


**ORIGINAL ARTICLE**

# Hepatitis B virus-regulated growth of liver cancer cells occurs through the microRNA-340-5p-activating transcription factor 7-heat shock protein A member 1B axis

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Hepatocellular carcinoma (HCC) is a common cancer with poor prognosis. Hepatitis B virus (HBV) is one of the leading causes of HCC, but the precise mechanisms by which this infection promotes cancer development are not fully understood. Recently, miR-340-5p, a microRNA (miRNA) that has been identified as a cancer suppressor gene, was found to inhibit the migration and invasion of liver cancer cells. However, the effect of miR-340-5p on cell proliferation and apoptosis in HBV-associated HCC remains unknown. In our study, we show that miR-340-5p plays an important role during HBV infection and hepatocellular carcinoma development. Specifically, this miRNA directly binds to the mRNA encoding activating transcription factor 7 (ATF7), a protein that both promotes cell proliferation and suppresses apoptosis through its interaction with heat shock protein A member 1B (HSPA1B). We further found that miR-340-5p is downregulated by HBV, which enhances ATF7 expression, leading to enhanced cell proliferation and inhibition of apoptosis. Notably, ATF7 is upregulated in HCC tissue, suggesting that HBV may target miR-340-5p *in vivo* to promote ATF7/HSPA1B-mediated proliferation and apoptosis and regulate liver cancer progression. This work helps to elucidate the complex interactions between HBV and host miRNAs and further suggests that miR-340-5p may represent a promising candidate for the development of improved therapeutic strategies for HCC.

**KEYWORDS**

apoptosis, ATF7, HBV-related HCC, miR-340-5p, proliferation

**Abbreviations:** ATF7, activating transcription factor 7; co-ip, coimmunoprecipitation; EdU, 5-ethynyl-2'-deoxyuridine; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HSPA1B, heat shock protein A member 1B; HSP, heat shock protein; IP, immunoprecipitation; miR-340-5p-in, miR-340-5p inhibitor; miR-340-5p-m, miR-340-5p mimic; miRNA, microRNA; MS, mass spectrometry; PRMT1, protein arginine methyltransferase 1; qRT-PCR, quantitative RT-PCR; XRCC6, X-ray repair cross-complementing protein 6.

Song and Wei equally contributed to this study.

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## 1 | INTRODUCTION

Hepatocellular carcinoma is a widespread cancer with a poor prognosis and mortality among all malignancies, with approximately half of HCC cases occurring in China. Chronic HBV infection has been closely linked with the initiation and development of HCC, and those infected with HBV have a 15-20-fold greater risk for HCC development compared to the uninfected population.<sup>1,2</sup> However, the precise molecular mechanisms involved in HBV-mediated hepatocarcinogenesis remain unclear.

A large number of studies have investigated the role of HBV in HCC development, and these have highlighted numerous processes by which this infection may contribute to the complex pathogenesis of liver carcinoma. For example, the HBV virus can integrate its DNA into the host genome, which may lead to mutation and chromosomal instability.<sup>3,4</sup> Other studies have also indicated that HBV can directly interact with host cell molecules in order to modulate critical liver cell functions, such as proliferation, apoptosis, migration, invasion, and metabolism.<sup>5,6</sup> In addition, there is published evidence that HBV may promote carcinogenesis by stimulating inflammatory responses.<sup>7,8</sup>

An increasing number of studies have shown that miRNAs are involved in multiple processes that affect the development, progression, and prognosis of HCC. miRNAs are a class of short, endogenous, non-coding RNAs that play important roles in gene regulation by binding to, and repressing the expression of target mRNAs in a sequence-dependent method. In particular, a number of reports have identified miR-340 as a tumor suppressor in HCC<sup>9,10</sup> as well as several other cancers including ovarian cancer,<sup>11</sup> breast cancer,<sup>12</sup> colon cancer,<sup>13</sup> glioblastoma,<sup>14</sup> and pancreatic cancer.<sup>15</sup> Consistent with these data, in a previous study, we found that miR-340 expression was significantly downregulated in HCC tissues compared with normal liver tissues. Furthermore, we showed that HBV could downregulate miR-340 in hepatoma cells, which facilitates their migration.<sup>10</sup> Critically, in addition to invasion and metastasis, HCC development and progression also involve other important processes, such as enhanced cell proliferation and apoptotic inhibition. However, the role that HBV-mediated miR-340-5p inhibition may play in these processes has not been elucidated.

Herein, we found that ATF7 is a target of miR-340-5p, and provided experimental evidence that HBV can regulate HCC cell proliferation and apoptosis through the miR-340-5p-ATF7-HSPA1B axis. Thus, in the present study, we provide further insight into the molecular mechanisms by which HBV promotes HCC development and progression, and our findings suggest new potential therapeutic targets for treatment of this devastating disease.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines and transfection

HepG2 and Huh7 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and HepG2.2.15 cells were preserved in our laboratory until use.

All cells were cultured in DMEM (Gibco/Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), containing 10% FBS (Gibco/Life Technologies), and maintained at 37°C with 5% CO<sub>2</sub> in a humidified chamber. Cell transfection was done using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol when cells reached 80% confluence in complete growth medium.

### 2.2 | RNA interference and plasmid construction

Knockdown of ATF7 and HSPA1B expression was carried out using siRNAs that were synthesized by RiboBio (Guangzhou, China); negative control siRNAs (si-NC) containing unrelated sequences were also generated. To generate the FLAG-ATF7, FLAG-XRCC6, FLAG-PRMT1, FLAG-HSPA1B, and HA-ATF7 constructs, we amplified the corresponding cDNAs and cloned these into the pEF-FLAG or pXJ40-HA vectors. The miR-340-5p mimic and inhibitor, miRNA-NC were also designed and synthesized by RiboBio. Negative control miRNA (miR-NC) contained a random sequence that was distinct from that of miR-340-5p. The pUC18 and pHBV1.3 plasmids were used as described,<sup>16</sup> and all siRNA and primer sequences are listed in Table 1.

### 2.3 | RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and reverse transcription was carried out using the Prime Script RT Reagent Kit with gRNA Eraser (TaKaRa, Shiga, Japan), according to the manufacturer's instructions. qRT-PCR analyses were carried out as previously described;<sup>16</sup> primer sequences are listed in Table 1. For detection of expression in clinical cancer samples, the target gene fold change was normalized to the average of expression in adjacent non-cancerous tissues. All reactions were carried out in duplicate. GAPDH was set as an internal control for protein-coding gene expression in each cell line and sample, and relative expression of miR-340-5p was normalized to U6 snRNA (U6 small nuclear RNA). Expression results were calculated using the 2<sup>-ΔΔC<sub>T</sub></sup> method.

### 2.4 | Western blot, co-immunoprecipitation, and mass spectrometry analyses

Cells were seeded into six-well plates and transfected with individual plasmids as described above. After 48 hours, transfected cells were harvested and resuspended in RIPA lysis buffer (Beyotime, Shanghai, China) to obtain whole protein extracts. Protein concentrations were measured using the enhanced BCA protein assay kit (Beyotime), and equal amounts of protein were separated by SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes, and these were blocked with 5% skim-milk in TBS with Tween 20 (TBS-T), containing 120 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 to prevent non-specific binding. Blots were then incubated with specific primary antibodies overnight at 4°C; these included antibodies to detect FLAG, Actin, HA and GAPDH (Sigma, St Louis, MO, USA),

**TABLE 1** Sequences and oligonucleotides used in the present study

Gene	Forward	Reverse	GeneBank no.
ATF7	AAGTATCCGTTCCGCCAAGG	GCATTGCACACAAACGGTCT	NM_006856.2
HSPA1B	TCCGAAGGACTGAGCTCTTG	GAGTAGGTGGTGCCAGGT	NM_005346.4
GAPDH	GAGAAGGCTGGGGCTCATT	TAAGCAGTTGGTGGTGACAG	NM_002046.6
HSPA1B	CCCAAGCTTATGGCCAAAGCCGCGGCGATC	GGGGTACCATCCACCTCCTCAATGGTAGG	NM_005346.4
XRCC6	CGGGATCCATGTCAGGGTGGGAGTCATAT	TCCCCCGGGTCTCGGAAGTGCTTGGTGAG	NM_001469.4
PRMT1	CCCAAGCTTATGGCGGCAGCCGAGGCCGCG	GGGGTACCAGCGCATCCGGTAGTCGGTGGA	NM_001536.5
ATF7-3'UTR	GGACTAGTCTCAGGACTCAGGTCTCCCA	AGCTTTGTTTAAACAGAGAGACAGGGGCGGTAAT	NM_006856.2
ATF7-3'UTR-Mut	AACTCTCTGTAAACATAAAGGAATCG	ATGTTTACAGAGAGTTTACCGGCCAA	NM_006856.2
si-HSPA1B-1#	GGTTCTGGACAAGTGTCAA	TTGACACTTGTCCAGAACC	NM_005346.4
si-HSPA1B-2#	CGGACAAGAAGAAGTTTCT	AGAACCTTCTTCTTGCCG	NM_005346.4
si-HSPA1B-3#	TACCATTGAGGAGGTGGAT	ATCCACCTCCTCAATGGTA	NM_005346.4
si-ATF7-1#	GACAGAGATTTACATTTCTGA	TCGAAATGTAAATCTCTGTCT	NM_006856.2
si-ATF7-2#	CGAACTGACTCAGTCATCA	TGATGACTGAGTCAGTTCCG	NM_006856.2
si-ATF7-3#	GCAGATCAAACGCCTACTC	GAGTAGGCGTTTGATCTGC	NM_006856.2
miR-340-5p mimic	UUAUAAAGCAAUGAGACUGAUU	AAUCAGUCUCAUUGCUUUUAUA	MIMAT0004692
miR-340-5p inhibitor		AAUCAGUCUCAUUGCUUUUAUA	MIMAT0004692

ATF7, caspase-9, poly ADP ribose polymerase (PARP) and HSPA1B (Proteintech, Wuhan, China), then HRP-conjugated secondary antibody (Sigma) at room temperature for 1 hour. Immunoreactivity of protein was visualized through ECL western blotting kit (Millipore, Hercules, CA, USA) according to the manufacturer's instructions.

For co-ip assays, cells were first lysed in WB or IP lysis buffers (Beyotime). Cell suspensions were then centrifuged at 13 000 g for 15 minutes, and either FLAG-beads or a mixture of HA antibody and Protein A/G agarose was added to the supernatant. After incubation at 4°C overnight, the FLAG or Protein A/G beads were washed, and the immune complexes were eluted from the beads. SDS-PAGE and western blot analysis were then used to separate and identify the eluted proteins.

For mass spectrometry analysis, SDS-PAGE gels were stained using Coomassie brilliant blue and then destained in a solution containing 40% purified water, 40% ethanol, and 20% acetic acid. Individual bands were excised and analyzed by mass spectrometry.

## 2.5 | Human specimens and histology

Clinical HCC samples were obtained from Zhongnan Hospital of Wuhan University. For immunohistochemical staining, samples were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Primary antibodies for ATF7 or HSPA1B were used at a concentration of 1:100. All samples were independently re-evaluated by two experienced clinical pathologists before further analysis, and the study protocol was approved by the Ethics Committee of Wuhan University (150013, Wuhan, China).

## 2.6 | Cell viability assays

For CCK-8 assay, Huh7 cells were seeded into six-well plates and transfected with the indicated plasmid when they reached 80% confluence. Transfected cells were digested with 0.25% trypsin, and 3000 cells were seeded into each well of a 96-well plate. The WST-1 Cell Proliferation and Cytotoxicity Kit (Beyotime) was used to measure proliferation according to the manufacturer's protocol. In brief, 10% WST-1 solution was added to cell culture medium in each well, and the optical density at 450 nm was measured at 0, 24, 48, and 72 hours. Six duplicate wells for each group were measured.

For key Fluor488-EdU staining assay, briefly, Huh7 cells were transfected with individual plasmids and incubated with 1× EdU working solution for 30 minutes, 4% PFA was used to fix cells, and Hoechst 33342 staining solution was incubated with cells for 15 minutes. At least four random fields of each well were obtained at 480 nm (green) and 350 nm (blue). Green represented proliferating cells and blue represented cell nucleus. Ratio of EdU staining = (proliferating cells/whole cells) × 100.

## 2.7 | Cell apoptosis assays

Hoechst 33258 staining was used to measure apoptosis. Briefly, Huh7 cells were plated onto cover slips that were placed at the bottom of six-well plates and then transfected with the indicated plasmid. Cells were fixed in 4% PFA for 15 minutes; Then Hoechst 33258 (10 µg/mL) was added to the media, and samples were incubated for 5 minutes at room temperature. Images were obtained under a

fluorescence microscope (Olympus BX61, Tokyo, Japan) at 340 nm, and five fields were randomly chosen for analysis from each well.

## 2.8 | Luciferase reporter assays

Putative target genes for miR-340-5p were predicted using the TargetScan human computational method ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)). We then amplified the 3'-UTR fragment (1994-2581 bp) of *ATF7*, which contained the predicted binding site for miR-340-5p (2461-2483 bp), and cloned this into the pMIR miRNA Expression Reporter Vector (Applied Biosystems, Foster City, CA, USA). The pMIR-ATF7-3'-UTR-Mut plasmid construct containing the mutated miR-340-5p binding site was generated using site-directed mutagenesis on the WT pMIR-ATF7-3'-UTR plasmid. All primer sequences are shown in Table 1, and luciferase activity was measured according to the manufacturer's protocols.

## 2.9 | Statistical analysis

All statistical analyses were carried out using GraphPad Prism 6 software. Data are presented as mean  $\pm$  standard deviation (SD), and two-tailed Student's *t* test (two-sample equal variance) was used to measure the significance of observed differences between two groups, with  $P < 0.05$  considered to be statistically significant. In all cases, at least three independent experiments were carried out. NS, no significant difference. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

# 3 | RESULTS

## 3.1 | Hepatitis B virus-modulated proliferation and apoptosis by miR-340-5p inhibition in HCC cell lines

In our previous study, we showed that miR-340-5p expression was negatively associated with HBV infection in HCC samples and cell lines. Furthermore, HBV promoted the migration and invasion of HCC cells through miR-340-5p inhibition.<sup>10</sup> Therefore, to further investigate the function of miR-340-5p in HCC, we treated Huh7 cells with miR-340-5p-m or miR-340-5p-in and carried out cell proliferation and apoptosis assays. We found that, compared with control miRNA, miR-340-5p-m inhibited cell proliferation, whereas miR-340-5p-in promoted proliferation (Figure 1A,B). In addition, we observed increased apoptosis in cells treated with miR-340-5p-m, and decreased apoptosis occurred when cells were treated with miR-340-5p-in (Figure 1C). These data suggest that miR-340-5p functions to inhibit proliferation and to promote apoptosis in the Huh7 cell line.

We then tested the role of miR-340-5p in HBV-mediated HCC growth by transfecting Huh7 cells with either HBV vector alone (pHBV) or pHBV plus miR-340-5p-m. As expected, we found that transfection with pHBV promoted cell proliferation compared with vector control (pUC18), whereas miR-340-5p-m reversed this effect (Figure 1D,E). We further observed decreased apoptosis in Huh7 cells transfected with pHBV compared to controls, whereas apoptotic levels were restored in cells cotransfected with pHBV/miR-340-5p-m

(Figure 1F). Moreover, we also confirmed that some important proteins are involved in proliferation and apoptosis, such as cyclin D1, PARP, and caspase-9. Results shown that miR-340-5p inhibited protein expression of cyclin D1 and promoted cleaved PARP, and cleaved caspase-9 (Figure S1). Thus, these results indicate that miR-340-5p is involved in HBV-mediated growth regulation in HCC cells.

## 3.2 | ATF7 confirmed as a target of miR-340-5p

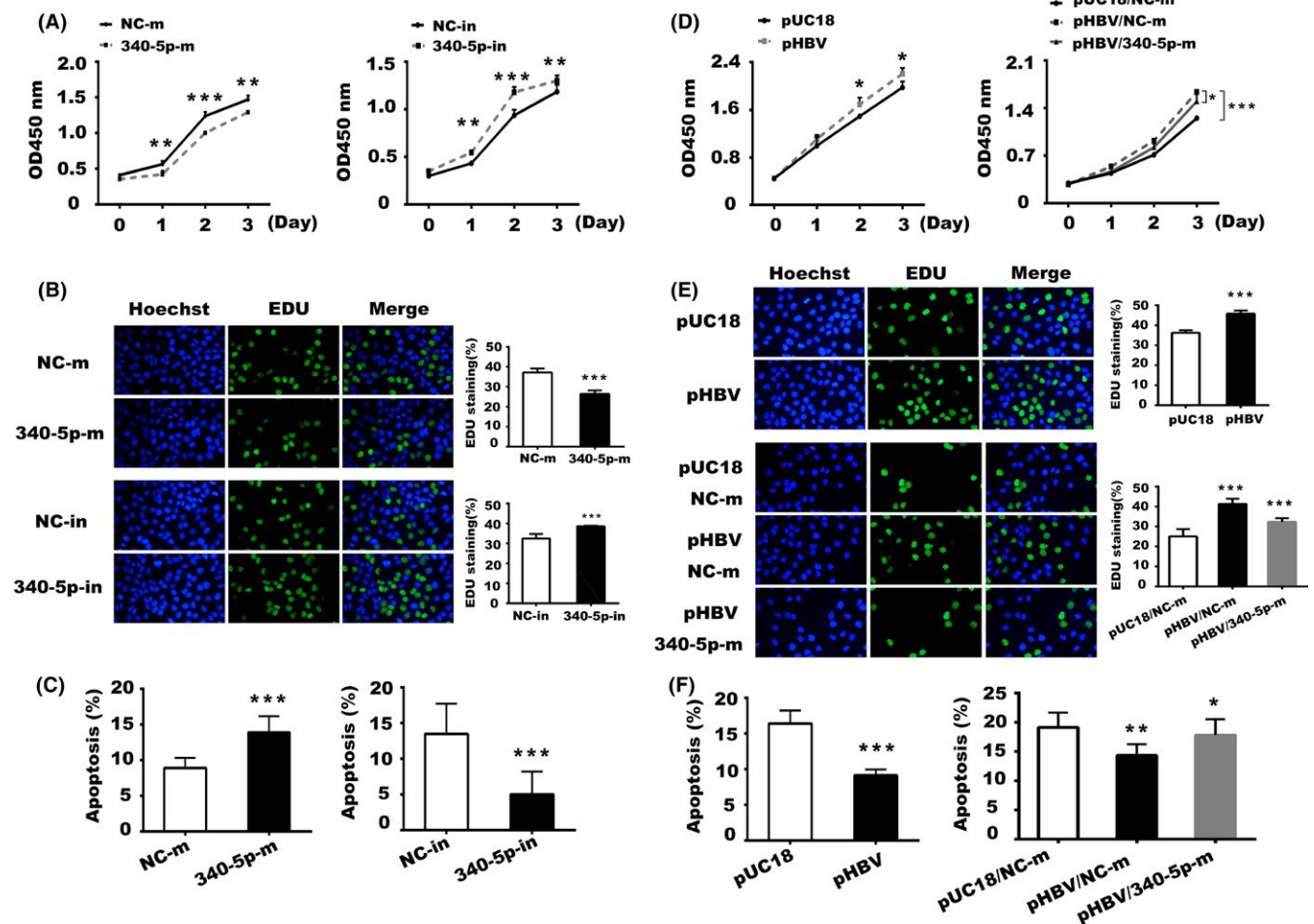
In order to identify potential targets of miR-340-5p that could be mediating its effect on proliferation and apoptosis, we used four different tools, namely miRanda, TargetScan, miRBase, and PicTar. Our results indicated that *ATF7* mRNA was a putative target of miR-340-5p. We therefore carried out qRT-PCR and western blot analysis to measure *ATF7* mRNA and protein levels, respectively, in cells transfected with miR-340-5p-m or miR-340-5p-in. We found that, in both cases, *ATF7* expression was negatively correlated with miR-340-5p expression (Figure 2A). To further validate whether *ATF7* is a direct target of miR-340-5p, we generated two reporter constructs containing either the partial WT 3'-UTR of the *ATF7* gene (*ATF7*-3'-UTR) or a mutated version containing a UUAU to ACAG substitution in the predicted miR-340-5p binding site (*ATF7*-3'-UTR-Mut) (Figure 2B). Luciferase reporter assays showed that the *ATF7*-3'-UTR construct showed markedly reduced reporter activity after transfection with miR-340-5p-m, whereas there was no detectable effect on *ATF7*-3'-UTR-Mut (Figure 2B), indicating that miR-340-5p inhibits *ATF7* by binding to its 3'-UTR region.

A number of studies have suggested a role for *ATF7* in oncogenesis, but its precise function in liver cancer has not been reported. Therefore, to further investigate the role of *ATF7* in HCC cells, we silenced endogenous *ATF7* using siRNA and measured its effect on proliferation and apoptosis. Notably, we observed decreased proliferation (Figure 2C,D) and enhanced apoptosis (Figure 2E) in Huh7 cells transfected with siRNA targeting *ATF7* (si-*ATF7*) compared to control siRNA (si-NC). Conversely, overexpression of FLAG-tagged *ATF7* (FLAG-*ATF7*) exerted the opposite effect (Figure 2C-E). Thus, these data indicate that *ATF7* is a direct target of miR-340-5p and exerts pro-growth effects on the Huh7 cell line.

To determine whether miR-340-5p inhibits proliferation and promotes apoptosis through its effect on *ATF7*, Huh7 cells were transfected with miR-340-5p-in plus siRNA-*ATF7* or miR-340-5p-in plus si-NC (or the double control NC-in/si-NC). We found that miR-340-5p-in promoted cell proliferation and decreased apoptosis in the presence of si-NC, whereas this oncogenic effect was relieved by endogenous *ATF7* silencing (Figure 2F-H). Thus, these data suggest that miR-340-5p regulates proliferation and apoptosis in HCC cells through its effect on *ATF7*.

## 3.3 | Hepatitis B virus promoted cell proliferation and inhibited apoptosis by downregulation of miR-340-5p expression to induce ATF7 expression

The above data indicated that miR-340-5p inhibited the growth of HCC by modulating expression of *ATF7*. To further investigate the relationship among the expression of *ATF7*, miR-340-5p,



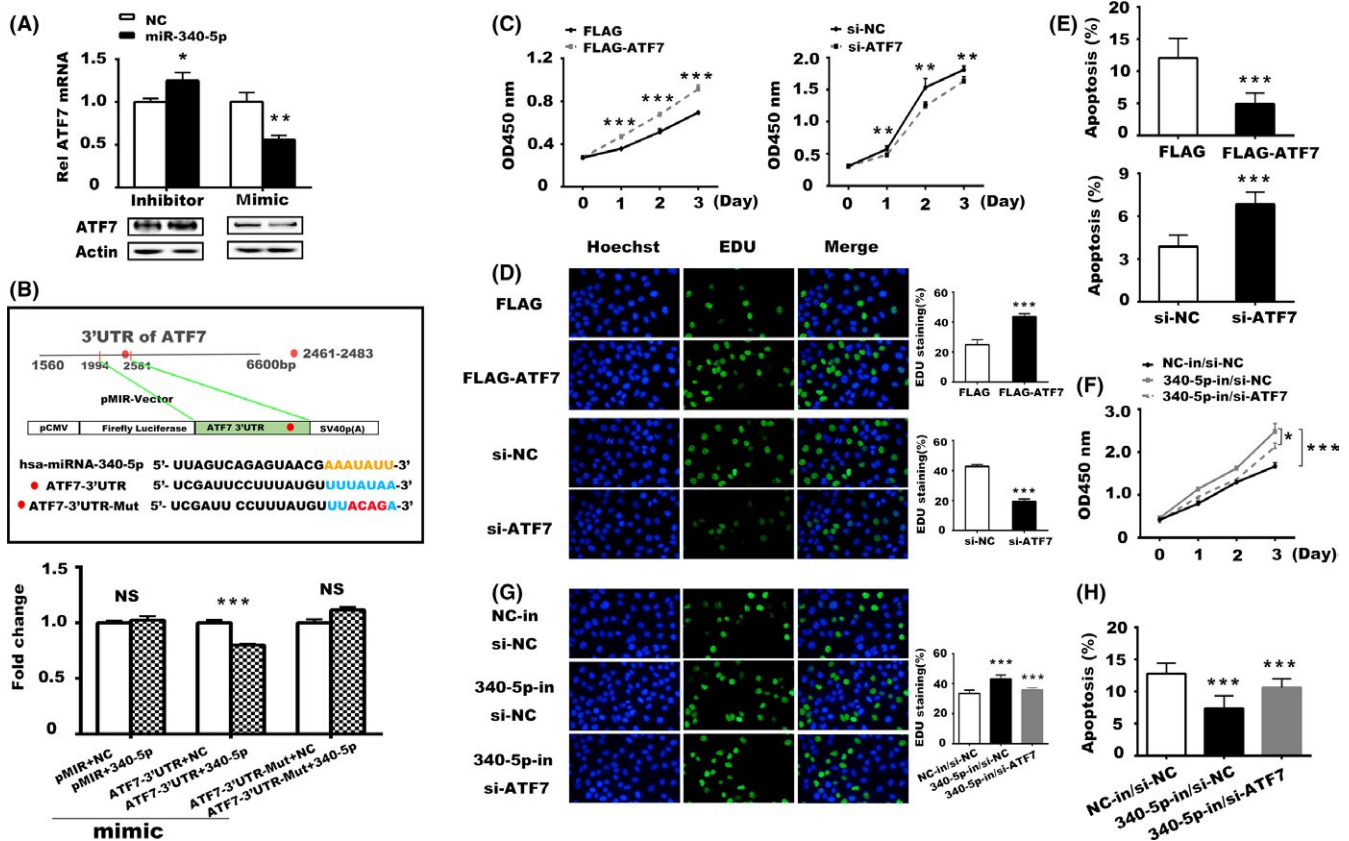
**FIGURE 1** MicroRNA-340-5p (miR-340-5p)-mediated hepatitis B virus (HBV) induced proliferation and inhibition of apoptosis in Huh7 cells. (A, B) miR-340-5p inhibits proliferation and (C) promotes apoptosis of Huh7 cells. (D, E) HBV induces proliferation of Huh7 cells, but this effect was ameliorated by cotransfection with miR-340-5p mimic (miR-340-5p-m). (F) HBV inhibited apoptosis in Huh7 cells; however, apoptotic levels were restored by cotransfection with miR-340-5p-m. \* $P < .05$ ; \*\* $P < .01$ , \*\*\* $P < .001$ . EdU, 5-ethynyl-2'-deoxyuridine

and HBV at the mRNA and protein levels, we used HepG2 and HepG2.2.15 cells, which are HBV negative and positive, respectively. Expression analysis with qRT-PCR and western blot assays showed that miR-340-5p was decreased, and ATF7 was increased, in HepG2.2.15 cells compared to HepG2 cells (Figure 3A, left panel). We observed a similar pattern in Huh7 cells, transfected with either pHBV or a control plasmid (Figure 3A, right panel), but the elevated ATF7 expression in cells transfected with pHBV was abolished by cotransfection with miR-340-5p-m (Figure 3B). Thus, these data indicate that ATF7 is regulated by the HBV-miR-340-5p pathway in Huh7 cells. We then measured cell proliferation and apoptosis in Huh7 cells cotransfected with pHBV and miR-340-5p-m alone or in combination with FLAG-ATF7 (and the associated controls) to further elucidate the biological function of ATF7 in the context of the HBV-miR-340-5p pathway. We found that the enhanced growth (increased proliferation and decreased apoptosis) induced by HBV was ameliorated by miR-340-5p-m, but was partially rescued by overexpression of FLAG-ATF7 (Figure 3C,D). Thus, this may indicate that there are other partially redundant pathways that carry out the same

functions. We achieved similar results using western blot analysis whereby HBV could increase the protein expression of cyclin D1 and inhibit cleaved PARP and caspase-9, but miR-340-5p with HBV cotransfection decreased the expression of cyclinD1 and increased cleaved PARP and caspase-9 protein level (Figure 3E). Furthermore, we found that ATF7 rescued protein expression of cyclin D1, PARP and caspase-9 (Figure 3F).

### 3.4 | ATF7 regulated growth of HCC through interaction with HSPA1B

To more precisely elucidate the molecular mechanisms by which ATF7 modulates cell growth in HCC, Huh7 cells were transfected with FLAG-ATF7 or the control plasmid, and both IP and MS analyses were carried out. From the results of MS, we found XRCC6, PRMT1, and HSPA1B as potential binding partners of ATF7 (Figure 4A). We then carried out co-ip assays to validate the potential binding of each of these proteins with ATF7 and found that ATF7 showed a strong interaction with endogenous and exogenous HSPA1B, but showed no detectable interaction with XRCC6 or PRMT1 (Figure 4B). Based



**FIGURE 2** Activating transcription factor 7 (ATF7) was confirmed as a direct target of microRNA-340-5p (miR-340-5p) and mediated its effect on proliferation and apoptosis in huh7 cells. A, mRNA and protein levels of ATF7 were measured by qRT-PCR and western blot analysis, respectively. Results indicated that miR-340-5p mimic (miR-340-5p-m) negatively regulated ATF7 expression at the mRNA and protein levels. B, Dual-luciferase reporter assays were used to validate the putative miR-340-5p binding site in the 3'-UTR of ATF7. Inhibition of reporter luciferase activity was observed when miR-340-5p-m was cotransfected with the WT ATF7-3'-UTR reporter. No repression was observed for ATF7-3'-UTR-mut, in which four key nucleotides in the miR-340-5p binding site were mutated. C-E, ATF7 promoted proliferation of Huh7 cells as measured by (C) CCK-8 and (D) EdU staining assays. E, ATF7 suppressed apoptosis of Huh7 cells by Hoechst 33258 assay; quantification was obtained by calculating the ratio of apoptotic cells to whole cells in every photograph. F-H, ATF7 was involved in miR-340-5p-mediated inhibition of proliferation in Huh7 cells, as measured by (F) CCK-8 and (G) EdU staining assay. H, Hoechst 33258 staining showed that ATF7 is involved in the miR-340-5p-mediated induction of apoptosis in Huh7 cells. \* $P < .05$ ; \*\* $P < .01$ , \*\*\* $P < .001$ , NS, no significant difference. EdU, 5-ethynyl-2'-deoxyuridine

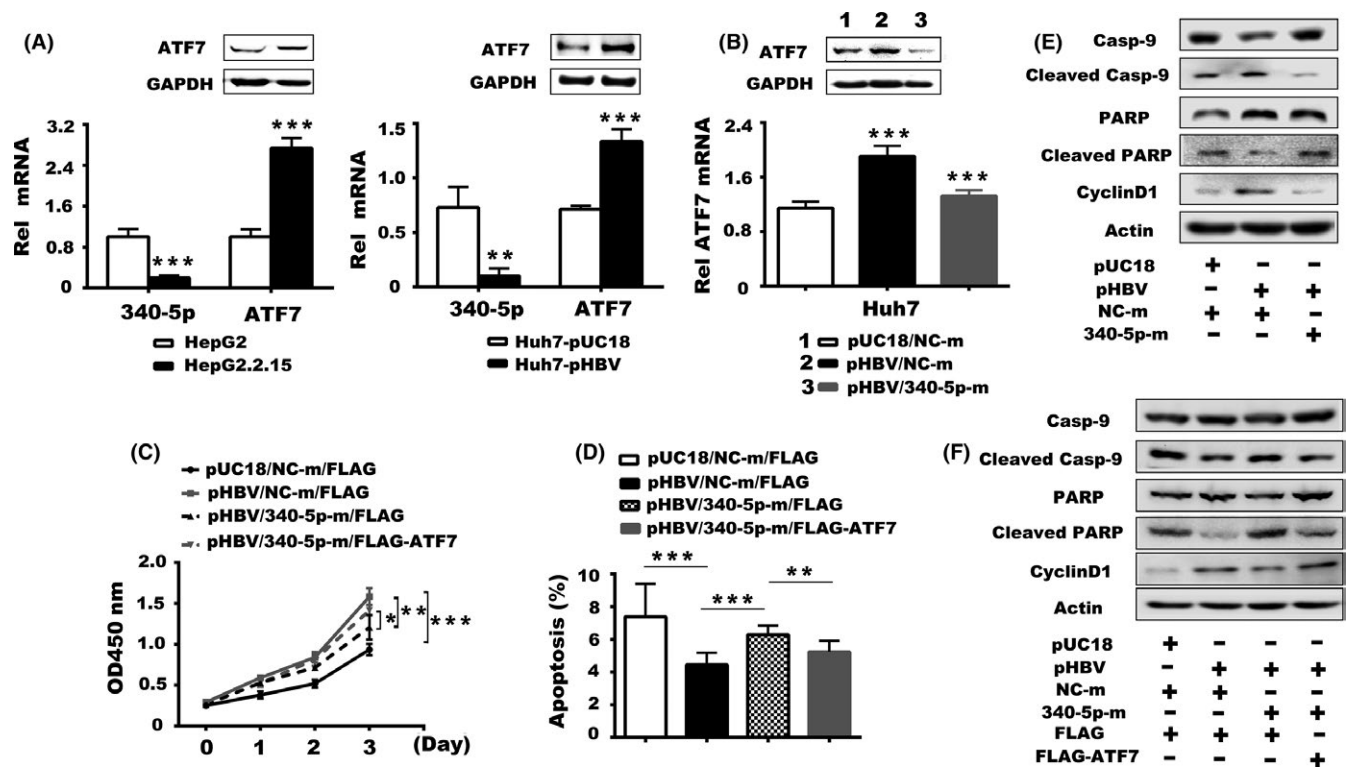
on these data, we therefore conclude that ATF7 interacts with HSPA1B in Huh7 cells.

Heat shock proteins (HSP) are stress-inducible molecules that modulate cellular responses to conditions such as chronic inflammation, fibrosis, and carcinogenesis. Notably, HSPA1B (also referred to as HSP70-2), a member of the HSP family, had been shown to promote cell proliferation, migration, and invasion and to suppress apoptosis in various cancers.<sup>17,18</sup> Elevated expression of HSPA1B was also reported in HCC tissue and had been shown to play a role in HBV-related HCC development.<sup>19</sup> We therefore further investigated the functional role of HSPA1B in HCC by silencing endogenous HSPA1B expression using siRNA (si-HSP). We found that HSPA1B knockdown inhibited cell proliferation (Figure 4C,D), and promoted apoptosis (Figure 4E). In addition, the proliferation and apoptotic effects of HBV or miR-340-5p transfection were ameliorated by HSPA1B knockdown (Figure 4F-I). Collectively, these data show that HSPA1B exerts a positive

effect on cell growth and may be involved in the HBV-miR-340-5p-ATF7 axis.

### 3.5 | ATF7 was upregulated in HCC tissues in vivo

In our previous study, we found that the expression of miR-340-5p was significantly lower in HCC tissues than in matched non-cancerous liver tissue.<sup>10</sup> To determine how ATF7 expression relates to that of miR-340-5p in HCC patients, we carried out a search for ATF7 and HSPA1B transcription in the TCGA database (<https://cancergenome.nih.gov/>). As shown in Figure 5A,E, we found that ATF7 and HSPA1B appeared to be upregulated in tumor tissues, as compared to normal tissues. We further used qRT-PCR to measure mRNA levels of ATF7 in tumor tissue and detected notably higher expression in HCC tumors relative to paired adjacent tissues (Figure 5B). Last, we measured ATF7 and HSPA1B protein levels by immunohistochemistry (IHC) in HCC tissue samples and matched adjacent



**FIGURE 3** Activating transcription factor 7 (ATF7) was involved in the hepatitis B virus (HBV)/microRNA (miR-340-5p) pathway-mediated modulation of cell proliferation and apoptosis. A, Expression levels of miR-340-5p and ATF7 were measured by qRT-PCR and western blot analysis (ATF7 only) in HepG2 cells, an HBV-negative cell line, and in HepG2.2.15 cells, an HBV-positive cell line. B, ATF7 mRNA and protein levels were measured in Huh7 cells. ATF7 levels were increased in cells transfected with pHBV, but decreased to control levels in cells cotransfected with HBV and miR-340-5p mimic (miR-340-5p-m). C, D, Effects on (C) cell proliferation and (D) apoptosis induced by HBV in Huh7 cells were ameliorated by miR-340-5p-m, but partially rescued by overexpression of FLAG-ATF7. E, F, Western blotting results show protein expression of cyclin D1, poly ADP ribose polymerase (PARP) and caspase-9. \* $P < .05$ ; \*\* $P < .01$ , \*\*\* $P < .001$

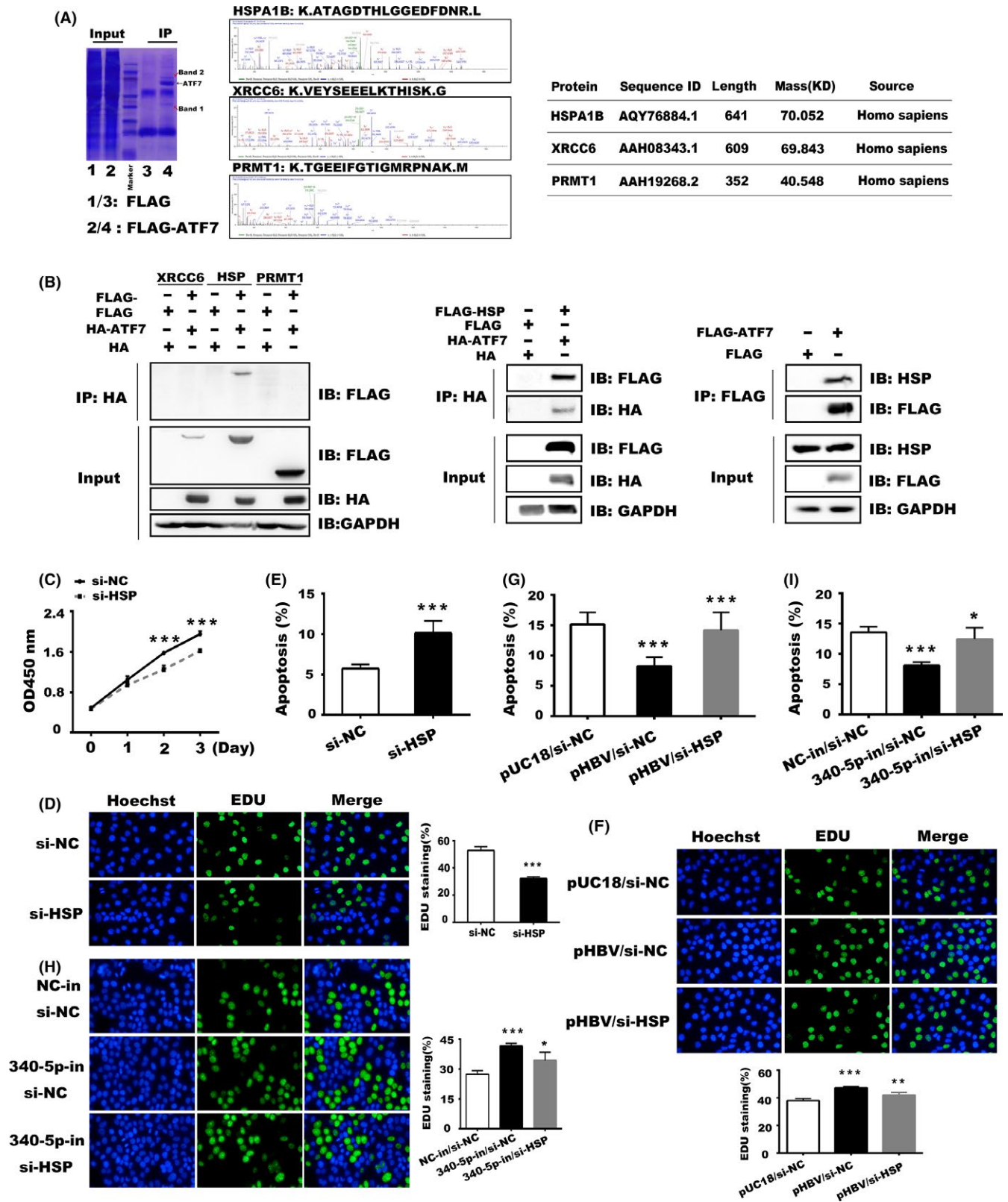
tissues. We found that in non-cancerous tissues, ATF7 and HSPA1B were barely detectable, whereas expression was much stronger in HCC tissues (Figure 5C,D,F). These *in vivo* data further support the conclusion that ATF7 is a target of miR-340-5p, and that miRNA-340-5p-mediated ATF7-HSPA1B repression functions to inhibit liver cancer growth.

## 4 | DISCUSSION

An increasing number of published reports have shown that miRNAs are downregulated in different cancers.<sup>20,21</sup> These non-coding RNAs play important roles in gene regulation and, thus, the identification of cancer-specific miRNAs and their targets is critical for understanding their role in the development and progression of oncogenesis. Furthermore, the effect of any given miRNA on its various target genes can often produce a range of different pathophysiological effects. To our knowledge, the previous study is the first to examine miR-340-5p regulation by HBV and to investigate the role that miR-340-5p plays in regulating migration and invasion of liver cancer cells as it pertains to the development and progression of HCC.<sup>10</sup> In this report, we further show that miR-340 functions as a tumor growth suppressor in

liver cancer cells, most likely through the downregulation of its target ATF7, which physically interacts with the heat shock protein, HSPA1B.

To confirm that miR-340 acts as a tumor growth suppressor in HCC, we carried out proliferation and apoptosis assays in Huh7 cells expressing either a miR-340-5p mimic or an inhibitor. It is well known that miR-340 plays a role in the regulation of different cancers by modulating the expression of various target genes. For example, a study by Xie et al<sup>22</sup> found that inhibition of miR-340 in endometrial carcinoma promoted a significant increase in proliferation and a decrease in apoptosis through the activity of a number of different proteins. Another study by Fernandez et al<sup>23</sup> showed that miR-340 inhibited tumor cell proliferation and induced apoptosis in non-small cell lung adenocarcinoma by regulating pumilio RNA binding family members 1 and 2 (PUM1 and PUM2), which functioned as negative regulators of p27. In addition, Yuan et al<sup>9</sup> showed that miR-340 suppressed HCC cell proliferation and invasion by regulating the JAK1/STAT3 pathway. Our data, which indicate that miR-340 can inhibit proliferation and induce apoptosis in HCC cells, are consistent with previous reports. However, we further found that downregulation of miR-340 by HBV modulates proliferation and apoptosis of hepatocellular carcinoma cells by promoting the expression of its target ATF7.

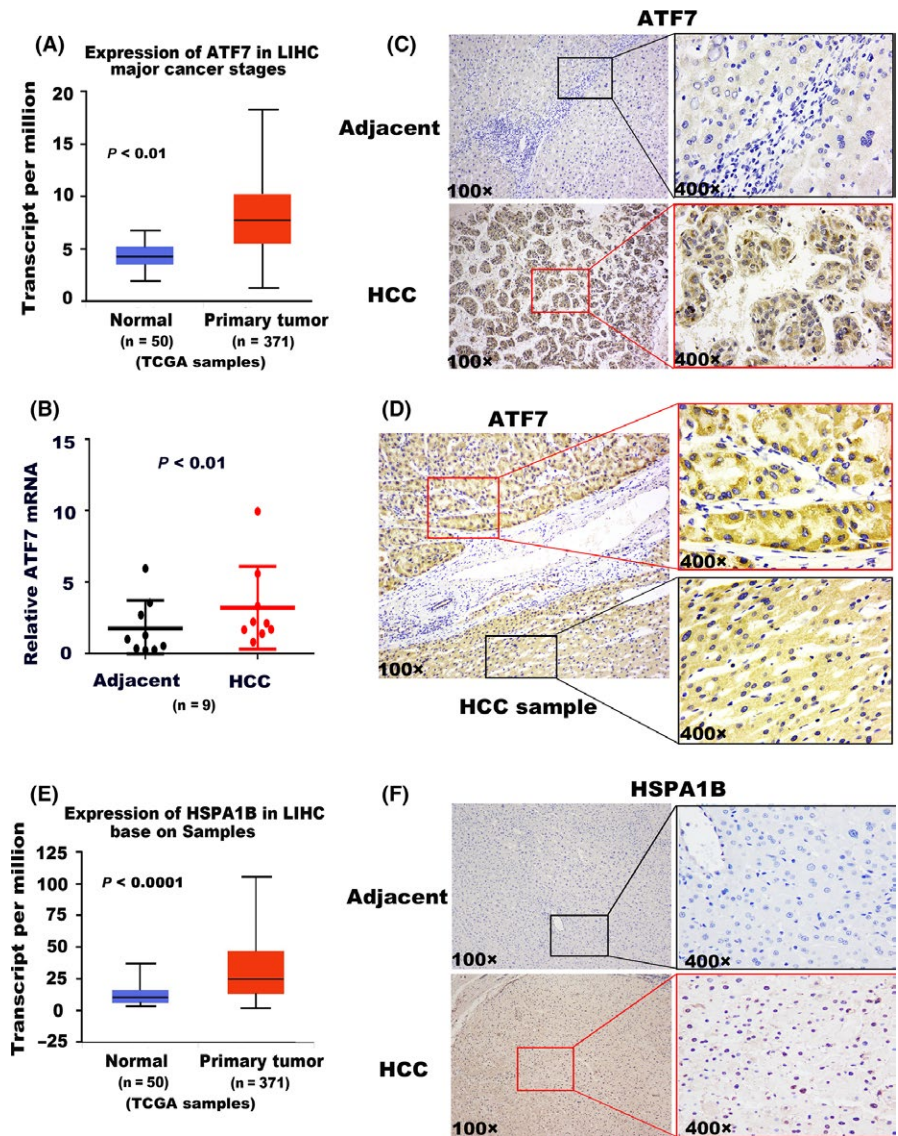


ATF7 belongs to the ATF/cAMP response element-binding (CREB) protein family<sup>24</sup> whose members show diverse physiological functions in the regulation of cell survival.<sup>25</sup> Notably, there is increasing evidence indicating that ATF7 may function to regulate proliferation and the cell cycle in various cancers. For example,

ATF7 was shown to promote cell cycle progression by regulating cyclin D1, as well as the expression of stress-activated MAPK in G1 and S phases and Cdk1-cyclin B1 in the G2/M phase.<sup>26,27</sup> In addition, ATF7 has been shown to form a heterodimeric complex with the AP-1 family member, c-jun, through its C-terminal



**FIGURE 4** Heat shock protein A member 1B (HSPA1B) interacted with activating transcription factor 7 (ATF7) to regulate proliferation and apoptosis in Huh7 cells. A, Immunoprecipitation (IP) and mass spectrometry (MS) analyses were carried out in Huh7 cells transfected with FLAG-ATF7 or control plasmid (FLAG), and bands of approximately 40 kDa (band 1) and 70 kDa (band 2) were identified by MS. B, Coimmunoprecipitation (co-ip) assays were used to verify the interaction between ATF7 and HSPA1B, X-ray repair cross-complementing protein 6 (XRCC6), and protein arginine methyltransferase 1 (PRMT1) in Huh7 cells. ATF7 interacted with endogenous and exogenous HSPA1B, but showed no detectable interaction with XRCC6 or PRMT1. C, CCK-8 and D, EdU cell proliferation assays in Huh7 cells. Results indicate that HSPA1B knockdown inhibited proliferation. E, Hoechst 33258 assay. si-HSPA1B increased apoptosis in Huh7 cells. F, G, Cotransfection with si-HSPA1B and the HBV vector, pHBV1.3 (pHBV), (F) inhibited HBV-induced cell proliferation and (G) restored HBV-mediated inhibition of apoptosis in Huh7 cells. H, I, EdU and Hoechst staining assay in Huh7 cells. Results show that miR-340-5p-inhibitor (miR-340-5p-in) increased proliferation and inhibited apoptosis but was restored by si-HSPA1B. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ . EdU, 5-ethynyl-2'-deoxyuridine



**FIGURE 5** Activating transcription factor 7 (ATF7) and heat shock protein A member 1B (HSPA1B) were upregulated in hepatocellular carcinoma (HCC) samples. A, E, The Cancer Genome Atlas (TCGA) database results indicated that expression of ATF7 and HSPA1B was higher in HCC samples than in paratumor normal liver samples. B, Expression of ATF7 mRNA was measured by qRT-PCR in nine paired HCC clinical samples and the adjacent tissue controls, showing significantly increased expression in HCC tissue relative to controls. C, D, F, Immunohistochemistry analysis of ATF7 and HSPA1B protein expression in nine HCC clinical samples and in matched control samples showed that expression levels are higher in tumor tissue relative to controls. Black box shows adjacent tissues, and red box represents HCC tumor tissues

leucine-zipper region, which functions to promote cell growth.<sup>28</sup> Mouse knockout studies have further shown that ATF7 knockout, combined with key mutations in ATF2, results in embryonic lethality that is characterized by high levels of apoptosis in developing hepatocytes and hematopoietic cells.<sup>29</sup> Here, our results are consistent with published reports and show that ATF7 promotes Huh7

cell proliferation and blocks apoptosis through its interaction with HSPA1B.

Heat shock protein A member 1B is a member of HSP which is involved with cell survival and cellular growth in various cancer cell lines.<sup>30</sup> A number of reports showed that HSPA1B overexpression was associated with early spread and progression of different cancers, such

as bladder urothelial carcinoma,<sup>31</sup> ovarian cancer,<sup>32</sup> and cervical carcinoma.<sup>18</sup> Jeng et al suggested that HSPA1B was highly expressed in HBV-related HCC compared to non-tumor tissue, Its single nucleotide polymorphism (site 1267) play important role in risk and prognosis of HCC.<sup>33</sup> Our result indicated miR-340 affected HCC by ATF7 interaction with HSPA1B. In summary, our study showed that downregulation of miR-340 by HBV induces liver cancer cell proliferation and inhibits apoptosis by inducing expression of its target, *ATF7*, which further interacts with HSPA1B. Specifically, our in vitro studies indicate that overexpression of miR-340 protects against HBV-induced cell growth through inactivation of *ATF7*, and thus suggests that functional miR-340 overexpression may represent a possible therapeutic strategy for HCC. Additionally, from our analysis of miR-340 expression in tissue from cancer patients and in vitro assays,<sup>10</sup> we conclude that miRNAs, such as miR-340, are required for protection against HCC. Based on the strong miR-340-mediated tumor suppression observed in our study, and together with the highly promising therapeutic approaches that involve promoting expression of tumor suppressor miRNAs, we propose that further studies are warranted to explore the potential of miR-340 as a therapeutic target for prevention and/or treatment of HBV-dependent HCC.

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## CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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#### SUPPORTING INFORMATION

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