired Student t test between two groups. The data were expressed as mean ± SD and $p < 0.05$ was considered to be statistically significant ($0.01 < {}^{*}P < 0.05$; $0.001 < {}^{**}P < 0.01$; and ${}^{***}P < 0.001$).

Results

SERUM HBV DNA IS INVERSELY CORRELATED WITH miR-192-3p LEVELS IN HUMAN PATIENTS AND HBV INFECTION DOWN-REGULATES miR-192-3p EXPRESSION IN CULTURED CELLS

Earlier studies have suggested that miR-192 is regulated by HBV,[21,22] while another study found no significant difference between HBV-infected hepatocellular

carcinoma tissues and normal hepatoma tissue.[1] To determine whether there is any correlation between HBV infection and miR-192-3p expression, we collected a large number of human patients and control serum samples and determined the levels of miR-192-3p in the control and different patient groups (Fig. 1A). The results revealed that at the low levels of HBV, there was no significant difference between the patients and control (normal), whereas patients with high levels of HBV had reduced miR-192-3p. Our findings indicate that miR-192-3p is inversely correlated with serum HBV levels and provides a possible explanation for the different findings in the earlier studies.

To investigate whether HBV infection affects the expression of miR-192-3p, we turned to culture cells. We transfected different hepatic cell lines with a HBV plasmid and examined the cellular miR-192-3p levels. The results showed that miR-192-3p expression was significantly decreased in HBV-transfected cells compared with the control (Fig. 1B middle and Supporting Fig. S1A). In addition, an analysis of the supernatant of the transfected cells revealed that the levels of miR-192-3p were also decreased in the supernatant of HBV-transfected cells compared with that in the control (Supporting Fig. S1B), consistent with the reduced expression in the transfected cells. Furthermore, miR-192-3p expression was also lower in the HBV-expressing stable cell line HepG2.2.15 compared with that in HepG2 (Supporting Fig. S1C). Finally, the down-regulation of miR-192-3p was HBV-dose-dependent (Fig. 1B right). Thus, HBV infection represses miR-192-3p expression.

HBV INHIBIT miR-192-3p EXPRESSION THROUGH HBx INTERACTION WITH C-MYC

To investigate the mechanism by which HBV down-regulates miR-192-3p, we transfected the plasmid-expressing viral proteins HBc, HBp, HBs, HBx, or its deletion mutant alone or with pGL3-miR-192-3p, and analyzed the miR-192-3p promoter activity and endogenous miR-192-3p expression. The results showed that HBx significantly decreased miR-192-3p promoter activity (Fig. 1C left) and endogenous miR-192-3p level (Fig. 1C middle), but HBx deletion mutant failed to inhibit miR-192-3p expression (Fig. 1C right). HBx was reported to indirectly regulate gene expression through its interactional transcriptional factors; for example, HBx

suppresses cellular miR-148a expression through interaction with p53.[23] It has been reported that HBx also interacted with c-myc.[24] To test whether HBx affected the expression of miR-192-3p through c-myc, we first confirmed the interaction of HBx and c-myc (Fig. 1D) and showed that HBx increased the c-myc protein level (Fig. 1E bottom). Importantly, our result demonstrated that knockdown of c-myc abrogated the ability of HBx to down-regulate the expression of miR-192-3p (Fig. 1E upper), and conversely, increasing levels of HBx led to a greater reduction in miR-192-3p expression both in the presence (Fig. 1E bottom) or absence (data not shown) of c-myc. To determine whether c-myc directly regulated miR-192-3p, reporter promoter constructs with various lengths of the miR-192 5′-flanking region (Fig. 1F upper-left panel) were generated and transiently co-transfected with c-myc expression plasmid or vector into cells. Analysis of the promoter activity revealed that the region of -476/-115 had potential binding sites of c-myc (Fig. 1F upper-right panel). Earlier studies suggested that c-myc bound to enhancer (E)-box sequences, typically CACGTG or CATGTG.[25] To explore the exact binding site for c-myc, we designed four pair primers flanking the potential c-myc binding site based on the (E)-box sequences (Fig. 1F, left panel) for ChIP assay. We showed that c-myc was recruited to a region around -193 to about -158 upstream of the miR-192 promoter, which contained CAATTG and CATGTG, but not HBx binding sequence (Fig. 1F right middle panel). The two-step cross-linking ChIP results indicated that HBx was also recruited to the same region through binding c-myc (Fig. 1F bottom-right panel). Taken together, these data suggest that HBV inhibits miR-192-3p expression through HBx interaction with c-myc.

HBV PROMOTES AUTOPHAGY BY DOWN-REGULATING miR-192-3p IN HEPATOMA CELLS

It has been reported that HBV replication involves autophagy,[4,5] and the HBV protein HBx, SHBs, or HBV itself promote autophagy in hepatocytes through a different mechanism.[4,12,15] However, so far, the effect of HBV on cell autophagy is still ambiguous.

To investigate whether the down-regulation of miR-192-3p is involved in HBV-induced autophagy, we first determined whether HBV transfection induced autophagy. As shown in Fig. 2A,

The nucleotide sequences of the 5'-flanking region (-724/+110) of the miR-192 gene

when Huh7 cells were transfected with GFP-LC3, a marker for autophagy, and treated with rapamycin, a well-known autophagy inducer, extensive autophagosome formation was observed (GFP+ puncta) (Fig. 2A). Importantly, when Huh7 cells were transfected with pUC18 (control) or pHBV1.3, autophagy was strongly induced in the HBV-transfected cells but not the control cells (Fig. 2A). Furthermore, western blot analysis showed an increase in the conversion of LC3-I to LC3-II in the rapamycin-treated or HBV-transfected cells but not the control cells. Similar results were obtained with HepG2 (Supporting Fig. S2). Thus, HBV could efficiently induce autophagy in hepatoma cells.

Because HBV transfection represses the expression of miR-192-3p, it is possible that miR-192-3p may affect HBV-induced autophagy. Thus, we next investigated whether miR-192-3p affected autophagy. We pretransfected the HepG2.2.15 with GFP-LC3 and subsequently transfected these cells with either a miR-192-3p mimic or inhibitor or the corresponding nonspecific controls. The results revealed that miR-192-3p mimic repressed HBV-induced autophagy, whereas miR-192-3p inhibitor increased autophagy in the transfected cells (Fig. 2B,C). To analyze the signaling pathways involved in miR-192-3p-induced autophagy, we used 3-MA, an inhibitor for PI3KC3, which diminishes autophagosome by reducing autophagy beginning,[26] and bafilomycin A1 (BafA1), a vacuolar ATPase inhibitor, which suppresses autophagic

flux involved in blocking the fusion between autophagosomes and lysosomes and as an inhibitor of lysosomal degradation.[27] After miR-192-3p mimics or inhibitor were transfected into cells, the cells were treated with or without 3-MA or BafA1. The results showed that no difference in LC3-II/LC3-I levels was observed in 3-MA treating cells (Fig. 2D upper panel, columns 3, 4, 7, and 8), and obviously different LC3-II/LC3-I levels were observed in BafA1-treating cells (Fig. 2D lower panel, columns 3 4, 7, and 8). These results suggest that miR-192-3p affected autophagy at the initiation stage.

Having demonstrated that miR-192-3p can regulate autophagy, we wanted to know whether it also affects HBV-induced autophagy in hepatoma cells. We co-transfected Huh7 with pHBV1.3 or control pUC18 and miR-192-3p mimics or negative mimics. Analyses of the autophagic proteins in the resulting cells showed that, as expected, HBV increased autophagic proteins Beclin-1 and LC3II, and reduced p62 (Fig. 2E). Importantly, the miR-192-3p mimics drastically reduced the effect on autophagy of HBV transfection (Fig. 2E). Furthermore, miR-192-3p mimics drastically reduced the levels of both the HBV-secreted proteins and the cellular HBV DNA (Fig. 2F upper), whereas miR-192-3p antagomir had the opposite effects (Fig. 2F bottom). These findings suggest that miR-192-3p inhibits HBV-induced autophagy, consequently leading to reduced HBV replication and propagation in the host cells.
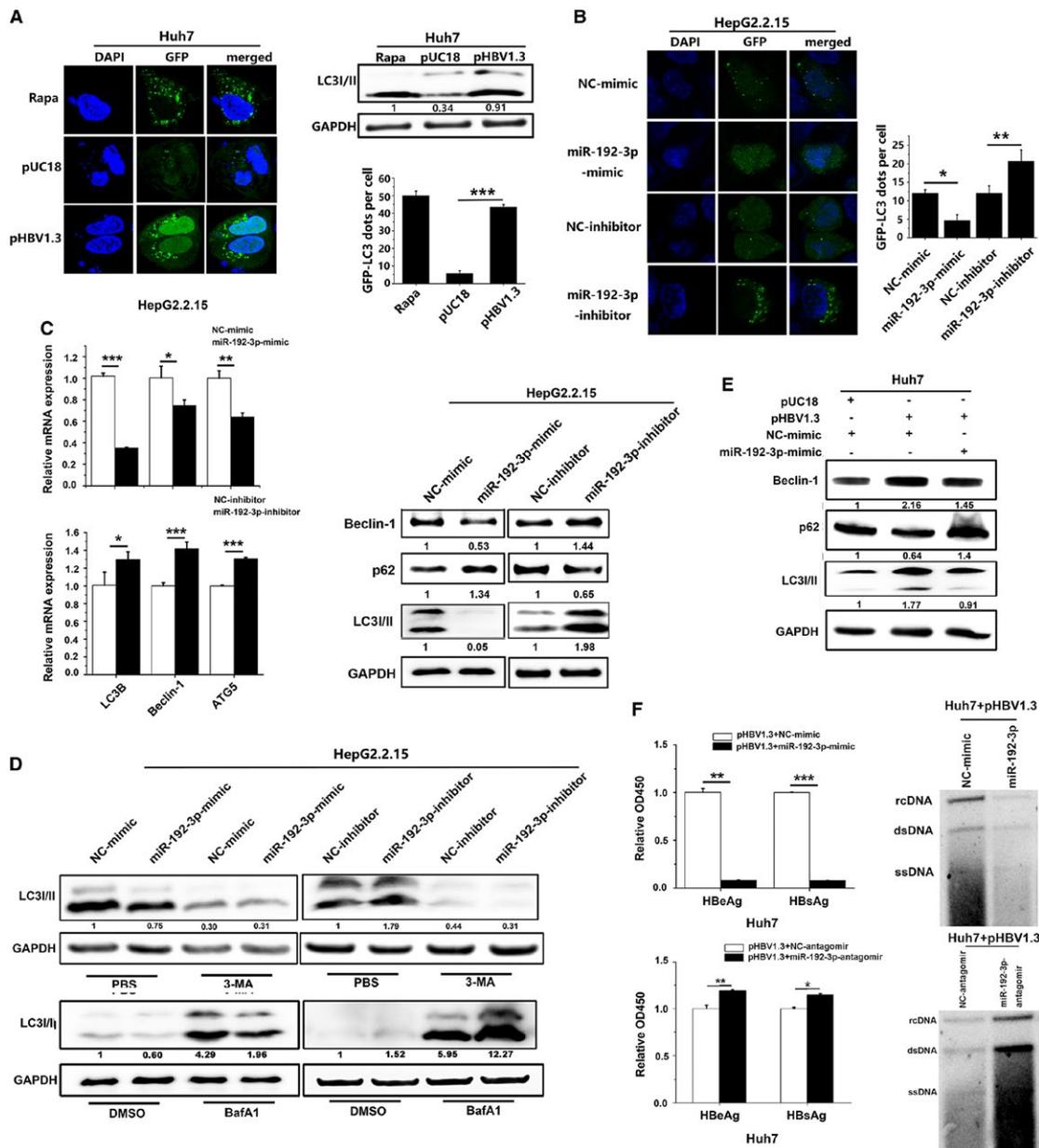
FIG. 2. HBV promotes autophagy by down-regulating miR-192-3p. (A) HBV induces autophagosome formation in hepatoma cells. The cells were stained with DAPI for DNA (blue nuclei) or the distribution of GFP-tagged LC3 was examined under a confocal fluorescence microscope. Representative confocal images are shown on the left. Quantification of autophagosomes by calculating the average number of green dots are shown in the lower right. Additionally, the level of LC3I/II was determined by western blot analysis (top right). (B) miR-192-3p inhibits autophagy. HepG2.2.15 cells, pretransfected with GFP-LC3 as in (A), were transfected with miR-192-3p mimic or inhibitor or the corresponding nonspecific controls. The LC3 puncta were visualized by confocal microscopy (left) and quantified by calculating (right). (C) The mRNA levels of ATG5, LC3B, and Beclin-1 (left panel) or the protein levels of Beclin-1, p62, and LC3I/II (right panel) were detected. (D) HBV promotes complete autophagic progression by down-regulating miR-192-3p. Western blot analysis of LC3-I/II accumulation after treatment with autophagy inhibitors (10 mM 3-MA [upper panel] and 100 nM BafA1 [lower panel]). (E) HBV-induced autophagy can be inhibited by miR-192-3p mimic. (F) miR-192-3p reduces the levels of HBV DNA and HBs/HBe Ag. Abbreviations: PBS, phosphate-buffered saline; and Rapa, rapamycin.

A

NC-inhibitor
miR-192-3p-inhibitor

HepG2.2.15

Relative mRNA expression

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

**

***

*

TRAF6 XIAP CXCR5 AP1S3 STAT3 ATF7 HNF4G MAVS IL15 IL11 NFAT5

B

293T

NC-mimic
miR-192-3p-mimic

Relative luciferase activity

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

*

Vector NFAT5 IL11 XIAP

C

HepG2.2.15

NC-mimic miR-192-3p-mimic NC-inhibitor miR-192-3p-inhibitor

XIAP

1   0.5   1   1.79

GAPDH

D

Site 1

5939   5959
5′ cacaauaaauaaaUAGGUCAa 3′   XIAP-UTRwt
            | | | | | | |
3′ ccgacaguuaaguAUCCAGUc 5′   hsa-miR-192-3p

5′ cacaauaaauaaaCGCCGGCc 3′   XIAP-UTRmt

Site 2

6112   6132
5′ aucucuaaauaaaUAGGUCAa 3′   XIAP-UTRwt
            | | | | | | |
3′ ccgacaguuaaguAUCCAGUc 5′   hsa-miR-192-3p

5′ aucucuaaauaaaCGCCGGCc 3′   XIAP-UTRmt

Huh7

NC-mimic
miR-192-3p-mimic

Fold change

1.2
1.0
0.8
0.6
0.4
0.2
0.0

**   **

pMIR XIAP-WT XIAP-M1 XIAP-M2 XIAP-DM

E

Relative XIAP mRNA expression

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

*

XIAP

1   1.27

GAPDH

pUC18 pHBV1.3

Huh7

F

Huh7

pUC18   +   -   -
pHBV1.3   -   +   +
NC-mimic   +   +   -
miR-192-3p-mimic -   -   +

XIAP

1   3.36   1.77

GAPDH

FIG. 3. XIAP is a direct target of miR-192-3p. (A) Regulation of candidate target genes by miR-192-3p. The miR-192-3p inhibitor increased the expression of XIAP, IL-11, and NFAT5. (B) XIAP but not IL-11 or NFAT5 is a direct target of miR-192-3p. A Firefly luciferase reporter construct (pMIR-REPORT) containing wild-type (WT) 3′-UTR of NFAT5 or IL-11 or XIAP was co-transfected with the control Renilla luciferase construct in the presence of miR-192-3p mimic or control. The relative Firefly luciferase activity was determined. (C) XIAP protein level is regulated by miR-192-3p in HepG2.2.15 cells. (D) Two miR-192-3p target sites are present in the XIAP 3′-UTR. Reporter constructs containing XIAP 3′-UTRs with or without mutations in one or both of the two potential miR-192-3p-binding sites (XIAP-UTR WT, M1[mut1], M2[mut2], or XIAP-UTR DM) were transfected into Huh7 cells in the presence of miR-192-3p mimic or scrambled control. The relative reporter activity was determined. (E) HBV induces XIAP expression. The amount of XIAP mRNA and protein were quantified by real-time quantitative PCR and western blot, respectively. (F) miR-192-3p suppresses the induction of XIAP by HBV.

XIAP IS A TARGET OF miR-192-3p

To investigate how miR-192-3p regulates autophagy, we searched the candidate target genes by TargetScan (www.targetscan.org) and miRbase (www.mirbase.org/). We analyzed putative targets gene expression in HepG2.2.15 cells treated with miR-192-3p inhibitor or nonspecific control and found that three of them, XIAP (X-linked inhibitor of apoptosis), interleukin (IL)-11 and nuclear factor of activated T cells 5 (NFAT5), were significantly up-regulated by miR-192-3p inhibitor (Fig. 3A). To determine whether these three candidate targets are regulated by miR-192-3p at the translation level, we cloned their 3′-UTRs into a luciferase reporter vector and analyzed their regulation by miR-192-3p. The results in Fig. 3B suggest that miR-192-3p is capable of binding to the 3′-UTR of XIAP messenger RNA (mRNA) to regulate translation. Consistently, when XIAP protein level was analyzed in HepG2.2.15 cells transfected with miR-192-3p mimic or inhibitor or the corresponding controls, it was repressed by the mimic but enhanced by the inhibitor (Fig. 3C).

To further analyze the translational regulation of XIAP by miR-192-3p, we mutated the putative target sites individually or altogether and analyzed the effect on the regulation by miR-192-3p (Fig. 3D). The results showed that mutating site 1 had little effect on the regulation by miR-192-3p, whereas site 2 was critical for the regulation by miR-192-3p, as mutating site 2 or both sites abolished the regulation by miR-192-3p (Fig. 3D).

This raises an interesting possibility that HBV infection regulates XIAP expression through miR-192-3p. Thus, we analyzed XIAP levels in Huh7 and HepG2 cells that were transfected with pHBV1.3 or control pUC18. The results showed that HBV infection increased XIAP levels (Fig. 3E and Supporting Fig. S3). The further study showed that when Huh7

cells were transfected with pHBV1.3 or control pUC18 in the absence of miR-192-3p mimic, XIAP was indeed up-regulated by HBV (Fig. 3F). More importantly, this up-regulation was drastically reduced by miR-192-3p mimics (Fig. 3F). Together these results show that HBV down-regulates miR-192-3p, leading to enhancement of XIAP expression.

HBV-INDUCED AUTOPHAGY THROUGH REGULATION OF THE miR-192-3p-XIAP AXIS

XIAP has long been well known for its anti-apoptotic function. More recently, it has been reported that XIAP also participates in the regulation of autophagy.[28] To investigate whether miR-192-3p regulates XIAP to affect apoptosis or/and autophagy during HBV infection, we studied both apoptosis and autophagy in cell cultures. By assaying apoptosis with flow cytometry, we showed that knocking down miR-192-3p did not affect apoptosis in HepG2.2.15 cells (data not shown). Similarly, when Huh7 cells were co-transfected with miR-192-3p mimic and pHBV1.3 plasmids, there was no effect on apoptosis by the miR-192-3p mimic (data not shown). These results indicate that miR-192-3p does not affect apoptosis by regulating XIAP under HBV infection condition.

We next investigated whether miR-192-3p regulated autophagy through XIAP. First, we transfected XIAP expression plasmid or interference RNA (siXIAP) into HepG2.2.15 pretransfected with GFP-LC3. Analysis of the autophagosomes with the GFP-LC3 fluorescence showed that XIAP overexpression promoted autophagy, whereas siXIAP inhibited the basal level of autophagy in the cells (Fig. 4A). The results were also confirmed by real-time quantitative PCR and western blot analyses of the autophagic proteins (Fig. 4B,C).
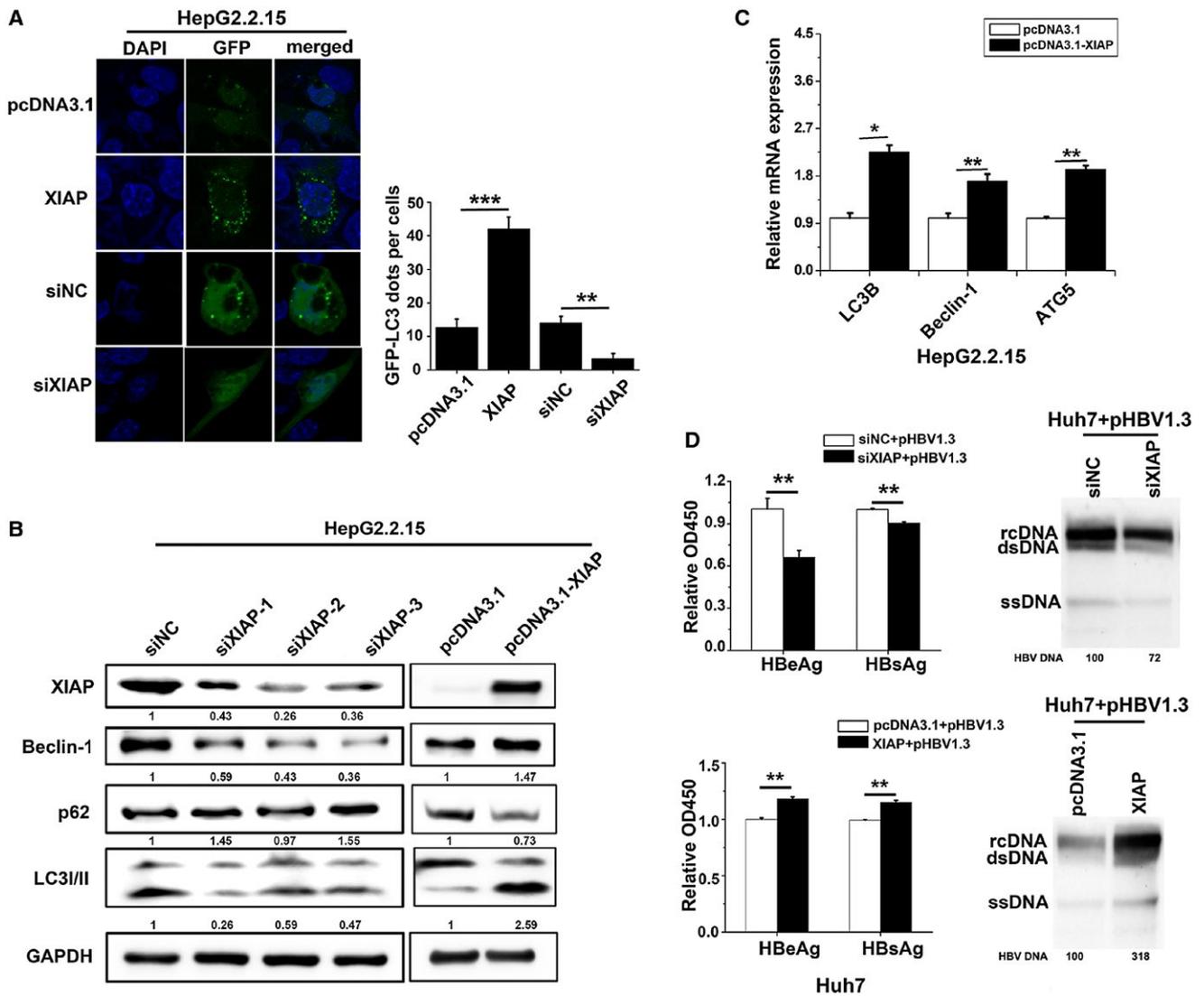
FIG. 4. XIAP promotes autophagy. (A) Autophagy is regulated by XIAP. Confocal microscopy images showed that overexpressing XIAP increased autophagy, whereas knocking down XIAP reduced it. The right panel shows quantification of GFP-LC3 puncta numbers. (B,C) XIAP affects the levels of autophagic mRNAs and proteins. (D) XIAP promotes HBV replication and protein expression. ELISA analysis of HBsAg and HBeAg in the supernatant, the cells were isolated for southern blot analysis of HBV DNA level. The southern blots on the right were scanned to quantify the relative DNA level as shown in the figure.

To determine whether XIAP has the similar function as miR-192-3p inhibitor in cells transfected by HBV, we co-transfected Huh7 cells with pHBV1.3 and XIAP overexpression or knockdown plasmid. The results showed that XIAP knockdown reduced viral protein secretion and viral DNA level in the cells, whereas XIAP overexpression had the opposite effects (Fig. 4D).

These findings suggest that HBV represses miR-192-3p, leading to increased XIAP expression, which in turn facilitates HBV replication and propagation. To

test this hypothesis, we transfected both miR-192-3p inhibitor and siXIAP plasmids into HepG2.2.15 cells pretransfected with the autophagy marker GFP-LC3. The results showed that siXIAP abrogated the increase in autophagy by miR-192-3p inhibitor (Fig. 5A). Similar conclusions were obtained with western blot analyses of the autophagic proteins (Fig. 5B). Furthermore, when miR-192-3p inhibitor and siXIAP plasmids were co-transfected into HepG2.2.15 cells, we found that as expected, inhibiting miR-192-3p

A

B

C

D

E

F
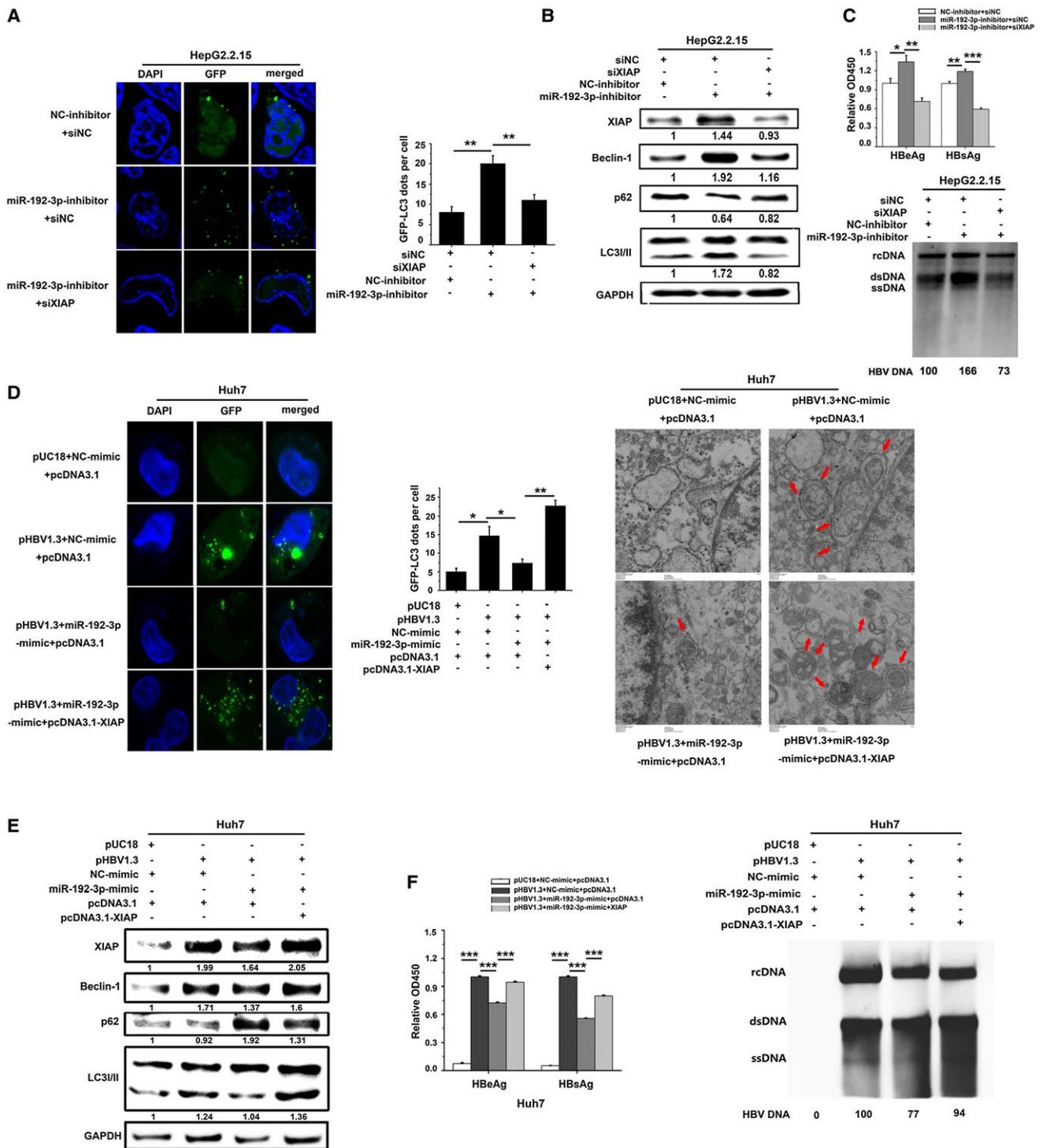
FIG. 5. HBV induces autophagy through the miR-192-3p-XIAP axis. (A) XIAP is required for miR-192-3p-induced autophagy. The GFP-LC3 puncta were visualized by confocal microscopy and quantified (right). (B) The miR-192-3p-XIAP axis regulates the autophagic protein levels. The levels of XIAP, Beclin-1, p62, and LC3-I/II proteins were determined by western blot in HepG2.2.15 cells. (C) miR-192-3p impairs HBV replication by targeting XIAP. The supernatant was assayed for HBsAg or HBeAg (upper panel). the amounts of nucleocapsid-associated HBV DNA were determined by southern blot analyses (bottom panel). The southern blot was scanned to quantify the relative DNA level as shown in the figure. (D) miR-192-3p inhibition of HBV-induced autophagy was rescued by XIAP overexpression. LC3 puncta were visualized by confocal microscopy (left) and quantified (middle). Images from transmission electron microscopy showing characteristic autophagosomes (red arrows) (right). (E) Autophagic protein levels. Cells were transfected as in (D), and the levels of XIAP, Beclin-1, p62, and LC3-I/II were examined by western blot. (F) miR-192-3p inhibits, whereas XIAP enhances, HBV replication. The supernatants of the cells above were assayed for HBsAg/HBeAg (left panel). The relaxed-circle, double-strand, and single-strand HBV DNA were analyzed by southern blot (right panel).

enhanced the levels of both HBV-secreted protein and DNA, and more importantly, knocking down XIAP simultaneously in such cells reduced the levels of both HBV secreted protein and DNA (Fig. 5C). In addition, in Huh7 cells with co-transfected HBV plasmid, miR-192-3p mimics reduced autophagosome formation induced by HBV as revealed by either fluorescence microscopy or electron microscopy (Fig. 5D). This inhibition by miR-192-3p mimics was reversed when XIAP was also overexpressed (Fig. 5D). Again, western blot analyses of the autophagic proteins confirmed these findings (Fig. 5E). Finally, southern blot and ELISA analyses of the HBV DNA and secreted protein levels showed that miR-192-3p mimic reduced their levels, whereas overexpression of XIAP prevented this inhibition (Fig. 5F). Taken together, these results indicate that HBV induces autophagy through miR-192-3p targeting to XIAP and that autophagy may be important for HBV replication.

miR-192-3p-XIAP AXIS REGULATES NF-κB SIGNALING TO AFFECT AUTOPHAGY

In addition to XIAP functioning as an E3 ubiquitin ligase, Wu et al. reported that XIAP inhibited autophagy through the XIAP-Mdm2-p53 pathway.[28] However, Lin et al. found that XIAP activated NF-κB to trigger Beclin-1-dependent autophagy.[29] Our findings showed that XIAP promoted autophagy, accompanied by increased Beclin-1 expression. This suggests that HBV-induced autophagy may involve NF-κB signaling. Thus, we investigated whether miR-192-3p-XIAP regulated NF-κB signaling. Western blot analyses showed that in HepG2.2.15 cells, miR-192-3p mimics increased the level of total IκBα but decreased the levels of p-IκBα and Beclin-1 (Fig. 6A, left panel), and miR-192-3p inhibitor decreased the level of total

IκBα and increased the levels of p-IκBα and Beclin-1 (Fig. 6A, middle panel). In addition, siXIAPs had similar effects as miR-192-3p mimics (Fig. 6A, right panel). When miR-192-3p inhibitor and siXIAP were co-transfected into HepG2.2.15 cells, siXIAP reduced the effects of miR-192-3p inhibitor on the levels of total IκBα, p-IκBα, and Beclin-1 (Fig. 6B).

In Huh7 cells transfected with HBV, the levels of p-IκBα and Beclin-1 were increased compared with the control cells, whereas the level of total IκBα was reduced. Co-transfection of miR-192-3p mimics inhibited these effects of HBV (Fig. 6C). When both miR-192-3p mimics and XIAP overexpression plasmid were co-transfected with HBV, XIAP countered the effects of miR-192-3p mimics (Fig. 6C). These findings indicate that HBV transfection leads to activation of IκBα signaling through the miR-192-3p-XIAP axis.

To test the function of the IκBα signaling pathway in HBV-induced autophagy, we investigated the effect of a specific inhibitor of the NF-κB pathway, SC-514.[30] Huh7 cells transfected with GFP-LC3 were pretreated with SC-514 and then transfected with HBV. Microscopic analysis of autophagosome showed that inhibiting IκBα signaling with SC-514 resulted in drastically reduced HBV-induced autophagy (Fig. 6D). This was also confirmed by western blot analyses of autophagic proteins (Fig. 6E). Interestingly, SC-514 had little effect on XIAP expression (Fig. 6E), suggesting that IκBα signaling is downstream of XIAP. Finally, the inhibitor also inhibited HBV replication, leading to reduced secreted HBV proteins and DNA (Fig. 6F). The results were further confirmed with another specific inhibitor of the NF-κB pathway, SN50 (Supporting Fig. S4).[31] These results indicate that HBV induces autophagy through the miR-192-3p-XIAP-NF-κB axis and that the HBV-inducing autophagy is crucial for HBV propagation.
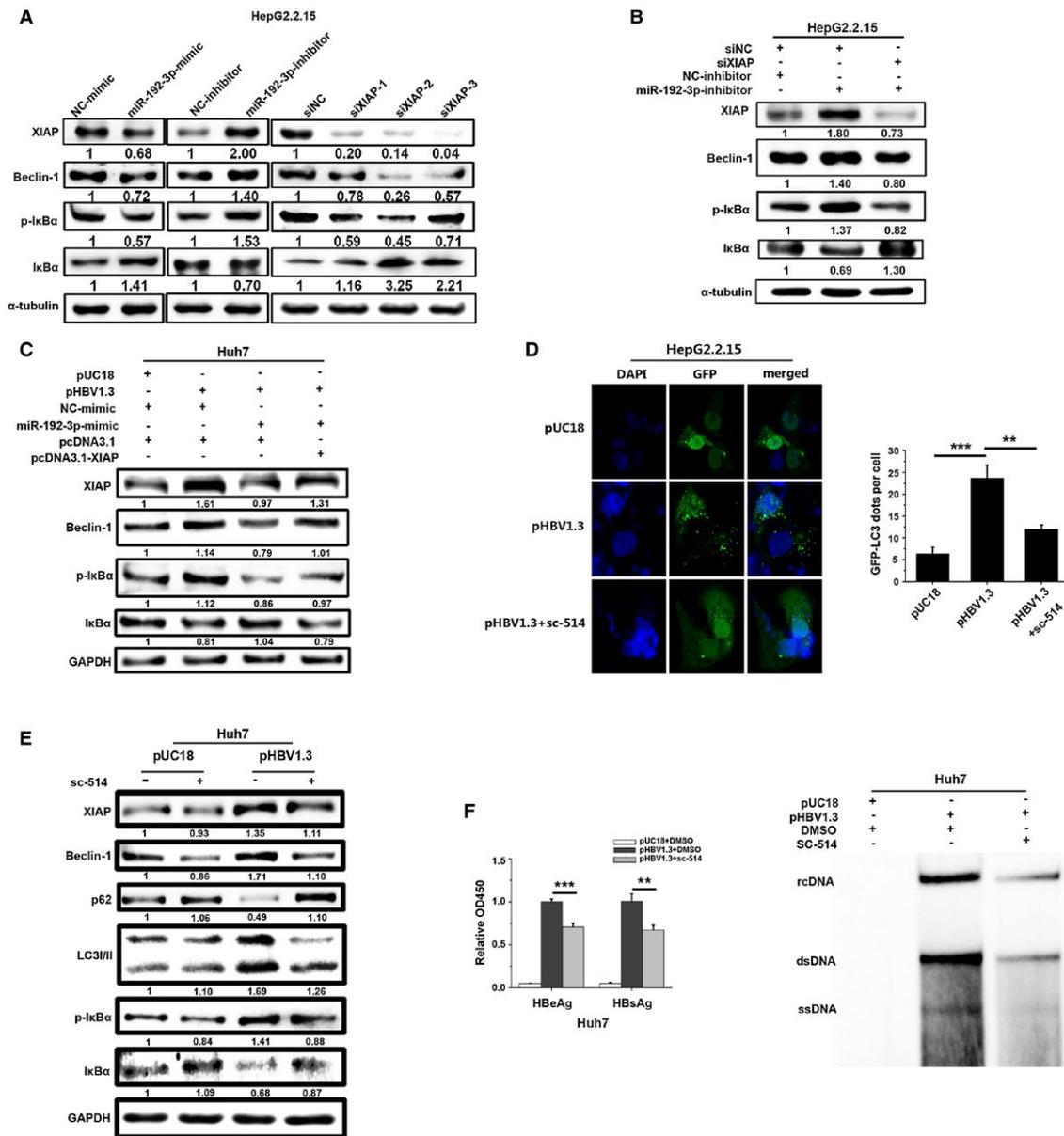
FIG. 6. HBV promotes autophagy through regulation of the NF-κB by the miR-192-3p-XIAP axis. (A) miR-192-3p or XIAP siRNAs suppresses the phosphorylation of IκBα. The expression of XIAP, Beclin-1, p-IκBα, and IκBα were explored by western blot analysis. (B) XIAP siRNAs-inhibited miR-192-3p-inhibitor induced phosphorylation of IκBα. The levels of XIAP, Beclin-1, p-IκBα, and IκBα were detected. (C) Huh7 cells were co-transfected with pHBV1.3, miR-192-3p mimic and pcDNA3.1-XIAP, or their corresponding controls, as indicated. Forty-eight hours later, XIAP, Beclin-1, p-IκBα, and IκBα levels were examined by western blot analysis. (D) Inhibiting the NF-κB signaling pathway reduces HBV-induced autophagy. Huh7 cells were pretreated with 5-mM NF-κB inhibitor SC-514 or DMSO for 4 hours followed by co-transfection with GFP-LC3 and pHBV1.3 or control pUC18. Forty-eight hours later, the cells were visualized by confocal microscopy for GFP-LC3, and GFP-LC3 puncta were quantified in the right panel. (E) Proteins extracted from the cells above were subjected to western blot analysis of XIAP, p62, LC3I/II, Beclin-1, p-IκBα, and IκBα. (F) NF-κB inhibitor reduces HBV replication. The supernatants of the cells above were assayed for HBs/HBe Ag (left). Viral DNA were analyzed by southern blot analysis (right).
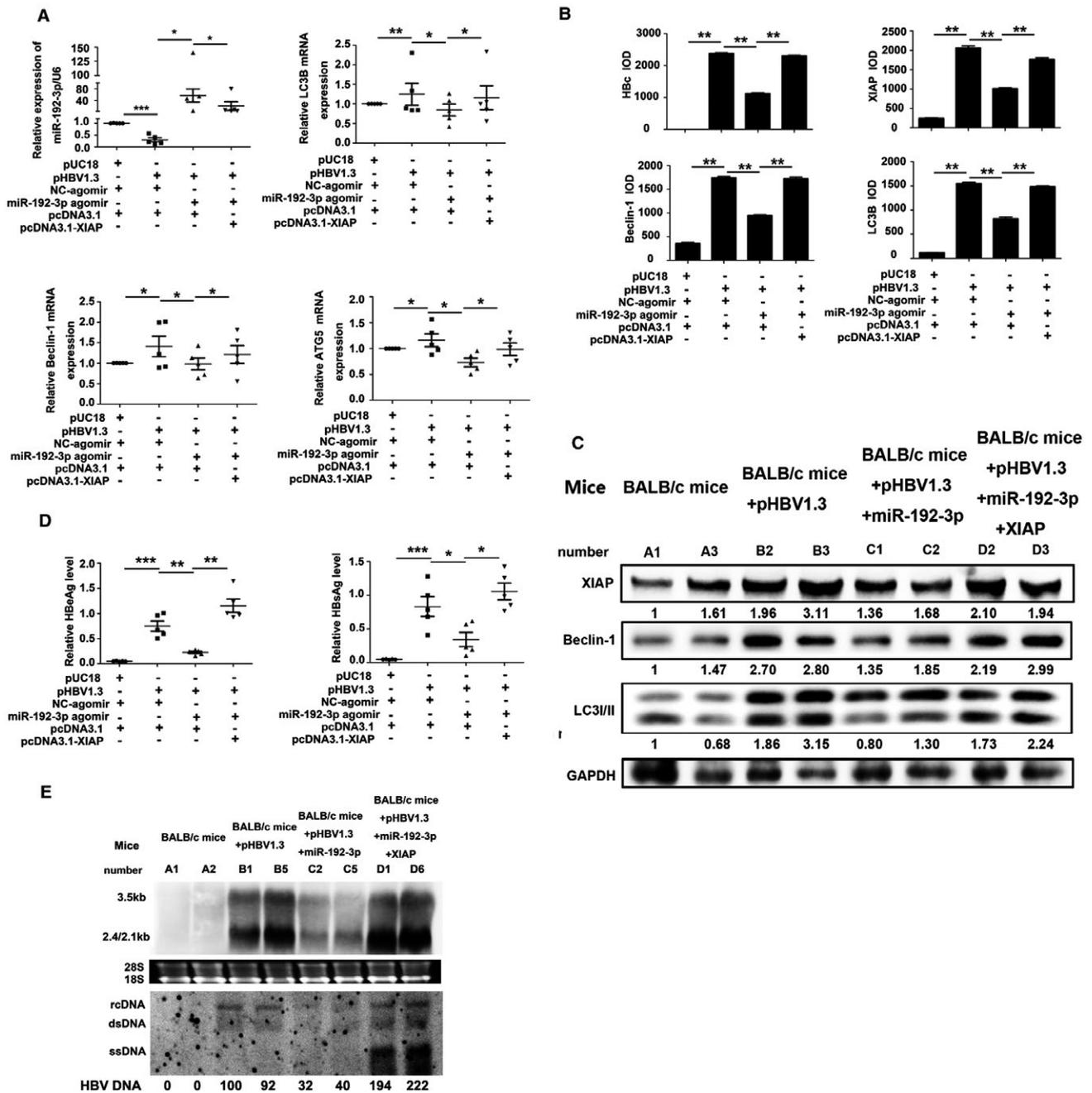
FIG. 7. HBV replication and autophagy are regulated by the miR-192-3p-XIAP axis *in vivo*. (A) Regulation of miR-192-3p, XIAP, and autophagy by HBV *in vivo*. The expression of miR-192-3p, Beclin-1 and LC3B, and ATG5 in the mouse liver were determined by real-time quantitative PCR. (B,C) The autophagic pathway is activated by HBV *in vivo*. Quantitative analysis of immunohistochemical staining is shown for HBc Ag, XIAP, Beclin-1, and LC3I/II (B), and western blot (C) for XIAP, Beclin-1, and LC3I/II in the liver of the mice above. (D) miR-192-3p inhibits the expression of HBsAg and HBeAg *in vivo*. Their levels in the blood of the HBV-infected mice above were quantified by ELISA. (E) miR-192-3p reduces the levels of HBV DNA and RNA transcripts *in vivo*. Their levels in the liver were analyzed by southern blot or northern blot analysis, respectively. The rRNAs (18S and 28S) were used as loading controls. The steady-state levels of HBV RNA were remarkably inhibited following miR-192-3p overexpression, whereas XIAP overexpression overcame the effect of miR-192-3p. Abbreviations: BALB/c, Bagg albino.
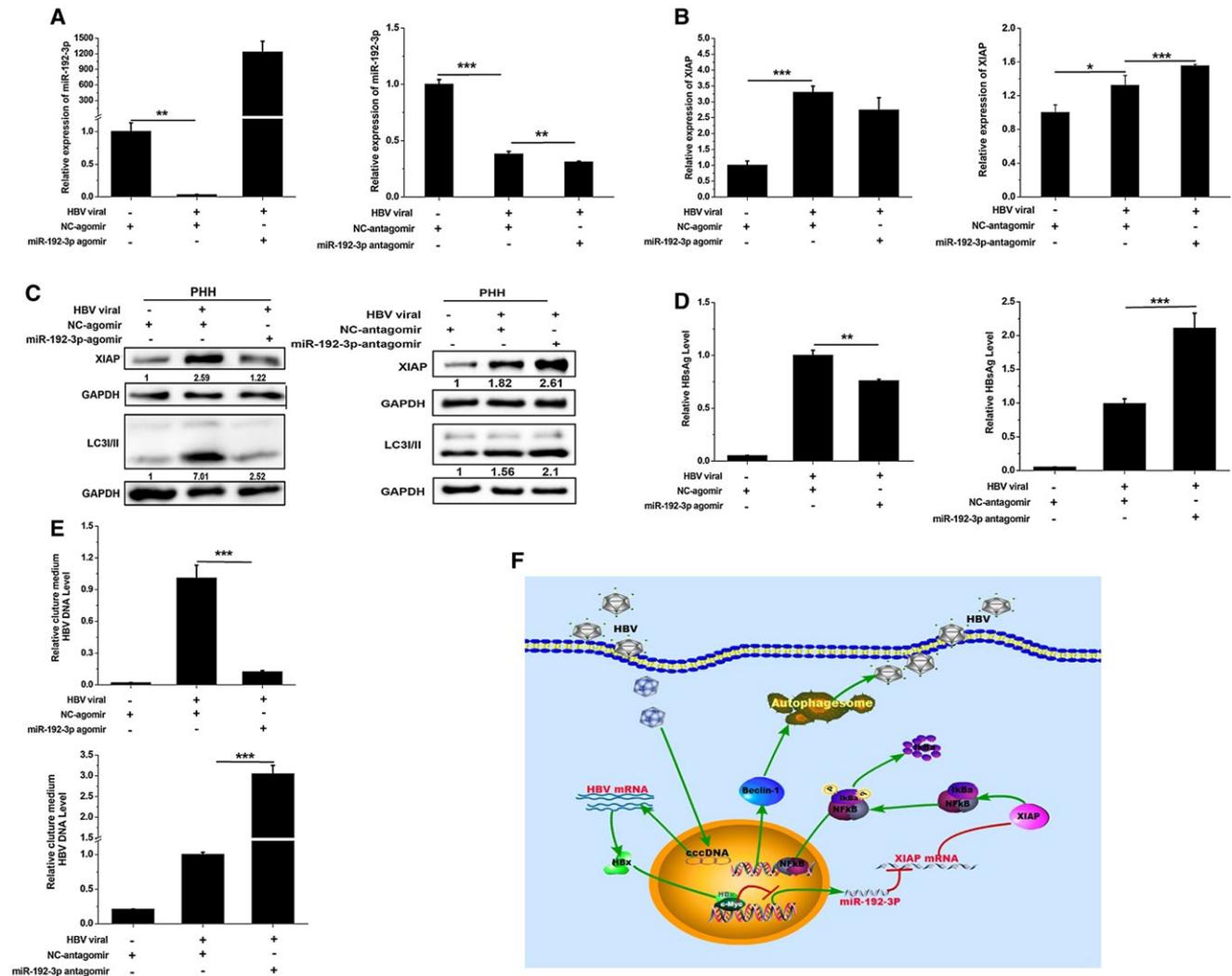
FIG. 8. HBV replication and autophagy are regulated by the miR-192-3p-XIAP axis in PHHs. (A-C) The levels of miR-192-3p (A), XIAP (B,C), and autophagy, as reflected by LC3I/II level (C), were regulated by HBV in an miR-192-3p-dependent manner in PHHs. The cells were infected with HBV and treated with miR-192-3p agomir/antagomir or control before analyses. (D,E) miR-192-3p reduces HBV replication in PHHs. The supernatants of the cells above were assayed for HBsAg (D) and viral DNA (E). (F) A proposed model describing HBV down-regulating miR-192-3p, which targets XIAP to induce autophagosome, leads to increased HBV transcription and HBV replication through the NF-κB pathway.

HEPATITIS B VIRUS USES THE miR-192-3p-XIAP AXIS TO INDUCE AUTOPHAGY FOR ITS PROPAGATION IN A MOUSE MODEL AND PRIMARY HUMAN HEPATOCYTES

Having discovered a pathway by which HBV regulates autophagy for its propagation in cell cultures, we next asked whether the mechanism functions *in vivo* with primary human hepatocytes. We introduced

pHBV1.3, miR-192-3p mimics, and XIAP or control plasmids into mice through hydrodynamic injection and sacrificed the mice 4 days later. The mouse liver was obtained to examine the markers for autophagy (LC3B and Beclin-1) and the HBV propagation (HBcAg). Consistent with the results in the cells, real-time quantitative PCR, western blot, and immunohistochemical analyses of the liver samples showed that miR-192-3p inhibited HBV-induced autophagy, whereas its target XIAP rescued the effects of miR-192-3p (Fig. 7A-C). In addition, analyses of

the HBV protein levels of HBeAg and HBsAg and DNA/RNA level in the blood and liver of the mice showed that miR-192-3p mimic reduced HBV propagation, whereas co-expression of XIAP abolished the effects of miR-192-3p mimic (Fig. 7D,E).

To determine whether the findings are true for primary human cells, we infected PHHs with HBV viruses. The results were consistent with the findings in hepatoma cell lines (Fig. 8A-E). Taken together, these results demonstrate that miR-192-3p-XIAP regulates HBV-induced autophagy, which in turn enhances HBV propagation *in vivo* (Fig. 8F).

Discussion

Earlier studies have shown that HBV-mediated cell survival is associated with starvation-induced autophagy in hepatic cells. Hepatitis B virus[4,13] and one of its encoded proteins HBx,[14,32] SHBs,[12] can enhance the autophagic process to enhance viral replication. However, the detailed molecular pathway remains unexplored. Our results discovered a pathway in which HBV induces autophagy by inhibiting miR-192-3p expression, leading to enhanced expression of the miR-192-3p target XIAP, which in turn activates the NF-κB pathway to promote autophagy in HepG2.2.15 and in HepG2 or Huh7 cells transiently transfected with HBV. We further show that the miR-192-3p-XIAP-NF-κB-induced autophagy is critical for HBV replication in cell cultures as well as in mouse model (Fig. 8F).

HBV is known to alter cellular miRNA expression.[33] MicroRNAs are noncoding single-stranded RNA molecules. Pre-miRNA transcripts typically contain several miRNA precursors, and during the process of maturation, generally one arm of each processed double-stranded miRNA precursor becomes functional. The other "passenger" strand denoted with "-3p" is normally degraded; however, in some cases both strands of the duplex become functional miRNAs that target different mRNA populations.[34] Recently, it was shown that miR-192-3p could serve as a regulator of adipocyte differentiation and/or lipid metabolism.[35] We have demonstrated here that HBV level is inversely correlated with the expression of miR-192-3p in a large collection of human HBV infection patient serum samples and that HBV down-regulates the expression of miR-192-3p in liver

hepatoma cell lines. Some earlier studies suggested that the regulation of miR-192 by HBV,[21,22] even though another study failed to show any effect due to HBV infection.[1] These earlier studies had fairly small patient sample sizes, which may contribute to the different findings. Our analysis used a large number of human patients and control serum samples and revealed that at the low levels of HBV, there was no significant difference between the patients and control (normal). However, patients with high levels of HBV had reduced miR-192-3p. This suggests that the effect of HBV on miR-192-3p is HBV-dose-dependent, and only at a high dose of HBV is miR-192-3p inversely correlated with serum HBV levels. Our results therefore also provide a possible explanation for the different findings in the earlier studies.

Our studies demonstrate that XIAP is a direct target of miR-192-3p. XIAP is well known as an inhibitor of caspases, and its overexpression in cancer cells delays and prevents apoptosis.[36] Interestingly, our results indicate that under HBV infection conditions, miR-192-3p does not regulate apoptosis through XIAP, suggesting that XIAP may have a different function. Huang et al. reported that XIAP inhibited autophagy through XIAP-Mdm2-p53 signaling.[28] However, Lin et al. showed that XIAP induced autophagy by up-regulating the transcription of Beclin-1 through the NF-κB signaling pathway.[29] Therefore, XIAP may have different effects on cells depending on the cell types and/or biological processes. Our findings demonstrate that both in different hepatoma cells and *in vivo*, XIAP functions as a promoter of HBV-induced autophagy. Furthermore, we have demonstrated that HBV activates the NF-κB signaling pathway through miR-192-3p-XIAP. The NF-κB signaling in turn leads to increased autophagy in the HBV-infected cells. The role of NF-κB in HBV replication may be complex. There have been reports showing that the activation of NF-κB inhibits HBV replication,[37] although other studies have demonstrated that the activation of NF-κB induces HBV replication.[38] Clearly, further studies are needed to investigate this interesting question.

The accumulation of autophagosomes is an intermediate process within the autophagic flux, which reflects the balance between the rate of their generation and conversion into autolysosomes. Thus, HBV-induced autophagosome accumulation may reflect three possibilities: (1) HBV induces completed

autophagy; (2) HBV induces incomplete autophagy; or (3) HBV simply suppress basic autophagic flux. Tang et al. believed that HBx induces autophagy in a Beclin-1-dependent manner at the initiation stage of autophagic progression.[15] However, Liu et al. showed that HBx induced autophagosome formation on the late stage of autophagy, in which HBx dramatically impaired the lysosomal degradative capacity.[32] Tang et al. suggested that HBx fails to stimulate the formation of autophagosome but sensitizes starvation-induced autophagosomes by up-regulating Beclin-1 expression.[15] In contrast, Liu et al. partly support the results of Sir et al. that HBx is sufficient to induce autophagosomes.[32] Until now, the molecular mechanism underlying HBV-induced autophagy has been a subject of much debate. Our results suggest that HBV induces autophagy at the initiation stage of the autophagic process through the interaction of HBx and c-myc to affect miR-192-3p-XIAP, which in turn regulates Beclin-1.

In summary, we discovered that miR-192-3p is a regulator of HBV-induced autophagy *in vitro* and *in vivo*. It functions as an autophagy inhibitor in host cells. When HBV infects the host cells, it increases autophagy through the miR-192-3p-XIAP-NF-κB pathway to facilitate HBV propagation (Fig. 8F). Furthermore, our analyses of clinical HBV patient samples indicate that the down-regulation of miR-192-3p may serve as a biomarker for HBV. More importantly, our discovery of an important role of the miR-192-3p-XIAP-NF-κB pathway in HBV-induced autophagy and propagation suggests possible therapeutic avenues to target any component of this pathway for the prevention and treatment of HBV-dependent human diseases.

Author contributions: Study design: G.S., M.G., and D.G.; data collection: J.W., J.C., Y.L., X.Z., M.W., S.W., Q.X., F.S., X.Y., Y.X., Y.C., C.L., and L.C.; statistical analysis: G.S., J.W., and J.C.; manuscript writing: G.S., J.W., and M.G.; critical revision of the manuscript: G.S., Y.B.S., D.G., and M.G. All authors read and approved the final manuscript.

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Supporting Information

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