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Long non-coding RNA HULC activates HBV by modulating HBx/STAT3/miR-539/APOBEC3B signaling in HBV-related hepatocellular carcinoma

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ABSTRACT

Long noncoding RNA HULC is identified and highly expressed in hepatocellular carcinoma (HCC). Hepatitis B virus (HBV) is a key driver of liver cancer. In the present study, we found that HULC remarkably elevated the levels of HBeAg, HBsAg, HBcAg, pgRNA, HBx, HBV DNA and covalently closed circular DNA (cccDNA), which activated the HBV replication in HBV-expressing hepatoma cells or *de novo* HBV-infected cell lines (PHH, HepG2-NTCP and dHepaRG). Mechanistically, HULC enhanced HBV cccDNA stability by down-regulating the APOBEC3B in hepatoma cells. HULC significantly up-regulated microRNA-539, which targeted the 3′UTR of APOBEC3B mRNA. Luciferase reporter gene assays revealed a putative STAT3-binding site located in the upstream of miR-539 promoter. Moreover, we identified that HULC was able to elevate HBx, which co-activated the STAT3 to stimulate the miR-539 promoter. Then, miR-539 down-regulated APOBEC3B and promoted HBV replication. Functionally, HULC enhanced the growth of hepatoma cells by activating HBV *in vitro* and *in vivo*, which could be blocked by overexpressing APOBEC3B. In conclusion, HULC activates HBV by modulating HBx/STAT3/miR-539/APOBEC3B signaling in HBV-related HCC.

1. Introduction

Accumulating evidence has indicated that long non-coding RNAs (lncRNAs) can participate in diverse physiological and pathological processes and affect disparate cellular functions [1–4]. LncRNAs are transcripts longer than 200 bp that do not have any apparent protein-coding ability [5,6]. The first lncRNA found to be specifically over-expressed in hepatocellular carcinoma (HCC) is known as highly up-regulated in liver cancer (HULC) [7]. HULC is thought to act as an endogenous sponge, because it down-regulates a series of microRNA activities, including those of miR-372 and miR-107 [8,9]. Moreover, our lab previously reported that HULC modulated abnormal lipid metabolism in hepatoma cells through a miR-9–mediated RXRA signaling pathway [10]. However, the role of HULC in hepatocarcinogenesis is poorly understood.

HCC is one of the most prevalent malignant tumors and a leading cause of cancer-related death, globally [11–13]. Hepatitis B virus (HBV) infection is closely related to the development of liver diseases [14–17]. More than 350 million people are chronically infected with HBV worldwide, and of these, about one-third develop severe HBV-related complications [18–20]. Upon HBV infection, cccDNA, which harbors a chromatin-like structure, dwells in the nuclei of infected cells. There, cccDNA serves as a template for the transcription of all viral RNAs, and thus, it sustains viral persistence [21–23]. The DNA cytidine deaminases, APOBEC3A (termed A3A) and APOBEC3B (termed A3B), are responsible for the deamination (i.e., destruction) of HBV cccDNA [24,25]. The interactions between HBV and host factors play crucial roles in the development of HCC [26]. However, the effect of lncRNAs on HBV is unclear.

In the present study, we are interested in the role of HULC in the modulation of HBV to enhance hepatocarcinogenesis. Interestingly, we identified that HULC could activate the HBV by HBx/STAT3/miR-539/APOBEC3B signaling, leading to the growth of liver cancer. Thus, our finding provides new insights into the mechanism by which HULC promotes HBV in hepatocarcinogenesis.

Abbreviations: APOBEC3B, Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B; cccDNA, Covalently closed circular DNA; ETV, Entecavir; HBV, Hepatitis B virus; HCC, Hepatocellular carcinoma; HULC, Highly up-regulated in liver cancer; LdT, Telbivudine; PHHs, Primary human hepatocytes; STAT3, Signal transducer and activator of transcription 3

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2. Material and methods

2.1. Patient samples

Thirty-one HCC tissue samples were obtained from Tianjin Tumor Hospital (Tianjin, China). The characteristics of the patients with HCC are presented in Supplementary Table S1. All patients approved the use of tissue samples for research purposes. The study protocol was approved by the Institute Research Ethics Committee at Nankai University.

2.2. Statistical analysis

The Gehan-Breslow-Wilcoxon test was used to analyze the relapse-free survival of 75 patients with HBV-related hepatomas and 267 patients with HBV-negative hepatomas. The HBV group was divided into low and high HULC expression subgroups. Pearson's correlation coefficient was used to determine correlations between HULC and APOBEC3B mRNA or miR-539 levels in tumor tissues. The student's *t*-test was used to compare independent groups. P-values were considered significant when $*p < 0.05$; $**p < 0.01$; or $***p < 0.001$, as appropriate. All results were replicated in three independent experiments.

3. Results

3.1. HULC elevates the levels of HBV indicated markers

Based on the data from The Cancer Genome Atlas (TCGA), we found that the high levels of HULC were significantly correlated with the poor relapse-free survival in patients with HBV-related HCC (n = 75, $p = 0.0279$, Supplementary Fig. S1A). Conversely, the low levels of HULC were not significantly related to the relapse-free survival in patients with HBV-negative HCC (n = 267, $p = 0.4212$, Supplementary Fig. S1B), suggesting that HULC promotes the malignant proliferation of HBV-related HCC. Moreover, we demonstrated that the expression levels of HULC were higher in HBV-expressing cells than HBV-free cells (Supplementary Fig. S1C). Importantly, the interactions between HBV and host play a key role in hepatocarcinogenesis [27]. We previously reported that HBx could up-regulate the HULC in liver cancer [28]. However, the effect of HULC on HBV replication has not been documented.

We first confirmed the overexpression (or interference) efficiency of HULC with real-time quantitative PCR (RT-qPCR) and RT-PCR analyses (Supplementary Fig. S1D–G). Interestingly, ELISA, RT-qPCR and qPCR assays indicated that the upregulation of HULC increased the levels of HBeAg, HBsAg, pgRNA, HBx and HBV DNA in HepG2-PCH9/3091 cells (Fig. 1A and Supplementary Fig. S1H–I). We performed cccDNA-specific qPCR and found that the overexpression of HULC increased HBV cccDNA in the nucleus of HepG2-PCH9/3091 cells (Fig. 1B). Conversely, the depletion of HULC led to the opposite results in HepAD38 (tet off) and HepG2.2.15 cells (Fig. 1C and D and Supplementary Fig. S1J-M). Southern blot analysis showed that HULC could increase the amount of HBV cccDNA in the cells (Fig. 1E), suggesting that HULC contributes to the HBV cccDNA stability during the activation of HBV replication in hepatoma cells. Moreover, we confirmed that the levels of HULC in clinical HCC tissues were higher in HBV cccDNA-positive (n = 21) than those in HBV cccDNA-negative tissues (n = 8; Supplementary Fig. 1P), supporting that HULC enhances HBV cccDNA in liver cancer. Furthermore, in *de novo* HBV-infected cells, including PHH, differentiated HepaRG (dHepaRG) and HepG2-NTCP cells, 7 days after the HBV infection, we found that the overexpression of HULC also significantly increased the expression of HBeAg (or HBsAg), pgRNA, HBx, HBV DNA and HBV cccDNA (Fig. 1F and Supplementary Fig. S1Q–W). The overexpression of HULC was determined by RT-qPCR and RT-PCR analyses in the cell (Supplementary Fig. S1N, O). Immunofluorescence analysis showed that the overexpression of HULC could increase the expression of HBcAg in HepG2-PCH9/3091 cells. Conversely, the interference of HULC resulted in the reduction of HBcAg in HepAD38 cells (Fig. 1G). Taken together, we conclude that HULC increases the expression of HBV indicated markers.

3.2. HULC down-regulates APOBEC3B to maintain HBV cccDNA stability

It is known that APOBEC3A and APOBEC3B can result in cytidine deamination, apurinic/apyrimidinic site formation and lead to partial cccDNA degradation without affecting the host genome [24,29]. Therefore, we hypothesized that HULC might modulate HBV cccDNA by regulating APOBEC3A or APOBEC3B. Then, we observed that the cccDNA amplification was successful at reduced denaturing temperatures when in HepAD38 and HepG2.2.15 cells were treated with small interference RNAs of HULC (si-HULC-1) (Fig. 2A), suggesting that HULC motivates HBV cccDNA cytidine deamination. RT-qPCR analysis confirmed that, in HepG2-PCH9/3091 cells, the HULC could down-regulate APOBEC3B, but not APOBEC3A (Fig. 2B). Conversely, the depletion of HULC up-regulated the expression of APOBEC3B, but not APOBEC3A (Fig. 2C), which was validated by Western blot analysis (Fig. 2D and Supplementary Fig. S2A and B). Furthermore, we confirmed the inhibitory effect of HULC on APOBEC3B in HBV-infected dHepaRG cells, HBV-free HepG2 cells and L-O2 cells (Supplementary Fig. S2C and D), suggesting that HULC can down-regulate APOBEC3B. Meanwhile, the effect of HULC knockdown on APOBEC3B was examined by RT-qPCR assays in HepG2.2.15 cells (Supplementary Fig. S2E). The silencing efficiencies of si-A3B-1 and si-A3B-2 were confirmed by RT-qPCR in HepAD38 and HepG2.2.15 cells (Supplementary Fig. S2F). Interestingly, we demonstrated that interfering with APOBEC3B could rescue the si-HULC-induced inhibition of HBV indicated markers (HBeAg, HBsAg and cccDNA) in HepAD38 and HepG2.2.15 cells (Fig. 2E). Conversely, HULC-induced upregulation of these markers was abolished by co-transfecting with APOBEC3B (Fig. 2F), supporting that HULC maintains HBV cccDNA stability by down-regulating APOBEC3B. The expression levels of APOBEC3B were confirmed by the Western blot analysis in HepAD38, HepG2.2.15 and HepG2-NTCP cells (Supplementary Fig. S2G–I). We also assessed the correlation between HULC and APOBEC3B in clinical HBV-related HCC tissues. RT-qPCR showed that the levels of HULC were negatively associated with those of APOBEC3B in clinical samples (Fig. 2G). Thus, we conclude that HULC down-regulates APOBEC3B to maintain HBV cccDNA stability.

3.3. HULC-elevated miR-539 down-regulates APOBEC3B by targeting its mRNA 3′UTR

We identified HULC-mediated miRNAs in three databases: TargetScan (http://www.targetscan.org/vert_71/), microRNA.org (http://34.236.212.39/microrna/home.d), and miRwalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html). We selected five high-score miRNAs from 22 miRNAs predicted to target the 3′UTR of APOBEC3B mRNA (Supplementary Fig. S3A and B).

Strikingly, RT-qPCR revealed that the overexpression of HULC dose-dependently increased the levels of miR-539 in HepG2-PCH9/3091 cells (Fig. 3A). Conversely, the knockdown of HULC reduced the expression of miR-539 in HepAD38 cells (Fig. 3B), suggesting that HULC may up-regulate miR-539. Moreover, we observed that the expression of HULC was significantly correlated with those of miR-539 in clinical HBV-related HCC tissues ($p < 0.0001$, Fig. 3C). Bioinformatics analysis indicated that miR-539 targeted a site in the 3′UTR of APOBEC3B mRNA (Fig. 3D). As expected, the luciferase reporter gene assay showed that the overexpression of miR-539 decreased the luciferase activities of APOBEC3B mRNA 3′UTR reporter (pGL3-A3B-3′UTR-wt) in HepAD38 and HEK293T cells (Fig. 3E and Supplementary Fig. S3C). Conversely, anti-miR-539 could result in the opposite results in the cells (Fig. 3F and Supplementary Fig. S3D). However, anti-miR-539 failed to work when

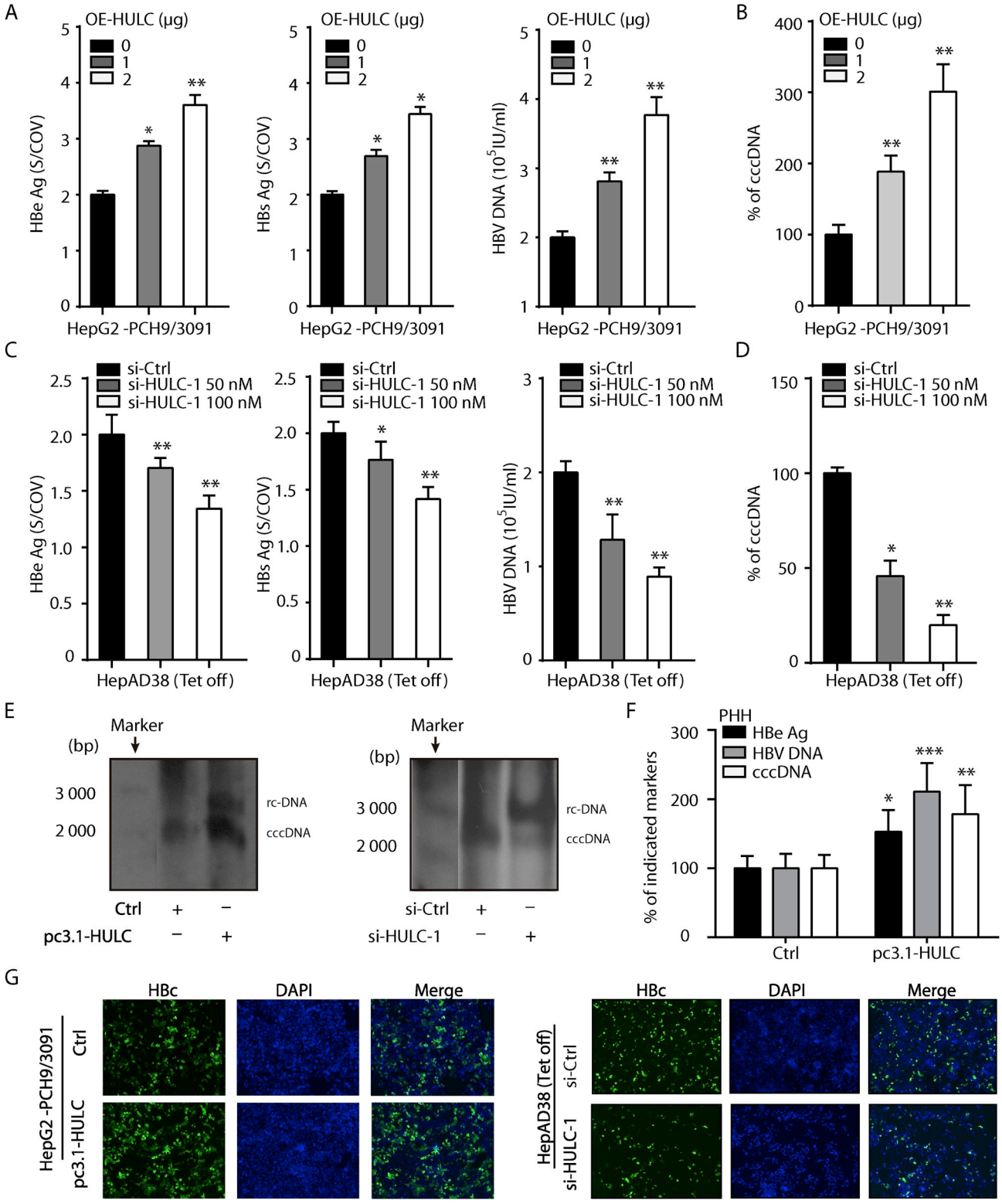

Fig. 1. HULC elevates the levels of HBV indicated markers. (A) The levels of HBeAg, HBsAg and HBV DNA were measured by ELISA assays and qPCR assays in the supernatant of HULC over-expressed HepG2-PCH9/3091 cells. (B) The cccDNA level was measured by qPCR assays in the nucleus of the HULC over-expressed HepG2-PCH9/3091 cells. (C) The levels of HBeAg, HBsAg and HBV DNA were measured by ELISA assays and qPCR assays in the supernatant of HULC knock-down HepAD38 cells. (D) The levels of cccDNA were measured by qPCR assays in the nucleus of the HULC knock-down HepAD38 cells. (E) The levels of cccDNA were examined by Southern blot in HULC over-expressed HepG2-PCH9/3091 cells (left) and HULC knock-down HepAD38 cells (right). (F) HBV indicated markers (HBeAg, HBV DNA, cccDNA) were tested by ELISA or qPCR assays in *de novo* HBV-infected PHH cells. (G) The HBc expression in HULC over-expressed HepG2-PCH9/3091 cells (left) and in HULC knock-down HepAD38 cells (right) was detected by immunofluorescence assays. Each experiment was repeated at least three times. Error bars represent means ± SD (n = 3). Statistically significant differences are indicated: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Student's *t*-test.

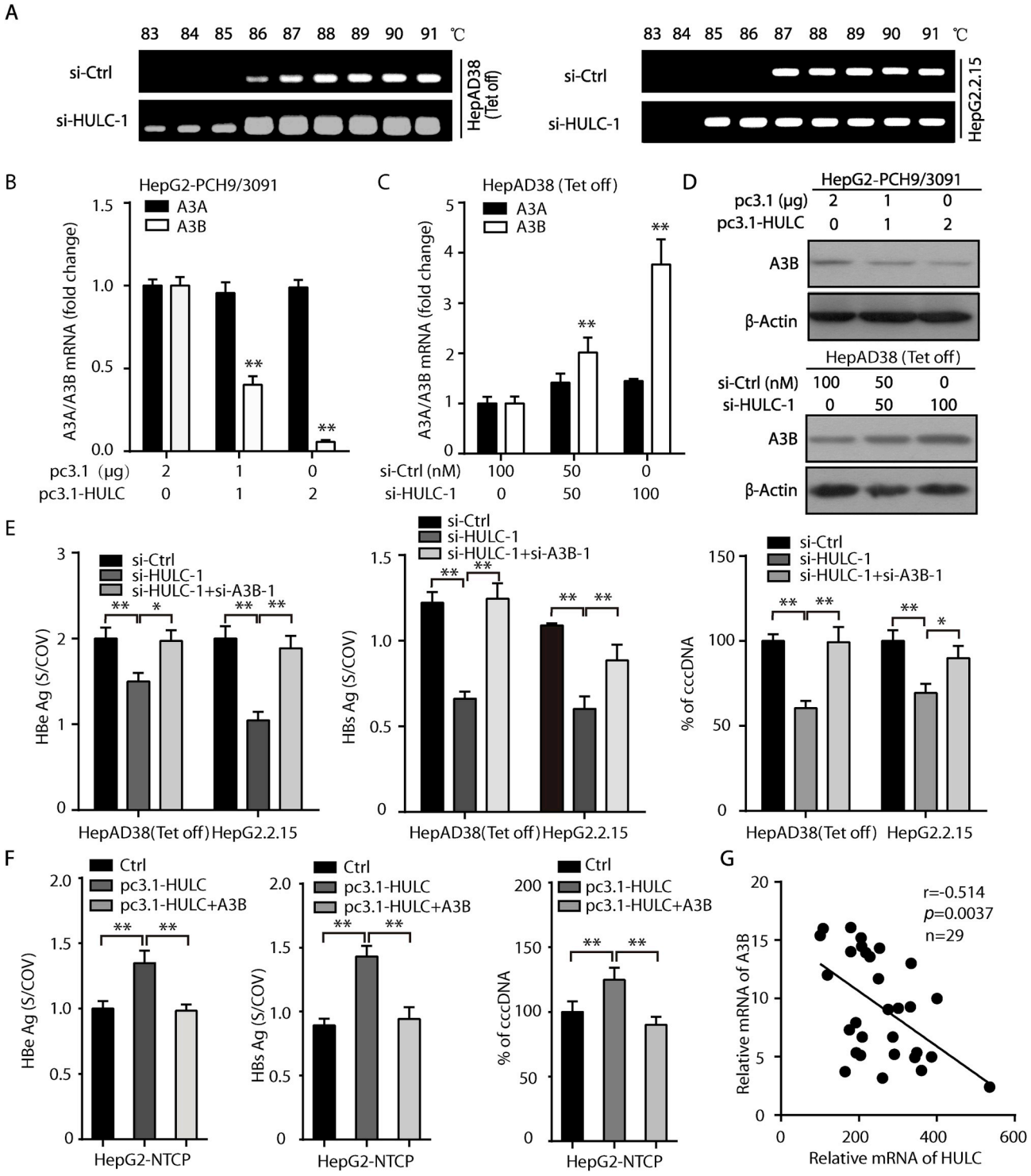

Fig. 2. HULC down-regulates APOBEC3B to maintain HBV cccDNA stability. (A) 3D-PCR analyses were performed for cccDNA in HepAD38 (left) and HepG2.2.15 cells (right). (B) The expression of APOBEC3A (A3A) and APOBEC3B (A3B) were measured by RT-qPCR in HepG2-PCH9/3091 cells. (C) The expression of A3A and A3B in HepAD38 cells were measured by RT-qPCR. (D) The expression of A3B was measured by Western blot analysis in HepG2-PCH9/3091 cells (up) and HepAD38 cells (down). (E) The levels of HBeAg and HBsAg were measured by ELISA assays in the supernatant of HepAD38 and HepG2.2.15 cells; the cccDNA level was measured by qPCR assays in the nucleus of the HepAD38 and HepG2.2.15 cells. (F) The levels of HBeAg and HBsAg were measured by ELISA assays in the supernatant of HBV infected HepG2-NTCP cells; the cccDNA level was measured by qPCR assays in the nucleus of the HepG2-NTCP cells. (G) Correlation between the HULC mRNA level and the A3B mRNA level was examined by RT-qPCR in the 29 cases of HBV-related HCC tissues (**$p < 0.01$, r = −0.514, Pearson's correlation coefficient). Each experiment was repeated at least three times. Error bars represent means ± SD (n = 3). Statistically significant differences are indicated: *$p < 0.05$; **$p < 0.01$; Student's *t*-test.

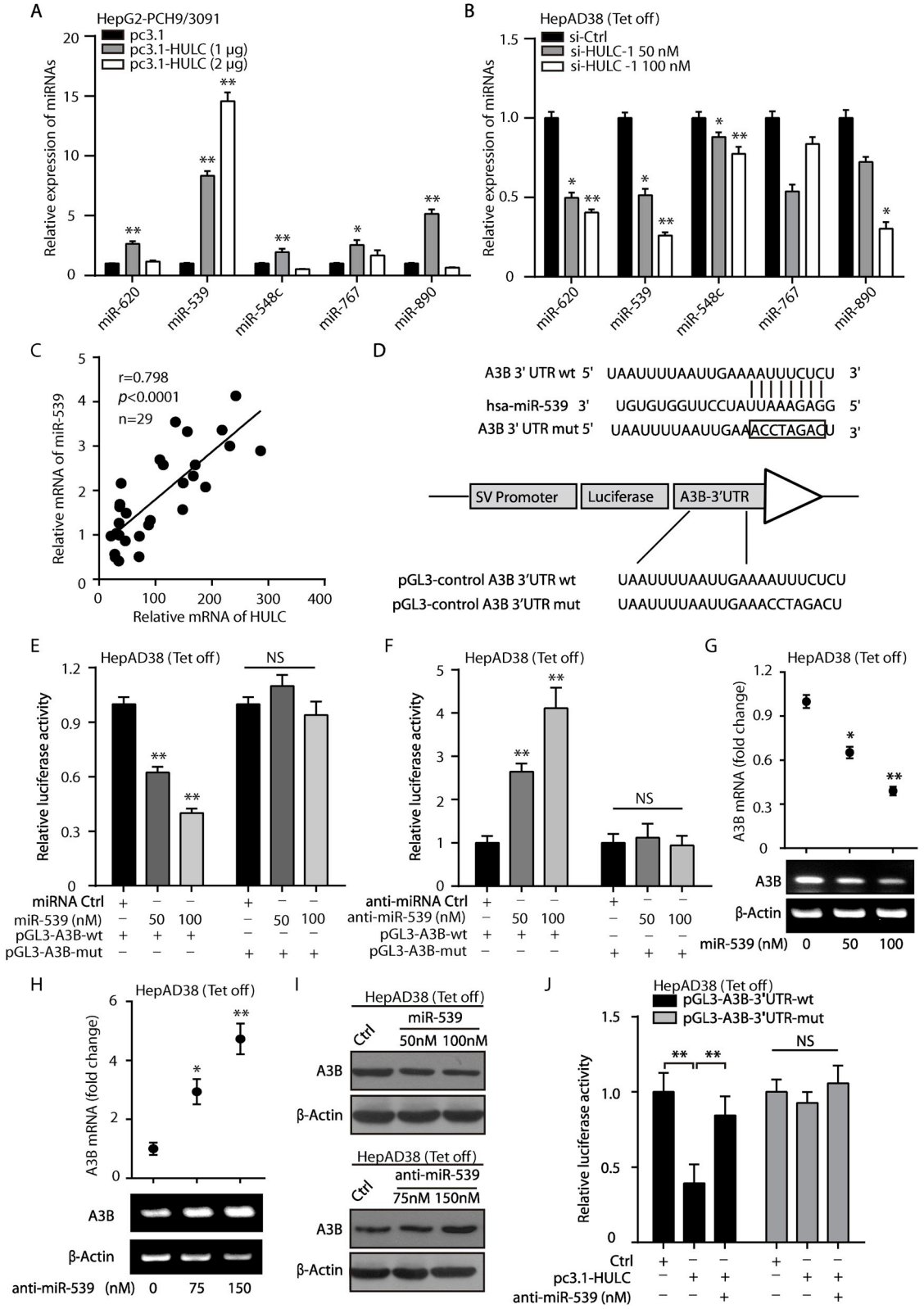

Fig. 3. HULC-elevated miR-539 down-regulates APOBEC3B by targeting its mRNA 3′UTR. (A) The expression of miRNAs (miR-620, miR-539, miR-548c, miR-767 and miR-890) was examined by RT-qPCR in HepG2-PCH9/3091 cells. (B) The expression of miRNAs (miR-620, miR-539, miR-548c, miR-767 and miR-890) was examined by RT-qPCR in HepAD38 cells. (C) Correlation between the HULC mRNA level and the miR-539 mRNA level was examined by RT-qPCR in the 29 cases of HBV-related HCC tissues (***$p < 0.001$, r = 0.798, Pearson's correlation coefficient). (D) A model demonstrates the predicted conserved miR-539 binding site at nucleotides 207–214 of the APOBEC3B (A3B) mRNA 3′UTR. The generated mutant sites at the A3B mRNA 3′UTR seed region were indicated. The wild-type A3B mRNA 3′UTR (or mutant) was inserted into the downstream of the luciferase reporter gene in the pGL3-control vector. (E and F) The effect of miR-539 (or anti-miR-539) on the pGL3-A3B-wt and pGL3-A3B-mut in HepAD38 cells was measured by luciferase reporter assays. (G and H) The effect of miR-539 (or anti-miR-539) on the expression of A3B in HepAD38 cells was measured by RT-qPCR. (I) The effect of miR-539 (or anti-miR-539) on the expression of A3B in HepAD38 cells was measured by Western blot analysis. (J) Luciferase activities were measured by luciferase reporter gene assays in HepAD38 cells. Each experiment was repeated at least three times. Error bars represent means ± SD (n = 3). Statistically significant differences are indicated: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS, no significant; Student's *t*-test.

the target sites were mutated (pGL3-A3B-3′UTR-mut; Fig. 3E and F and Supplementary Fig. S3C and D), suggesting that miR-539 directly targets the 3′UTR of APOBEC3B mRNA. The transfection efficiencies of miR-539 and anti-miR-539 were determined by RT-qPCR in the cells (Supplementary Fig. S3E and F). Moreover, RT-PCR and RT-qPCR showed that miR-539 dose-dependently suppressed APOBEC3B in HepAD38 cells at the mRNA levels (Fig. 3G). Conversely, the treatment with anti-miR-539 dose-dependently increased the expression of APOBEC3B mRNA (Fig. 3H). Western blot analysis validated the down- and up-regulation of APOBEC3B treated with miR-539 and anti-miR-539 in the cells, respectively (Fig. 3I), supporting that miR-539 can down-regulate the expression of APOBEC3B at the mRNA and protein levels. Moreover, RT-qPCR showed a negative association between miR-539 and APOBEC3B in clinical samples (Supplementary Fig. S3G).

Next, we found that the overexpression of HULC suppressed the luciferase activities of pGL3-A3B-3′UTR-wt in HepAD38 and HepG2.2.15 cells. The treatment with anti-miR-539 could rescue HULC-induced down-regulation of luciferase activities. HULC failed to influence the luciferase activities of pGL3-A3B-3′UTR-mut (Fig. 3J and Supplementary Fig. S3H). Taken together, we conclude that HULC-elevated miR-539 down-regulates APOBEC3B by targeting its mRNA 3′UTR in hepatoma cells.

3.4. HULC promotes the expression of miR-539 by stimulating HBx/STAT3 signaling

The luciferase reporter gene assays showed that HULC increased the activities of miR-539 promoter in HepG2.2.15 cells (Fig. 4A). Conversely, silencing of HULC reduced the activities of miR-539 promoter in HepAD38 cells (Fig. 4B), suggesting that HULC can up-regulate miR-539 at the transcription level. We searched for transcription factor binding sites in the miR-539 promoter with Alggen Promo (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?-dirDB = TF_8.3) and JASPAR (http://jaspar. binf.ku.dk/), and identified a putative STAT3-binding site (−2820bp ~ -2802bp). We cloned the miR-539 promoter fragments, including the STAT3 binding site, into a reporter vector (termed pGL3-539-wt), and we also created a mutated promoter (termed pGL3-539-mut; Fig. 4C). As expected, the relative luciferase activities of the pGL3-539-mut were clearly reduced relative to the activities of pGL3-539-wt in HepG2.2.15 and HepAD38 cells (Fig. 4D). Furthermore, we transfected a siRNA that interfered with STAT3 mRNA translation (pSi-STAT3), which suppressed the luciferase activities of pGL3-539-wt, but not pGL3-539-mut, in HepAD38 and HepG2.2.15 cells (Fig. 4E and Supplementary Fig. S4A), suggesting that STAT3 is involved in activation of the miR-539 promoter. The interference efficiency of pSi-STAT3 has experimentally shown by RT-qPCR in HepAD38 cells (Supplementary Fig. S4B).

Next, our data showed that the knockdown of STAT3 blocked the HULC-stimulated luciferase activities of pGL3-539-wt in HepG2.2.15 and HepAD38 cells (Fig. 4F), suggesting that HULC increases the miR-539 promoter activities by stimulating the transcription factor STAT3. However, we found that HULC failed to affect STAT3 expression in the cells (Supplementary Fig. S4C and D). Previous studies showed that HBx acted as a co-activator to induce differential expression of microRNAs (miRNAs), such as miR-22 and miR-221 [30,31], and HBx was able to co-activate STAT3 in the up-regulation of miR-21 [32]. In this study, we found that HBx exhibited a dose-dependent up-regulation of miR-539 expression in HepG2.2.15 cells (Supplementary Fig. S4E). Conversely, interfering with HBx, it down-regulated miR-539 expression in HepAD38 cells (Supplementary Fig. S4F). Thus, we hypothesized that HULC might up-regulate miR-539 by activating STAT3 on the miR-539 promoter (Fig. 4G). Chromatin immunoprecipitation (ChIP) assays indicated that STAT3 was recruited to the miR-539 promoter, and HBx increased STAT3 occupancy on the miR-539 promoter (Fig. 4H and Supplementary Fig. S4G). Moreover, we examined the HBx expression by Western blot analysis in HepG2 cells transfected with PCH9/3091 or PCH9/3091-HBx (HBx deletion) and HepAD38 cells in the presence or absence of tetracycline (Supplementary Fig. S4H). Interestingly, ChIP assays showed that HBx interference blocked the HULC-elevated STAT3 occupancy on the miR-539 promoter (Fig. 4I and Supplementary Fig. S4I). Functionally, luciferase reporter gene assays showed that the knockdown of HBx attenuated the HULC-mediated increase of pGL3-539 luciferase activities (Fig. 4J). Thus, we conclude that HULC activates the miR-539 promoter by stimulating HBx/STAT3 signaling.

3.5. HULC modulates HBV replication by stimulating miR-539 targeting APOBEC3B mRNA 3′UTR

ELISA and qPCR assays showed that anti-miR-539 caused reductions of HBV-specific markers (HBeAg, HBsAg and HBV-DNA) in HepAD38 and HepG2.2.15 cells (Fig. 5A). Moreover, anti-miR-539 blocked the HULC-mediated HBV-specific marker expression (HBeAg, HBsAg and HBV-DNA) in HepAD38 and HepG2.2.15 cells (Fig. 5A). The similar results were observed for cccDNA with qPCR in HepAD38 and HepG2.2.15 cells (Fig. 5B), suggesting that miR-539 is involved in the regulation of HULC-stimulated HBV replication. Furthermore, we performed 3D-PCR assays to examine the impact of miR-539 on cccDNA deamination in HepAD38 and HepG2.2.15 cells. Interestingly, we observed that the presence of anti-miR-539 lowered the denaturing temperatures for cccDNA amplification (Fig. 5C and Supplementary Fig. S5A), indicating that miR-539 leads to cccDNA deamination. Moreover, Southern blot analysis demonstrated that HULC could increase cccDNA levels, and the addition of anti-miR-539 or APOBEC3B could block this HULC-mediated increase in cccDNA (Fig. 5D), suggesting that HULC contributes to the stability of HBV replication through its effects on miR-539 and APOBEC3B.

Next, we examined the effect of anti-HBV drugs, such as ETV and LdT, on the expression of HULC, miR-539 and APOBEC3B. ELISA assays and immunofluorescence analysis confirmed the antiviral effects of ETV and LdT on HBV-specific markers (HBeAg, HBsAg and HBcAg) in HepAD38 cells (Supplementary Fig. S5B–D). However, ETV and LdT treatments failed to affect the expression of HULC, miR-539 or APOBEC3B in HepAD38 cells, when measured after 0, 24, 48 and 72 h (Fig. 5E and Supplementary Fig. S5E). Based on these findings, we conclude that HULC is able to modulate HBV replication by stimulating miR-539 targeting APOBEC3B mRNA 3′UTR.

3.6. HULC promotes hepatoma cell proliferation by modulating HBV in vitro

It is well known that HBV infection is a leading cause of HCC [33]. However, it remains unclear whether HULC promotes liver cancer by inducing HBV replication (Supplementary Fig. S6A). Here, we observed that HULC stimulated HepAD38 cell proliferation much more effectively in HBV-positive than in HBV-negative cells (Fig. 6A). Conversely, interfering with HULC reduced the cell proliferation ability of HBV-infected HepG2-NTCP cells (Supplementary Fig. S6B and C). Moreover, we found that infecting with HBV could promote HepG2-NTCP cell proliferation (Supplementary Fig. S6B and C). These findings were corroborated with EdU assays (Supplementary Fig. S6D), supporting that HULC enhances the proliferation of hepatoma cell by activating HBV replication.

MTT and colony formation assays showed that HULC promoted the proliferation of HBV-infected HepG2-NTCP cells, but the treatment with si-HBx, anti-miR-539 or pc3.1-A3B could block HULC-promoted cell proliferation (Fig. 6B). Conversely, the treatment with HBx, miR-539 or si-A3B-1 could rescue si-HULC-1-induced inhibition of HepAD38 cell proliferation (Fig. 6C), suggesting that HULC promotes hepatoma cell proliferation by stimulating HBx/miR-539/APOBEC3B signaling in HBV-expressing hepatoma cells.

To better understanding the effect of HBV on gene expression, we profiled HBV-modulated genes with cDNA microarrays. Surprisingly,

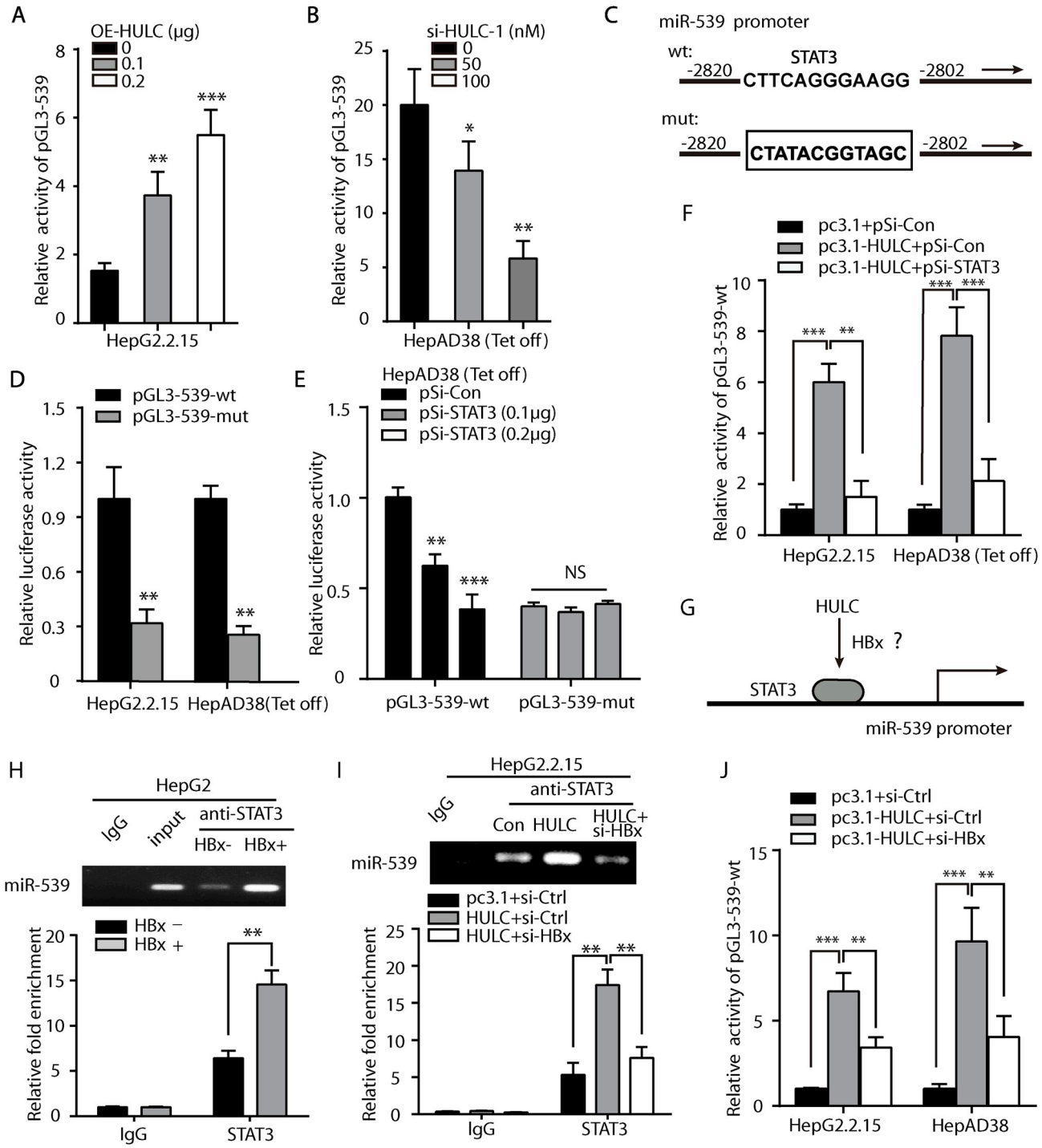

Fig. 4. HULC promotes the expression of miR-539 by stimulating HBx/STAT3 signaling. (A and B) Luciferase activities were measured by luciferase reporter gene assays in HepG2.2.15 and HepAD38 cells. (C) A model demonstrates the predicted conserved STAT3 binding site at nucleotides −2820 bp to −2802 bp of the miR-539 promoter. (D) Luciferase activities of pGL3-539 (or pGL3-539-mut) were detected by luciferase reporter gene assays in HepG2.2.15 and HepAD38 cells. (E) Luciferase activities were measured by luciferase reporter gene assays in HepAD38 cells (tet off) treated with pSi-STAT3 or pSi-Con. (F) Luciferase activities were measured by luciferase reporter gene assays in the HepG2.2.15 and HepAD38 cells. (G) A model of HULC up-regulating miR-539 promoter through HBx-co-activating STAT3. (H) ChIP assays were performed for STAT3 in the miR-539 promoter (up) and their quantitation (down) in HepG2. (I) ChIP assays were performed for STAT3 in the miR-539 promoter (up) and their quantitative (down) in HepG2.2.15 cells. (J) Luciferase activities were measured by luciferase reporter gene assays in HepG2.2.15 and HepAD38 cells. Each experiment was repeated at least three times. Error bars represent means ± SD (n = 3). Statistically significant differences are indicated: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; Student's *t*-test.

476 genes were up-regulated, and 914 genes were down-regulated, based on the criterion of a 2-fold difference in the absence and presence tetracycline (tet) in HepAD38 cells (Supplementary Fig. S6E; Supplementary Tables S2 and S3). We performed gene ontology (GO) functional, biological process and cellular component enrichment analyses to identify HBV target genes (Supplementary Fig. S6F and G), suggesting that HBV globally affects cellular gene expression. Moreover, we performed RT-qPCR to validate the expression of the identified regulated genes (Supplementary Fig. S6H). Thus, we conclude that HULC promotes hepatoma cell proliferation by activating HBV *in vitro*.

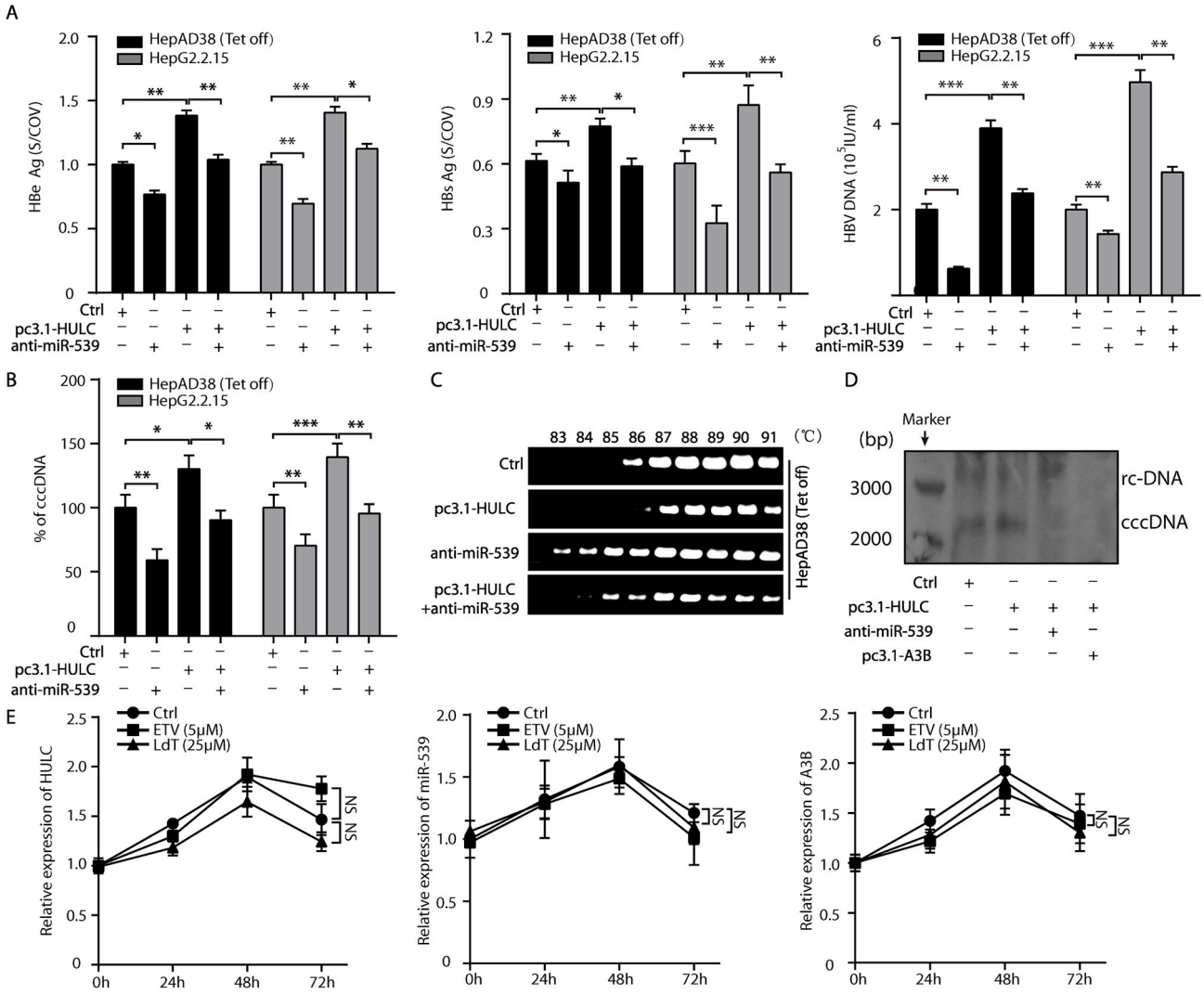

Fig. 5. HULC modulates HBV replication by stimulating miR-539 targeting APOBEC3B mRNA 3′UTR. (A) The HBeAg and HBsAg levels were measured by ELISA assays in the supernatant of HepAD38 and HepG2.2.15 cells; the HBV DNA levels were measured by qPCR assays in the supernatant of HepAD38 and HepG2.2.15 cells. (B) The cccDNA levels were measured by qPCR assays in the nucleus of HepAD38 and HepG2.2.15 cells. (C) HBV cccDNA denaturation analysis by 3D-PCR in HepAD38 cells. (D) The levels of cccDNA were examined by Southern blot analysis in HepG2-PCH9/3091 cells. (E) The expression of HULC, miR-539 and APOBEC3B (A3B) were examined by RT-qPCR in HepAD38 cells treated with indicated doses of ETV and LdT. Each experiment was repeated at least three times. Error bars represent means ± SD (n = 3). Statistically significant differences are indicated: $*p < 0.05$; $**p < 0.01$, $**p < 0.001$; NS, no significant; Student's *t*-test.

3.7. HULC facilitates hepatoma cell growth through HBV in vivo

To extend our understanding of the role of HULC in HBV-related HCC, we subcutaneously injected different pretreated cells into 4-week-old BALB/c athymic nude mice. As expected, we observed that the treatment with si-HULC-1 abolished the enhancement of HBV-mediated HepG2-NTCP cell growth in mice (Fig. 7A). Immunohistochemistry showed that the levels of Ki-67 expression in tumor tissues (Fig. 7B) were consistent with tumor growth among the different groups (Fig. 7A), suggesting that HULC is able to promote the growth of hepatoma cells through HBV *in vivo*. However, these different treatments did not affect mouse weight in the three groups (Supplementary Fig. S7A). The levels of HULC expression were confirmed by RT-qPCR analyses in the tumor tissues (Fig. 7C). Meanwhile, the efficiency of HBV infection was evaluated by RT-qPCR, and we found that the knockdown of HULC could reduce HBV mRNA *in vivo* (Supplementary Fig. S7B). Next, we demonstrated the effect of APOBEC3B on the growth of hepatoma cells *in vivo*. Then, we observed that the tumorigenicity was remarkably increased in nude mice injected with HepAD38 cells pretreated with si-A3B-1 *in vivo* (Fig. 7D), suggesting that APOBEC3B suppresses the growth of HBV-expressing hepatoma cells *in vivo*. Immunohistochemistry showed that the levels of Ki-67 expression in tumor tissues were consistent with tumor growth among the different groups (Fig. 7E), supporting that APOBEC3B suppressed the growth of HBV-expressing hepatoma cells *in vivo*. The si-A3B-1-mediated silencing efficiency of APOBEC3B was validated by Western blot analysis in the tumor tissues from the nude mice (Fig. 7F). RT-qPCR showed that HBV mRNA was up-regulated by si-A3B-1 in the tumor tissues, supporting that APOBEC3B is able to suppress HBV replication *in vivo* (Supplementary Fig. S7C). Taken together, we conclude that HULC facilitates the growth of hepatoma cells through HBV *in vivo*.

4. Discussion

HULC was the first identified lncRNA that was specifically over-expressed in liver cancer [7]. We previously reported that HULC played pivotal roles in aberrant lipid metabolism and tumor angiogenesis in HCC [8,10]. The interplay between HBV and host factor plays vital roles

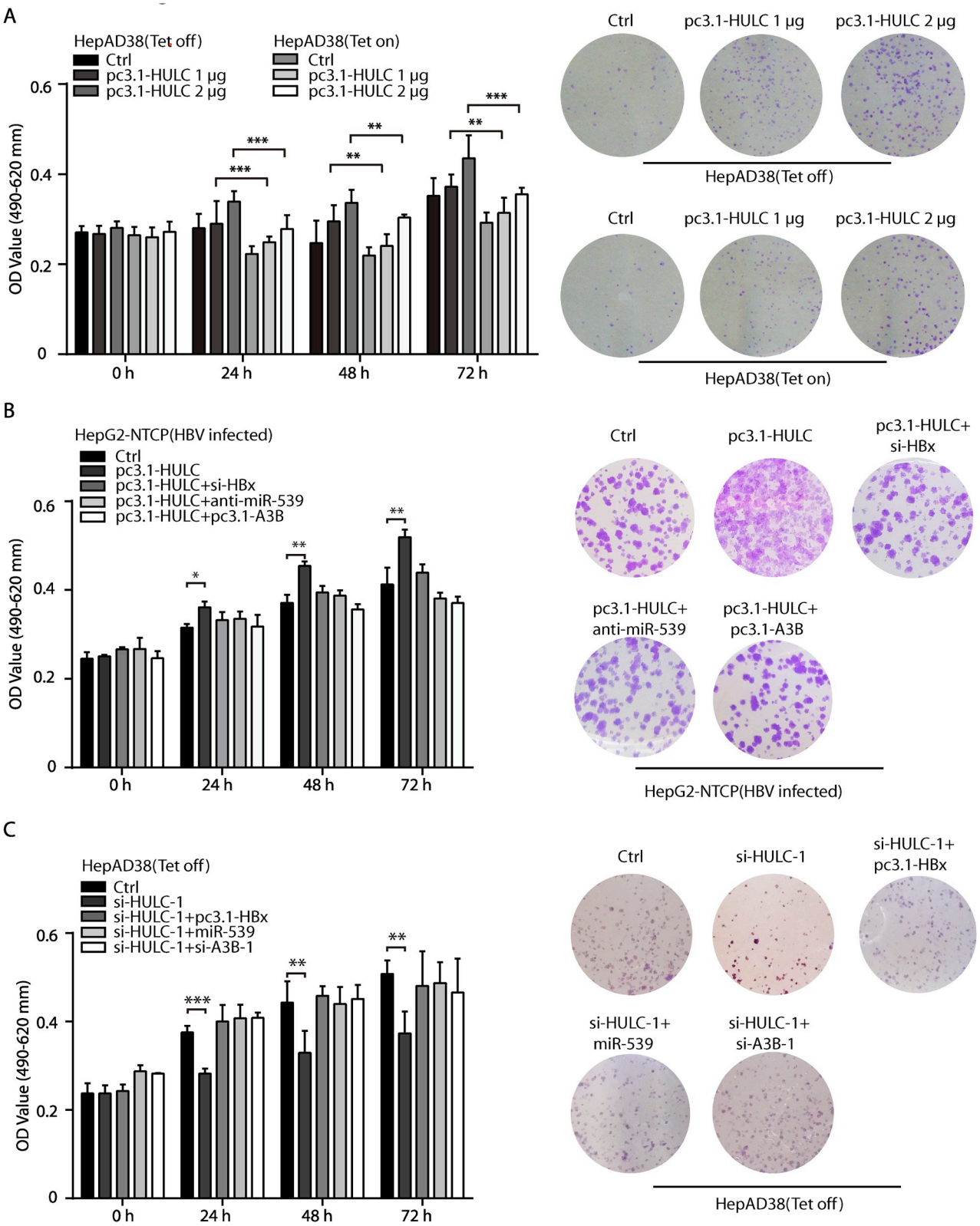

Fig. 6. HULC promotes hepatoma cell proliferation by modulating HBV *in vitro*. (A) The effect of pc3.1-HULC on cell proliferation was determined by MTT assays and colony formation assays in HepAD38 (tet off) and HepAD38 (tet on) cells, respectively. (B) The cell viability of HBV-infected HepG2-NTCP cells was performed by MTT assays and colony formation assays. (C) The cell viability of HepAD38 cells (tet off) was performed by MTT assays and colony formation assays. Each experiment was repeated at least three times. Error bars represent means ± SD (n = 3). Statistically significant differences are indicated: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; Student's *t*-test.

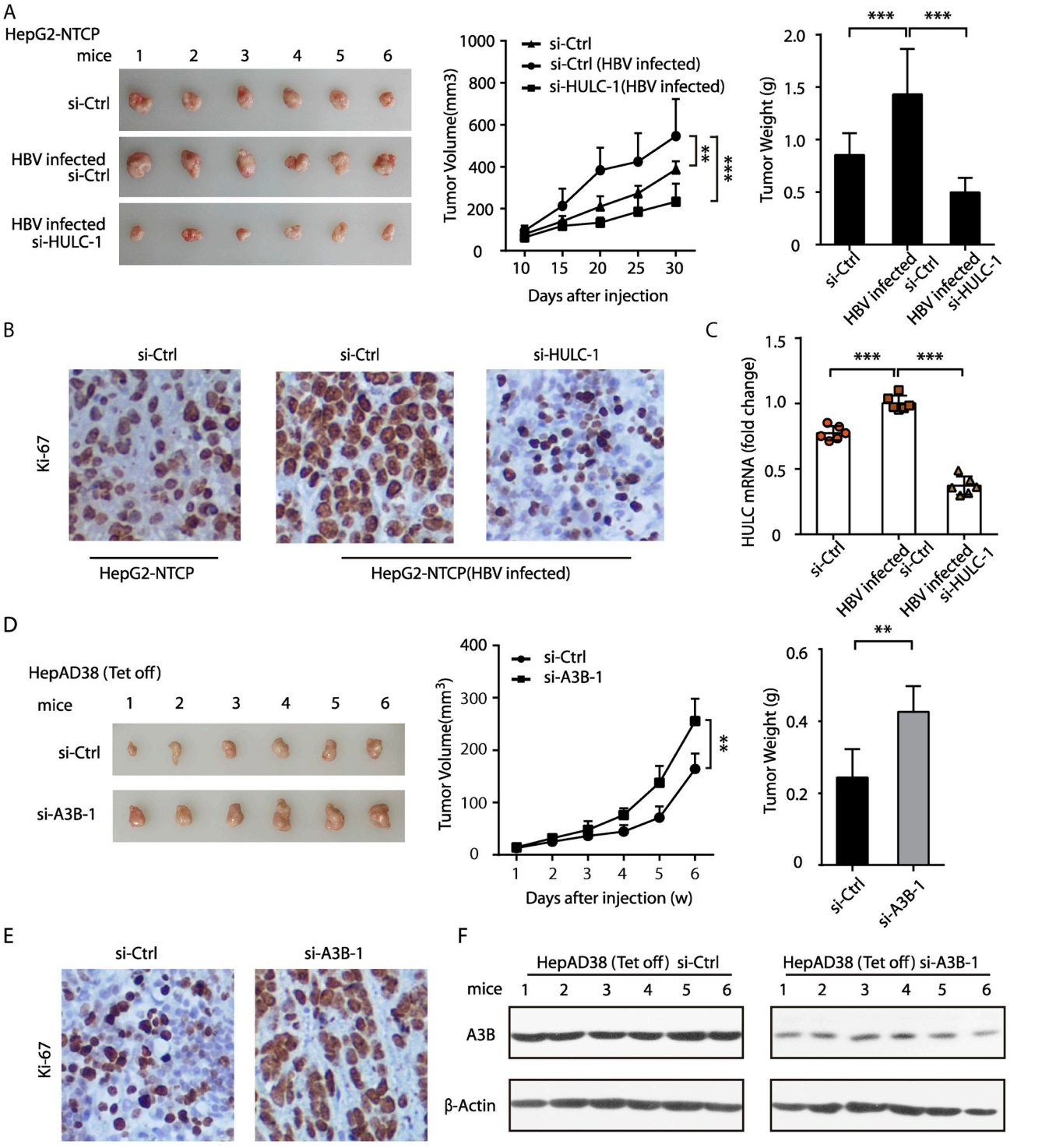

Fig. 7. HULC facilitates hepatoma cell growth through HBV *in vivo*. (A) The left panel displays the photographs of dissected tumors from nude mice; the right panel displays the growth curve and average weight of the tumors transplanted with HepG2-NTCP cells pretreated with different treatments in nude mice. (B) Immunohistochemistry staining for Ki-67 was performed in the tumor tissues from nude mice transplanted with different pretreated HepG2-NTCP cells. (C) The expression of HULC was evaluated by RT-qPCR in the tumor tissues from nude mice transplanted with different pretreated HepG2-NTCP cells. (D) The left panel displays the photographs of dissected tumors from nude mice; the right panel displays the growth curve and average weight of the tumors transplanted with different preprocessed HepAD38 cells (tet off) in nude mice. (E) Immunohistochemistry assays for Ki-67 were measured in tumor tissues from nude mice transplanted with HepAD38 cells (tet off) pretreated with different treatments in nude mice. (F) The protein expression of APOBEC3B (A3B) was examined by Western blot analysis in the tumor tissues from nude mice, respectively. Each experiment was repeated at least three times. Error bars represent means ± SD (n = 3). Statistically significant differences are indicated: $^{**}p < 0.01$; $^{***}p < 0.001$; Student's *t*-test.

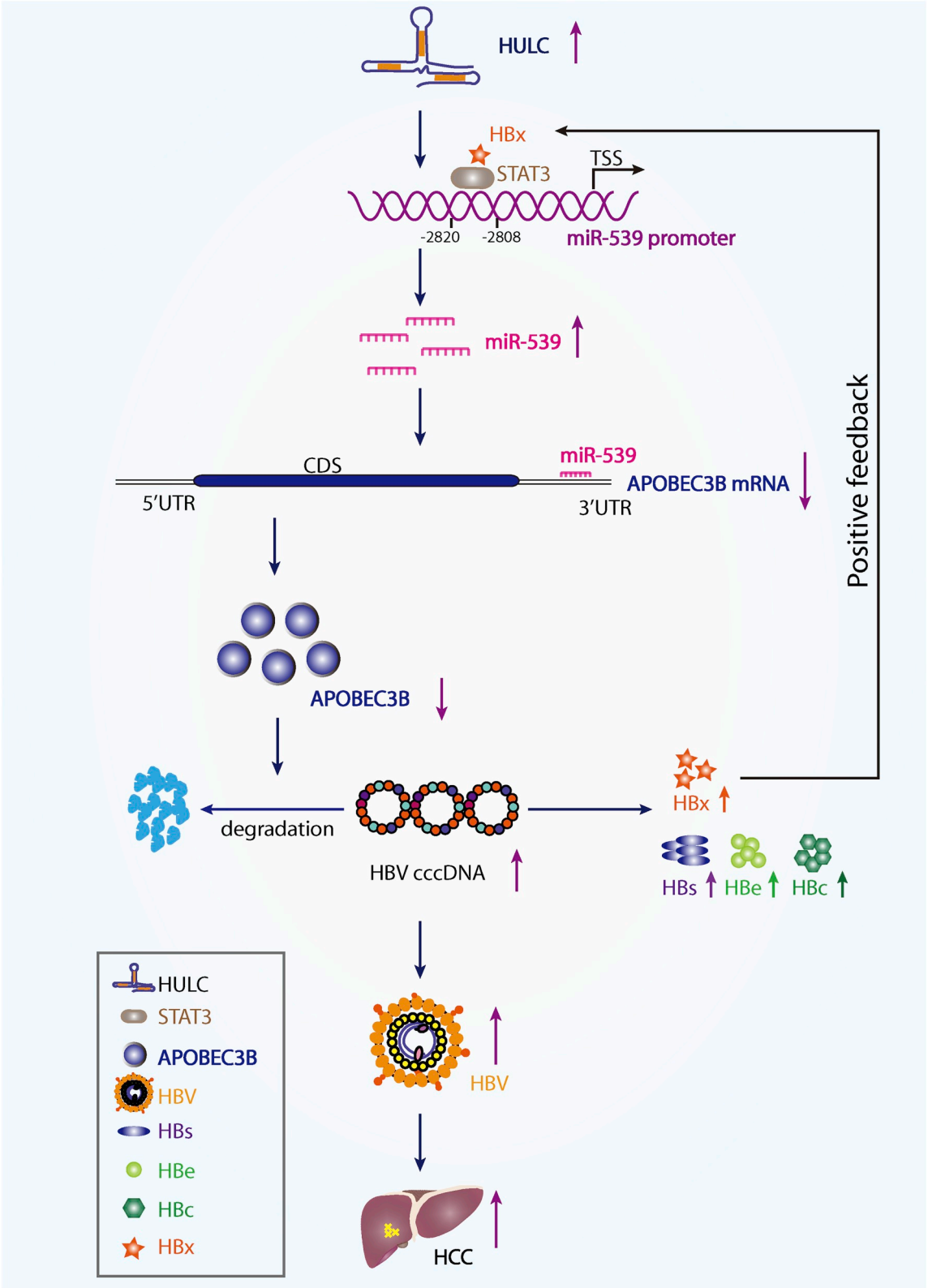

Fig. 8. A model of HULC enhancing hepatocarcinogenesis. HULC promotes the expression of miR-539 through HBx-co-activated STAT3. The miR-539 then down-regulates the expression of APOBEC3B, leading to an increase of HBV cccDNA which is the template of HBV replication. Thus, HULC activates HBV by HBx/STAT3/miR-539/APOBEC3B signaling in HBV-related HCC.

in the development of hepatoma. However, the significance of HULC in HBV-related HCC is poorly understood. In this study, we investigated the mechanism underlying the HULC enhancement of hepatocarcinogenesis in HBV-related-HCC.

We first observed that HULC could activate HBV replication in hepatoma cells. It has been known that the cytosine deaminization activity of APOBEC3A or APOBEC3B is essential for inducing cccDNA degradation [24]. Strikingly, we demonstrated that HULC was able to

maintain the stability of HBV cccDNA by down-regulating APOBEC3B. We showed that HULC increased miR-539 -expression, which targeted the APOBEC3B mRNA 3′UTR. It was previously reported that HULC might act as an endogenous sponge, which down-regulated the miR-372 and miR-107 [8,9]. Moreover, our previous data showed that HULC suppressed the miR-9 by inducing CpG island methylation of the miR-9 promoter [10]. It has been reported that HBx constitutively enhances tyrosine phosphorylation on STAT3 [32]. Here, our data revealed that HULC elevated HBx, which transcriptionally co-activated STAT3 to activate the miR-539 promoter. Functionally, we demonstrated that HULC could activate HBV replication by HBx/miR-539/APOBEC3B signaling, leading to the growth of hepatoma cell *in vitro* and *in vivo*.

To better understand the effect of HBV on gene expression, we profiled HBV-modulated genes with cDNA microarrays. Our data showed that HBV globally affected cellular gene expression. HBV is known to be a key driver in hepatocarcinogenesis. However, the deletion of APOBEC3B might promote persistent HBV infection, and therefore, increases the risk of developing HCC [25]. In this study, we found that APOBEC3B reduced the growth of HBV-expressing hepatoma cells *in vitro* and *in vivo*. It suggests that APOBEC3B may serve as a potential tumor suppressor in HBV-related HCC. Thus, we conclude that HULC is able to activate HBV by HBx/STAT3/miR-539/APOBEC3B signaling in HBV-related HCC.

Previous studies showed that antiviral treatments for viral hepatitis appeared to reduce the risk of HCC in patients chronically infected with HBV [34,35]. In this study, we failed to observe that ETV and LdT could affect the expression of HULC, miR-539 or APOBEC3B in HBV-expressing hepatoma cells. It implies that no-response to the HULC/miR-539/APOBEC3B signaling might contribute to the failure of nucleos(t)ide analogues in eliminating HBV. Therapeutically, HULC may serve as a therapeutic target for HCC. However, the detailed mechanism by which HULC modulates HBV cccDNA is not well documented, largely due to the lack of animal models that reproduce clinical HBV-associated HCC. Overall, our finding suggests that the interaction between host factors and HBV plays a crucial role in hepatocarcinogenesis.

In summary, we propose a model that explains the possible mechanism the HULC mediates activation of HBV in liver cancer cells (Fig. 8). In the model, HULC promotes the expression of miR-539 by stimulating HBx-co-activated STAT3. Then, miR-539 down-regulates APOBEC3B, leading to an increase of HBV cccDNA, which acts as a template for HBV replication. Thus, HULC activates HBV replication by HBx/STAT3/miR-539/APOBEC3B signaling, leading to the growth of liver cancer. Our finding provides new insights into the mechanism by which HULC promotes HBV in hepatocarcinogenesis.

Conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.04.008.

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