The microRNA-99 family modulates hepatitis B virus replication by promoting IGF-1R/PI3K/Akt/mTOR/ULK1 signaling-induced autophagy

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Abstract
MicroRNAs are small highly conserved noncoding RNAs that are widely expressed in multicellular organisms and participate in the regulation of various cellular processes including autophagy and viral replication. Evidently, microRNAs are able to modulate host gene expression and thereby inhibit or enhance hepatitis B virus (HBV) replication. The miR-99 family members are highly expressed in the liver. Interestingly, the plasma levels of miR-99 family in the peripheral blood correspond with HBV DNA loads. Thus, we asked whether the miR-99 family regulated HBV replication and analyzed the underlying molecular mechanism. Compared with primary hepatocytes, miR-99 family expression was downregulated in hepatoma cells. Transfection of miR-99a, miR-99b, and miR-100 markedly increased HBV replication, progeny secretion, and antigen expression in hepatoma cells. However, miR-99 family had no effect on HBV transcription and HBV promoter activities, suggesting that they regulate HBV replication at posttranscriptional steps. Consistent with bioinformatic analysis and recent reports, ectopic expression of miR-99 family attenuated IGF-1R/Akt/mTOR pathway signaling and repressed insulin-stimulated activation in hepatoma cells. Moreover, the experimental data demonstrated that the miR-99 family promotes HBV replication posttranscriptionally through IGF-1R/PI3K/Akt/mTOR/ULK1 signaling-induced autophagy.

KEYWORDS
autophagy, hepatitis B virus, IGF-1R/PI3K/Akt/mTOR/ULK1 signaling-induced autophagy

1 | INTRODUCTION

Hepatitis B virus (HBV) is an enveloped, double-stranded DNA virus and can cause acute and chronic hepatitis in humans (Tong & Revill, 2016). Chronic HBV infection remains a global public health problem, affecting an estimated 240 million individuals (Schweitzer, Horn, Mikolajczyk, Krause, & Ott, 2015). Patients with chronic HBV infection have a significantly increased risk from severe liver diseases, cirrhosis, and hepatocellular carcinoma (HCC; Aravalli, Steer, & Cressman, 2008; Schweitzer et al., 2015). The current treatments for patients with chronic HBV infection include interferon-a and nucleoside analogues; however, they are limited by low rates of sustained response, side effects, and the emergence of drug resistance (Zoulim & Durantel, 2015). The key obstacle against curing chronic hepatitis B is the inability to eradicate or inactivate covalently closed circular DNA (Lucifora et al., 2014; Nassal, 2015). Thus, it is urgent to understand HBV–host interactions at the molecular level and to identify novel molecular targets for HBV therapy.

HBV replication is regulated by many extracellular and intracellular factors, such as specific hormones, inflammatory cytokines, and intracellular signaling pathways (Ma et al., 2012; Prange, 2012; Schoggins & Rice, 2011; Wang, Yeh et al., 2012). Among the relevant intracellular pathways, the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is a major cellular pathway involved in regulating HBV infection (Guo et al., 2007; Rawat & Bouchard, 2015). Guo et al. (2007) reported that HBV replication could be inhibited by activation of the PI3K/Akt signal pathway. This mechanism is likely in part responsible for the reduced HBV replication observed in tumor cells, which may show
activation of the PI3K/Akt pathway. Moreover, hepatitis B surface antigen (HBsAg) synthesis may also be regulated through the PI3K/Akt/mTOR signaling pathway (Teng et al., 2011).

In recent years, some studies have reported that HBV could also be regulated by metabolic processes, including autophagy (Li et al., 2015; Sir, Ann, & Ou, 2010; Su et al., 2015; Wang, Guo, Wang, & Qian, 2013). Autophagy, an evolutionarily conserved intracellular process, engulfs long-lived cytoplasmic macromolecules and damaged organelles and delivers them to lysosomes for degradation and recycling (Mizushima & Komatsu, 2011). Autophagy is one of several cellular processes regulated by the PI3K/Akt/mTOR pathway. The inhibition of mTOR activity has been demonstrated to induce autophagy in eukaryotic cells (Akers, Loffler, Wesselborg, & Stork, 2012; Neufeld, 2010). Autophagy can act as a cell-autonomous defense, directly eliminating intracellular microbes or their products. However, this intracellular process can also be exploited by some viruses to benefit their replication, such as HBV, hepatitis C virus, human immunodeficiency virus, dengue virus, and influenza A virus (Chen et al., 2014; Dreux, Gastaminza, Wieland, & Chisari, 2009; Fischl & Bartenschlager, 2011; Kyei et al., 2009; Sir, Tian et al., 2010). For HBV, Sir et al. reported that the hepatitis B x (HBx) protein activated PI3K3 to enhance autophagy, which in turn activates viral DNA replication (Sir, Tian et al., 2010; Tian, Sir, Kuo, Ann, & Ou, 2011). The small HBV surface protein could also trigger unfolded protein responses and was found to be required in order for HBV to induce autophagy (Li, Liu, Wang et al., 2011).

MicroRNAs (miRNAs) may participate in the regulation of diverse biological processes, such as proliferation, differentiation, apoptosis, immune response, and viral replication (Bartel, 2004; Singaravelu et al., 2015; Skalsky & Cullen, 2010). To date, some cellular miRNAs have been shown to modulate HBV transcription through regulating cellular factors, such as transcription factors and nuclear receptors, at the transcription level (Huang et al., 2015; Liu, Yeh, & Chen, 2011; Zhang, Hou, & Lu, 2013). Our previous study showed that miR-1 and miR-449a overexpression by an epigenetic mechanism resulted in a marked increase in nuclear receptor farnesoid X receptor alpha expression, leading to enhanced activity of the HBV core promoter and subsequently HBV replication (Zhang et al., 2011; Zhang et al., 2016). miR-372 and miR-373 are able to promote HBV gene expression via a pathway involving nuclear factor I/B (Guo et al., 2011). MiR-122 may indirectly act on HBV replication via downregulation of its target cyclin G1, thereby blocking the interaction between cyclin G1 and p53 and abrogating p53-mediated inhibition of HBV replication (Wang, Qiu et al., 2012). In contrast, miR-141 suppresses HBV expression and replication by targeting peroxisome proliferator-activated receptor-α (Hu et al., 2012). Similarly, miRNA-130a targets the metabolic regulators peroxisome proliferator-activated receptor-γ and its coactivator PGC1-α and thereby inhibits HBV replication (Huang et al., 2015).

A set of circulating miRNAs are aberrantly expressed in the peripheral blood of chronically HBV-infected children and patients (Akamatsu et al., 2015; Brunetto et al., 2014; Winther, Bang-Berthelsen, Heiberg, Pociot, & Hogh, 2013). Among them, miR-125b had increased serum levels in the correlation with high HBV loads and was found to enhance HBV replication in a dose-dependent manner in vitro (Li et al., 2016). Interestingly, another miRNA, miR-99a, was consistently found to have elevated serum levels in chronic hepatitis B patients along with miR-122 and miR-125b, partly associated with hepatitis B e antigen (HBeAg) positivity (Akamatsu et al., 2015; Brunetto et al., 2014; Winther et al., 2013).

In the present study, we investigated the molecular and cellular mechanism of miR-99 family to modulate HBV replication in hepatoma cells. We found that ectopic expression of miR-99 family could promote HBV protein production, DNA replication, and progeny secretion. However, they did not have an effect on HBV transcription and promoter activities. Further analysis revealed that ectopic expression of miR-99 family attenuated IGF-1R/Akt/mTOR signaling pathway through the reduction of the target gene expression and repressed insulin-stimulated activation. Importantly, increased autophagic activity by mTOR inhibition was found to be the main reason for enhanced HBV replication, likely by promoting the posttranscriptional steps. Taken together, miR-99 family promoted autophagic flux through inhibiting the mTOR/ULK1 signaling, and thereby enhanced HBV replication.

## RESULTS

### 2.1 Low expression of mature miR-100 and miR-99a in hepatoma cells

Previous data have identified members of the miR-99 family, including miR-100, miR-99a, and miR-99b, as tumor suppressors (Cui et al., 2012; Di Leva, Garofalo, & Croce, 2014; Li, Liu, Lin et al., 2011; Qin, Huang, & Wang, 2015). Their expression levels in HCC tissue were lower than that observed in normal liver tissue (Li, Liu, Lin et al., 2011; Qin et al., 2015). To compare expression levels of miR-99 family members in normal human hepatocytes and hepatoma cells, we determined their expression levels in primary hepatocytes (PHHs) and three hepatoma cell lines Huh7, HepG2, and HepG2.215 cells by quantitative miScript real-time reverse transcriptase polymerase chain reactions (RT-PCR). Mature miR-100 and miR-99a were expressed at approximately 20- and 50-fold higher levels in PHHs than in hepatoma cells, respectively (Figure 1a,b). However, miR-99b expression was not decreased in hepatoma cells; indeed, it was significantly higher in HepG2.215 cells than in primary human hepatocytes. Collectively, the data suggest that the expression of the miR-99 family is strongly dysregulated in hepatoma cells, compared to that in PHHs.

### 2.2 The miR-99 family promotes HBV protein production, DNA replication and progeny secretion

Next, we investigated whether miR-99 family members regulated HBV replication in hepatoma cells. Synthetic mimics of miR-100, miR-99a, and miR-99b were transfected into HepG2.215 cells at a final culture supernatant concentration of 40 nM. HBV replicative intermediates (HBV RIs) were isolated at Day 4 posttransfection and analyzed by Southern blotting. As compared to transfection with a miRNA control (miR-C), the amount of HBV RIs, HBV DNA in supernatants, and HBsAg and HBeAg secretion significantly increased following ectopic expression of miR-99 family members (Figure 2a). In Huh7 hepatoma cells, miR-99 family mimics could also improve HBV replication and gene expression if cotransfected with a replication competent clone of
HBV plasmid pSM2 (Figure 2b). Furthermore, the miR-99 family members enhanced HBV replication and gene expression in a dose-dependent manner in HepG2.2.15 cells (Figure 2c). Based on these results, subsequent experiments were performed mainly with HepG2.2.15 cells. Western blotting analysis of cell lysates indicated the hepatitis B core antigen expression in HepG2.2.15 cells obviously increased after transfection with miR-99 family members (Figure 3d). The analysis of HBV genome with miR-99 family members (Figure 3b). Strikingly, ectopic expression of miR-99 family members strongly increased the amount of HBV capsid and capsid-associated HBV DNA detected by Western and Southern blotting analyses, respectively (Figure 3e). Taken together, these results suggest that the miR-99 family members promote HBV replication at posttranscriptional steps, such as capsid formation.

2.3 | The miR-99 family does not promote HBV transcription and promoter activities

Several cellular miRNAs have been shown to inhibit or enhance viral replication by directly targeting viral RNAs (Liu et al., 2011; Wu, Jin, Li, & Guo, 2011). Although HBV is a DNA virus, its transcripts might be targeted and modulated by cellular miRNAs (Liu et al., 2013; Zhang et al., 2013). However, no potential miRNA target sites (UACGGGU) of the seed sequence of the miR-99 family members were found in the HBV genomic sequencing of the available prediction software packages (MiRanda, TargetScan, and Pictar) for studying miRNA-mRNA interactions (Griffiths-Jones, Saini, van Dongen, & Enright, 2008). In agreement, cotransfection of pMIR-REPORT plasmids harboring the full-length HBV genome or HBV genome fragments with miR-99 family mimics into HepG2.2.15 cells did not decrease the luciferase activities of different HBV fragments (Figure 3a). Thus, we found no evidence for a direct interaction between HBV transcripts or the HBV genome with miR-99 family members, which may lead to regulation of HBV gene expression or replication.

Next, the effects of the miR-99 family members on the activities of HBV promoters was measured in Dual-Glo luciferase report assays. Consistently, we found that the luciferase activities of the HBV SP1, SP2, Core, and X promoters also were not significantly changed by ectopic expression of miR-99 family members (Figure 3b).

The effect of miR-99 family members on the different steps of HBV life cycle was further examined. HBV RNA levels were separately determined by Northern blotting (Figure 3c) and real-time RT-PCR (Figure 3d) in HepG2.2.15 cells. We observed that HBV RNA levels remained unchanged after transfection of miR-99 family members, indicating that these miRNAs did not promote HBV transcription, further implying that the miR-99 family enhanced HBV replication through some other mechanism(s).

Strikingly, ectopic expression of miR-99 family members strongly increased the amount of HBV capsid and capsid-associated HBV DNA detected by Western and Southern blotting analyses, respectively (Figure 3e). Taken together, these results suggest that the miR-99 family members promote HBV replication at posttranscriptional steps, such as capsid formation.

2.4 | The miR-99 family regulates the IGF-1R/PI3K/Akt/mTOR signaling pathway and thereby enhances HBV replication

Previous works have demonstrated that HBV replication and HBsAg production could be regulated through the PI3K/Akt/mTOR signaling pathway (Guo et al., 2007). Clearly, treatment of HepG2.215 cells for 48 hr by using PI3K, Akt, and mTOR chemical inhibitors (LY294002, Akti-1/2, and rapamycin, respectively) resulted in significant enhancement of HBV replication, as well as HBsAg and HBeAg secretion (Figure 5a). In parallel, these three inhibitors decreased phosphorylation of Akt, mTOR, and p70S6K, respectively, without significant affecting total expression levels of these proteins (Figure 4a). Moreover, HepG2.215 cells were transfected with specific small interfering RNAs (siRNAs) against Akt and mTOR or control siR-C. As expected, HBV replication, as well as HBsAg and HBeAg secretion (Figure 5b), was significantly increased when Akt or mTOR expression was silenced (Figure 5c).

Previous studies have identified the mRNA of IGF-1R, Akt, and mTOR as direct targets with binding sites for miR-99 family members (Guo et al., 2007; Hu, Zhu, & Tang, 2014; Jin et al., 2013; Li et al., 2013; Teng et al., 2011). Thus, we tested whether ectopic expression of the miR-99 family could promote HBV replication by targeting the cellular IGF-1R/PI3K/Akt/mTOR signaling pathway. Firstly, we tested whether miR-99 family members could regulate IGF-1R, Akt, and mTOR functions in
hepatoma cells. HepG2.2.15 cells were transfected with miR-99 family mimics at a final culture supernatant concentration of 40 nM for 72 hr, and total and phosphorylated IGF-1R, Akt, mTOR, and p70S6K proteins were detected by Western blotting. As shown in Figure 4b, the miR-99 family could significantly reduce the formation of total and phosphorylated forms of the IGF-1R, Akt, mTOR, and p70S6K proteins, in agreement with previous data obtained with other cell types (Ge et al., 2014).

FIGURE 2  The miR-99 family promotes hepatitis B virus (HBV) DNA replication, progeny secretion, and HBV protein production. (a) HepG2.2.15 cells were transfected with miR-99 family mimics or a nonspecific microRNA control (miR-C) at a final concentration of 40 nM and harvested 96 hr later. (b) Huh7 cells were cotransfected with 1.5 μg of pSM2 plasmid and microRNA mimics at a final concentration of 40 nM and harvested after 72 hr. (c) HepG2.2.15 cells were transfected with different doses of miR-99 family mimics (at 10 or 40 nM) or miR-C and harvested after 96 hr. Hepatitis B virus replicative intermediates in cells were isolated and detected by Southern blotting. HBV DNA levels in supernatants were determined by quantitative real-time polymerase chain reaction analysis. Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) levels in culture supernatants were determined by chemiluminescence immunoassay. (d) Cell lysates from HepG2.2.15 cells were harvested at 72 hr posttransfection, and the analysis of HBcAg expression was performed by Western blotting. Beta-actin was used as the loading control. (e) HepG2.2.15 cells were separately transfected with miR-99 family inhibitors or an microRNA inhibitor control (anti-miR-C) at 40 nM and harvested after 96 hr. RC = relaxed circular DNA; S/CO = signal to cutoff ratio; SS = single stranded DNA.* p < .05, ** p < .01, *** p < .001.
Many growth factors, such as insulin and epithelial growth factor, promote activation of PI3K/Akt/mTOR signaling in hepatic and hepatocellular cells (Whittaker, Marais, & Zhu, 2010). Consistently, treatment of HepG2.2.15 cells with insulin at concentrations ranging from 1 to 10 μM for 72 hr significantly reduced HBV replication in a dose-dependent manner (Figure 5a). Dual-Glo luciferase report assay was performed to measure the firefly and Renilla luciferase activities. The results were calculated by fold change and normalized to the miR-C samples. (b) HepG2.2.15 cells were cotransfected with miR-99 family mimics or miR-C at 40 nM, pMIR-REPORT plasmids including pMIR-Luc, pMIR-HBV FL, pMIR-HBV1, pMIR-HBV2, pMIR-HBV3, or pMIR-HBV 3’UTR, and Renilla as an internal control for 48 hr. Dual-Glo luciferase report assay was performed to measure the firefly and Renilla luciferase activities. The results were calculated by fold change and normalized to the miR-C samples. (b) HepG2.2.15 cells were cotransfected with miR-99 family mimics or miR-C at 40 nM, pMIR-REPORT plasmids including pMIR-Luc, pMIR-HBV FL, pMIR-HBV1, pMIR-HBV2, pMIR-HBV3, or pMIR-HBV 3’UTR, and Renilla for 48 hr. The determination of luciferase activities was described above. (c) HepG2.2.15 cells were transfected with miR-99 family mimics or the miR-C control (40 nM) and harvested after 72 hr. HBV RNAs were detected by Northern blotting analysis, using 28S and 18S RNAs as loading controls. (d) Real-time reverse transcriptase polymerase chain reaction analysis of HBV pregenomic RNA (pgRNA) and hepatitis B x (HBx) RNA levels, using primers matching to the pgRNA-specific region and HBx region (covering all transcripts), respectively. (e) Cell lysates from HepG2.2.15 cells were harvested at 72 hr posttransfection. Analysis of HBV nucleocapsid and the encapsidated HBV DNA was detected by Western and Southern blotting, respectively. Beta-actin was used as the loading control. *p < .05, **p < .01, ***p < .001

Many growth factors, such as insulin and epithelial growth factor, promote activation of PI3K/Akt/mTOR signaling in hepatic and hepatocellular cells (Whittaker, Marais, & Zhu, 2010). Consistently, treatment of HepG2.2.15 cells with insulin at concentrations ranging from 1 to 10 μM for 72 hr significantly reduced HBV replication in a dose-dependent manner (Figure 5a). Compared to mock control with culture medium, insulin exposure also increased the levels of Akt and mTOR phosphorylation (Figure 5b).

We investigated whether ectopic expression of miR-99 family members could inhibit the insulin-mediated activation of PI3K/Akt/mTOR signaling pathway and reverse the reduction of HBV replication. HepG2.2.15 cells were transfected with miR-99 family members at a final culture supernatant concentration of 40 nM. At 24 hr posttransfection, the cells were exposed to insulin at a concentration of 10 μM for 72 hr. Southern blotting analysis showed that treatment with insulin and miR-99 family members caused opposite effects on HBV replication and gene expression (Figure 5c). Furthermore, ectopic expression of miR-99 family promoted HBV replication, as well as HBsAg and HBeAg production in the presence of insulin. In contrast, the induction of Akt and mTOR phosphorylation by insulin was markedly reduced by miR-99 family members and also causing a significant decrease in total Akt and mTOR protein expression, as shown by
Western blotting analysis (Figure 5b). Altogether, these findings suggest that the miR-99 family members counteract insulin-mediated activation of the PI3K/Akt/mTOR signaling pathway and downregulation of HBV replication.

2.5 | The miR-99 family promotes HBV replication through mTOR/ULK1 signaling pathway-induced autophagy

The Akt/mTOR signaling pathway is known to regulate several downstream processes including protein synthesis, ribosome biogenesis, lipid synthesis, nutrient import, and autophagy (Alers et al., 2012; Dazert & Hall, 2011; Laplante & Sabatini, 2012). To assess which biological process downstream of the Akt/mTOR signaling pathway may affect HBV replication, we transfected HepG2.2.15 cells with six selected siRNAs at a final concentration of 20 nM targeting the downstream effectors of the Akt/mTOR signaling pathway. HBV replication and gene expression in HepG2.2.15 cells were analyzed at 96 hr post-siRNA transfection. Compared to the control, HBV replication and HBsAg and HBeAg secretion significantly decreased following knockdown of ULK1 expression (Figure 6a). These findings suggested that autophagy mediates, at least in part, the regulatory function of Akt/mTOR pathway on HBV replication, consistent with data from previous studies showing that autophagy played a major role in regulating HBV replication (Sir, Tian et al., 2010).

On the basis of the results of the gene-silencing experiment, we asked whether the positive effect of miR-99 family members on HBV replication may be abolished by 3-methyladenine (3-MA), an inhibitor of autophagy. Consistent with previous findings, treatment with 3-MA alone significantly reduced HBsAg and HBeAg secretion (data do not show), HBV replication, and capsid formation in HepG2.2.15 cells, but reverse results were obtained with rapamycin (Figure S3a). The expression of green fluorescence protein (GFP)-tagged LC3 was widely used to study autophagy. In this experiment, Huh7 cells were transfected with the GFP-LC3 plasmid, then treated with rapamycin or 3-MA. Indeed, rapamycin strongly promoted the formation of autophagic puncta, but 3-MA abolished it, as judged by using the immunofluorescence detection (Figure S3b and c). Thus, HepG2.2.15 cells were pretransfected with three miR-99 family members or a control miRNA at a final concentration of 40 nM and then treated with 3-MA for 48 hr or MHY1485, an mTOR activator, for 72 hr. Clearly, both the treatment of 3-MA (Figure 6b) and MHY1485 (Figure S4a) completely abolished the positive effect of the miR-99 family members on HBV replication. In contrast, treatment with rapamycin enhanced the effect of miR-99 family members on HBV replication in HepG2.2.15 cells (Figure S4b).

Next, we examined whether miR-99 family members were indeed able to modulate autophagy in hepatoma cells. Interestingly, a recent report demonstrated that miR-100 could induce autophagy in HCC cells (Ge et al., 2014). Thus, HepG2.2.15 cells were transfected with miR-99 family mimics or control miR-C and harvested after 72 hr.
Western blotting analysis confirmed that ectopic expression of the miR-99 family significantly elevated the level of autophagy marker LC3II and promoted the degradation of autophagic cargo receptor p62 (Figure 6c,d). Furthermore, Huh7 cells were cotransfected with three miR-99 family members or control miR-C and plasmid GFP-LC3, fixed, stained, and finally subjected to confocal microscopy. In concordance with the Western blotting results in HepG2.2.15 cells, the miR-99 family members strongly increased the formation of autophagic puncta in Huh7 cells (Figure 6e,f). In addition, Huh7 cells were transfected with plasmid GFP-LC3 and miRNAs and then treated with 10 μM chloroquine (CQ), an inhibitor that prevented the acidification of lysosomes. As indicated in Figure S5, ectopic expression of miR-99 family members could further increase the formation of autophagosomes in Huh7 cells, as CQ reduced autophagic degradation and preserved the formed autophagosomes. Collectively, all three miR-99 family members were capable of promoting autophagy in hepatoma cells; therefore, they likely enhance HBV replication by promoting autophagy.

2.6 | HBV replication can be regulated through the mTOR/ULK1 signaling pathway

As inhibition of mTOR activity led to the activation of autophagy via the ULK1-ATG13-FIP200 complex (Alers et al., 2012; Jung, Seo, Otto,
FIGURE 6  The miR-99 family promotes hepatitis B virus replication through mTOR/ULK1 signaling pathway-induced autophagy. (a) HepG2.2.15 cells were transfected with specific small interfering RNAs against p70S6K, 4E-BP1, SREBP1, HIF-1α, ULK1, or IRF7 mRNAs, or a control siRNA (siR-C) at 20 nM and harvested after 96 hr. Hepatitis B virus replicative intermediates in cells were isolated and detected by Southern blotting. HBsAg and HBeAg secretion in supernatant were determined by chemiluminescence immunoassay. (b) HepG2.2.15 cells were pretransfected with miR-99 family mimics or control miR-C at 40 nM, after which the cells were treated with 10 mM of 3-methyladenine for 48 hr. The levels of hepatitis B virus replicative intermediates, hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) production in supernatants were determined as described above. (c) HepG2.2.15 cells were transfected with miR-99 family mimics or microRNA control (miR-C) at 40 nM and harvested after 72 hr. The levels of LC3 and p62 expression were detected by Western blotting, with beta-actin as a loading control. (d) The LC3-II/beta-actin ratios of Western blotting bands were quantified by densitometric analysis using ImageJ software. (e) Representative images of Huh7 cells cotransfected with the green fluorescence protein (GFP)–LC3 plasmid and miR-99 family mimics or miR-C at 40 nM. The cells were fixed and stained with 6-diamidino-2-phenylindole (DAPI). Bars, 5 μm. (f) Statistical analysis of the numbers of LC3 puncta per cell. RC = relaxed circular DNA; S/CO = signal to cutoff ratio; SS = single stranded DNA. *p < .05, **p < .01, ***p < .001
we addressed the question whether the ULK1 complex participates in miR-99 family-induced autophagy and enhancement of HBV replication. Firstly, we clarified the role of ULK1 on autophagic formation and HBV replication, as ULK1 plays a key role in the initiation of autophagosome formation (Ro et al., 2013). HepG2.2.15 cells were transfected with miR-99 family mimics or control miR-C at a final concentration of 40 nM. Cell lysates were collected for Western blotting analysis. We observed that ectopic expression of miR-99 family members resulted in ULK1 dephosphorylation in hepatoma cells (Figure 7a). However, ULK1 silencing by specific siRNAs abolished the enhancing effect of the miR-99 family members on HBV replication, and HBsAg and HBeAg secretion (Figure 7b), suggesting that ULK1 is required for the action of the miR-99 family on HBV replication and gene expression.

Next, we examined the consequences of silencing ULK1, FIP200, and ATG13, three factors forming the complex for involved autophagy initiation, for HBV replication. Thus, HepG2.2.15 cells were transfected with specific siRNA against ULK1, FIP200, ATG13, ATG5 or control siR-C at 20 nM and harvested after 72 hr. ATG5 was used as positive control, as it combined with ATG12 to form the ATG12 conjugation system and to regulate autophagic vesicle formation. As shown in Figure 8a and 8b, the level of LC3II expression significantly decreased; however, the degradation of p62 was blocked following silencing of these four targets. Meanwhile, the effectiveness of siRNA duplexes was verified by real-time RT-PCR (Figure 8c). Moreover, Huh7 cells were cotransfected with the GFP-LC3 plasmid and siRNAs, after which they were treated with 10 μM CQ for 24 hr. Consistent with previous reports, we found that the frequencies of autophagic puncta of LC3 by confocal microscopy were significantly decreased by silencing ULK1, FIP200, ATG13, or ATG5 (Figure 8d,e).

We next sought to examine whether HBV replication was affected by silencing the components of the ULK1-ATG13-FIP200 complex. HepG2.2.15 cells were transfected with different specific siRNAs and harvested at 96 hr. As revealed in Figure 8f, HBV replication, and HBsAg and HBeAg secretion into the supernatant were significantly decreased by knocking down ATG13 or ATG5 expression; however, HBsAg and HBeAg secretion was not significantly decreased by silencing FIP200. Taken together, our data suggest that mTOR/ULK1 signaling pathway-induced autophagy is an important process that mediates the enhancing effect of miR-99 family members on HBV replication (Figure 9).

3 DISCUSSION

In the present study, we found that the miR-99 family members were able to promote HBV replication in hepatoma cells upon ectopic expression. All three members of the miR-99 family targeted components of the IGF-1R/PI3K/Akt/mTOR signaling pathway and negatively regulated this pathway. Among the downstream pathways under the control of the PI3K/Akt/mTOR signaling pathway, autophagy may play a major role in regulating HBV replication. Transfecting hepatocytes with miR-99 family members clearly promoted autophagy. Blocking the initiation of autophagy by 3-MA abolished the enhancing effect of the miR-99 family member on HBV replication. On the basis of the available data, we conclude that the miR-99 family...
FIGURE 8  Hepatitis B virus replication can be regulated through ULK1-ATG13-FIP200 complex. (a) HepG2.2.15 cells were transfected with specific small interfering RNAs against ULK1, FIP200, ATG13, ATG5, or control siR-C at 20 nM, and harvested after 72 h. The levels of LC3 and p62 expression were by Western blotting, and beta-actin was used as a loading control. (b) The LC3-II/beta-actin ratios of Western blotting bands were quantified by densitometric analysis using ImageJ software. (c) The levels of the corresponding mRNAs were determined by real-time reverse transcriptase polymerase chain reaction using specific primers. (d) Representative images of Huh7 cells cotransfected with the plasmid green fluorescence protein (GFP)–LC3 and different specific small interfering RNAs and harvested after 48 hr. The cells were fixed and stained with 6-diamidino-2-phenylindole (DAPI). Bars, 5 μm. (e) Statistical analysis of the number of LC3 puncta per cell. (f) HepG2.2.15 cells were transfected with specific small interfering RNAs against FIP200, ATG13, ATG5, or the siRNA control at 20 nM and harvested after 96 hr. The detection of hepatitis B virus replicative intermediates, hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) secretion in supernatant was performed as described above. RC = relaxed circular DNA; S/CO = signal to cutoff ratio; SS = single stranded DNA. *p < .05, **p < .01, ***p < .001
promoted HBV replication via the IGF-1R/PI3K/Akt/mTOR/ULK1 autophagy axis.

It is interesting that miR-99b is lowly expressed in PHHs, however, much more abundantly expressed than miR-100 and miR-99a in HepG2.2.15 cells. This observation could result from the fact that these three miRNAs are transcribed and supposedly regulated independently. The three miR-99 family members are derived from different pri-miRNA transcripts. The locations of the coding sequences of miR-100, miR-99a, and miR-99b are on chromosome 11, NC_000011.10 (122152229..122152308, complement), chromosome 21, NC_000021.9 (16539089..16539169), and chromosome 19, NC_000019.10 (51692612..51692681), respectively. This explains why the level of miR-99b was increased but that of miR-99a and miR-100 decreased in hepatoma cells. Although the level of miR-99b was increased in hepatoma cells, it did apparently not compensate the loss of miR-100 and miR-99a expression. Thus, ectopic expression of the miR-99 family members could target the cellular mRNAs with the corresponding seed sequence sharing by all the three miRNAs, finally with similar effect on the cellular gene expression and signaling, as well as on HBV replication.

Data from several studies have demonstrated that HBV can promote the autophagy in hepatoma cells (Lazar, Uta, & Branza-Nichita, 2014; Li, Liu, Wang et al., 2011; Liu et al., 2014; Sir, Tian et al., 2010; Tang et al., 2009; Tian et al., 2011). Sir, Tian et al. (2010) found that the HBx protein was able to bind to PI3KC3 and enhance its enzymatic activity, an enzyme critical for the initiation of autophagy. The HBx was also found to upregulate Beclin1 expression and thereby sensitize cells to induce autophagy by starvation (Tang et al., 2009). Furthermore, Liu et al. (2014) showed that HBV- or HBx protein-induced autophagosome formation was accompanied by unchanged mTOR activity and decreased degradation of autophagic cargo proteins. The HBx protein may prevent lysosomal acidification, in turn leading to reduced lysosomal degradative capacity and the accumulation of immature lysosomes, possibly through interaction with V-ATPase, affecting its lysosomal targeting. The repressive effect of HBx on lysosomal function inhibits autophagic degradation and may be linked to the development of HBV-associated HCC (Lan et al., 2014; Liu et al., 2014). In addition, Li, Liu, Wang et al. (2011) showed that HBsAg was required for HBV-stimulated autophagy.

Autophagy was recognized to have a significant impact on HBV replication (Huang et al., 2014; Sir, Tian et al., 2010; Tian et al., 2011). Independent research consistently demonstrated that autophagy inhibition strongly reduced HBV replication in hepatic cells (Li, Liu, Wang et al., 2011; Sir, Tian et al., 2010). Moreover, Tian et al. (2011) demonstrated that a deficiency in autophagy strongly reduced HBV replication in a transgenic mouse model. According to the available results, autophagy enhances HBV replication at the late stage, likely after a step of capsid formation and viral assembly. Li, Liu, Wang et al. (2011) provided evidence that autophagy was required for HBV envelopment, although it was not necessary for HBV release. In agreement with previous reports, our results demonstrated that the miR-99 family positively regulated HBV replication at the posttranscriptional level. The regulation of HBV promoter activity was not affected by ectopic expression of miR-99 family members. The levels of HBV RNAs, capsids, and HBsAg and HBeAg secretion in hepatoma cells markedly increased following the transfection of miR-99 family members. Early reports suggested that inhibition of the PI3K/Akt/mTOR signaling pathway could promote HBV transcription (Guo et al., 2007). In agreement with proposed mechanism, PI3K, Akt, and mTOR chemical inhibitors decreased the levels of phosphorylated
miR-99 family members decreased the levels of both total and phosphorylated IGF-1R, Akt, mTOR, and p70S6K protein expression but in a different extent compared to the chemical inhibitors. Our results suggest that miR-99 family members had likely milder inhibition on the IGF-1R/miR-99/Akt/mTOR signaling pathway than the chemical inhibitors.

Bioinformatics analysis of HBV genomic sequences and testing with reporter plasmids suggested that miR-99 family members did not directly act on HBV mRNAs. Recent data have shown that cellular miRNAs have the potential to inhibit or stimulate viral replication in host cells by directly targeting the HBV genome or indirectly by targeting cellular genes (Huang et al., 2015; Kohno et al., 2014; Wang et al., 2014; Zhang et al., 2011). The miR-99 family has been shown to play an important role in many cellular and biological functions. MiR-99 family members were reported to target many cellular genes such as IGF-1R, Akt, mTOR, Ago2, and several components of the Transforming growth factor (TGF)-β signaling pathway (Chen et al., 2012; Lerman et al., 2011; Zhang et al., 2014). The regulation of these important cellular pathways by the miR-99 family was clearly essential for controlling cell growth (Cui et al., 2012; Li, Liu, Lin et al., 2011). As shown previously, miR-99 family expression was reduced in primary human hepatocellular carcinoma, compared with that observed in matching normal liver tissues (Li, Liu, Lin et al., 2011).

We showed in the present study that miR-99 family members are expressed in PHHs and hepatoma cells. However, the expression levels of miR-99 family members in hepatoma cells were strongly reduced, compared with their expression levels in PHHs. Consequently, the dysregulation of cellular processes might decrease the ability of hepatocytes or hepatoma cells to support HBV replication. Consistent with this possibility, previous data showed that the loss of differentiation status of hepatocytes might greatly reduce the ability of cells to support HBV replication (Martinet-Peignon et al., 2002; Su et al., 2010; Zhang et al., 2011). Li, Hacker, Kopp-Schneider, Protzer, and Bannasch (2002) showed that replication of woodchuck hepatitis virus and viral antigen expression were gradually decreased in early preneoplastic cell line ages. In general, HBV replication is low or absent in HCC tissues, which are associated with the dedifferentiation of hepatocytes. We demonstrated that the ectopic expression of miRNAs (like miR-1) in hepatoma cells could promote cell differentiation and restore, at least partially, the hepatocyte phenotype (Zhang et al., 2011). The data presented in the present study suggest that more miRNAs may capable of facilitating HBV replication in hepatoma cells by promoting cell differentiation. Thus, the understanding of interactions between host miRNAs and HBV will continue to grow by incorporating new miRNAs and studying their new functions, which will potentially yield new insights of therapeutic relevance.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plasmids and reagents

The HBV plasmid pSM2 was kindly provided by Prof. Hans Will as previously reported (Stanssens et al., 1989). The sequences of all miRNAs and siRNAs used in the present study are shown in supplemental tables (Table S1 and S2). The PI3K inhibitor LY294002 (Calbiochem), Akt inhibitor Akti-1/2 (Calbiochem), and mTOR inhibitor rapamycin (Calbiochem) were purchased from Merck Millipore (Germany). The mTOR activator MHY1485 was provided by Selleckchem. Insulin, 3-MA, and CQ were acquired from Sigma-Aldrich.

4.2 | Cell culture and transfection

All cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2. The human HepG2.2.15 hepatoma cell line, which harbors integrated dimers of the HBV genome (GenBank Accession Number: U95551) and shows a constantly detectable level of HBV replication, was cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), and 500 μg/mL G418 (Merck Millipore). Another human hepatoma cell line Huh7 was grown in Dulbecco’s Modified Eagle’s Medium (Gibco) supplemented with 10% fetal bovine serum. Plasmids, miRNAs, or siRNAs were transfected into cells at indicated concentrations using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions.

4.3 | Analysis of HBV replication and gene expression

The levels of HBsAg and HBeAg in the culture supernatants were determined using the Architect System and the HBsAg and HBeAg CMIA kits (Abbott Laboratories, Germany) according to the manufacturer’s instructions. HBV RNAs from intracellular core particles and HBV transcripts were extracted from hepatoma cell lines and detected by Southern and Northern blotting, respectively, according to published protocols (Zhang et al., 2011). The encapsidated HBV DNA in nucleocapsids was detected by Southern blotting. HBV nucleocapsid in cell lysate was analyzed in a native agarose gel and then detected by Western blotting analysis (Yang et al., 2016). HBV RNA in cells was detected by quantitative real-time RT-PCR assays (Qiagen). The mRNAs levels were normalized to the beta-actin expression level. The following primer pairs were used HBV pgRNA: 5′-TGCCCTCATCTGGTTCT-3′ (sense) and 5′-CCCAAWACCCCATATA-3′ (antisense); HBx: 5′-CGCTGTGCTATCAC-3′ (sense) and 5′-TAATCTCTCAACTCC-3′ (antisense). HBV progeny DNA was extracted from cell culture supernatant using the DNA Blood Mini Kit (Qiagen) and quantified by quantitative polymerase chain reactions (Invitrogen) as described (Zhang et al., 2011).

4.4 | Western blotting analysis

Western blotting analysis was performed as described previously (Zhang et al., 2011). Antibodies against the following proteins were used hepatitis B core antigen (Abcam), Akt (Cell Signaling), phospho-Akt (Ser473; Cell Signaling), mTOR (Cell Signaling), phospho-mTOR (Cell Signaling), ULK1 (Abcam), phospho-ULK1 (Ser757; Cell Signaling), LC3 (Sigma), p62 (Abcam), and beta-actin (Sigma). The membranes were washed with 1 x Tris-buffered saline with tween 20 (TBST) and incubated with a secondary peroxidase-affiniPure Rabbit antismouse IgG antibody (Jackson ImmunoResearch) or a peroxidase-affiniPure
Goat antirabbit IgG antibody (Jackson ImmunoResearch). Immunoreactive bands were visualized using an enhanced chemiluminescence system (GE Healthcare).

4.5 | Luciferase reporter gene assay

The Dual-Glo luciferase reporter assay system (Promega, E2940) was used to detect the firefly luciferase activity and the internal control Renilla luciferase activity separately. The firefly luciferase reporter plasmids pSP1, pSP2, pCP, pXP (containing HBV promoters), and pmiR-HBV FL, pmiR-HBV1, pmiR-HBV2, pmiR-HBV3, and pmiR-HBV3’UTR (containing full length genome and partial genome fragments) were generated previously and used in the luciferase reporter assay as described (Zhang et al., 2011).

4.6 | Real-time RT-PCR assay

Total RNA was extracted and detected as described previously (Zhang et al., 2011). The expression of mature miRNA or mRNA levels was determined by quantitative real-time PCR analysis using commercial miScript Primer or QuantiTect Primer Assays (listed in Table S3) from Qiagen.

4.7 | Microscopy image acquisition and quantification

For fluorescence staining, Huh7 cells were grown on cover slips and cotransfected with the plasmid GFP-LC3 and miRNAs or siRNAs. After transfection for 48 hr, cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 10 min. The nuclei was stained with 4’, 6-diamidino-2-phenylindole, and the distribution of the GFP-tagged LC3 protein was visualized with confocal microscope (LSM 710; Carl Zeiss) with objectives Plan-Apochromat 63x/1.40 oil Iris M27. Images were acquired by ZEN acquisition software (2012; Carl Zeiss) and analyzed by ImageJ software. The images were not manipulated other than contrast and brightness adjustments. For quantification of the number of the GFP-LC3 puncta, approximately 50 cells were recorded and analyzed by ImageJ software (Liu et al., 2014).

4.8 | Statistical analysis

All statistical data are expressed as mean ± standard error of mean. GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) was used for difference analysis by Student’s t test. Differences were considered as statistically significant when p < .05. All the experiments were repeated independently for at least three times.

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