



ORIGINAL ARTICLE

Hepatitis B virus X protein enhances hepatocarcinogenesis by depressing the targeting of *NUSAP1* mRNA by *miR-18b*

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ABSTRACT

Objective: The aim of this study was to investigate the underlying mechanism whereby HBx modulates the targeting of *NUSAP1* by *miR-18b* to enhance hepatocarcinogenesis.

Methods: We employed an integrated approach of bioinformatics analysis and molecular experiments in hepatoma cells, HBV transgenic mice, and clinical liver cancer tissues to investigate the role of HBx-regulated *miR-18b* in the development of liver cancer.

Results: In this study, we report that the HBx-mediated tumor suppressor *miR-18b* modulates hepatocarcinogenesis during the host-HBV interaction. The expression levels of *miR-18b* were lower in clinical HBV-positive liver cancer tissues and liver tissues of HBV-transgenic mice. Interestingly, HBx inhibited *miR-18b* expression by inducing the methylation of CpG islands in its promoter. Accordingly, we tested the hypothesis that HBx enhanced hepatocarcinogenesis by increasing the expression of target genes of *miR-18b*. Moreover, we identified nucleolar spindle-associated protein 1 (*NUSAP1*) as one of the target genes of *miR-18b*. *NUSAP1* was expressed at high levels in liver cancer tissues. Interestingly, HBx up-regulated *NUSAP1* by suppressing *miR-18b*. Functionally, *miR-18b* significantly inhibited the proliferation of hepatoma cells by depressing *NUSAP1* levels *in vivo* and *in vitro*.

Conclusions: Thus, we conclude that the targeting of *NUSAP1* mRNA by the tumor suppressor *miR-18b* is controlled by HBx-modulated promoter methylation during the host-virus interaction, leading to hepatocarcinogenesis. Our findings provide new insights into the mechanism by which HBx-mediated miRNAs modulate hepatocarcinogenesis.

KEYWORDS

Hepatitis B virus; HBx; miR-18b; *NUSAP1*; HCC

Introduction

Hepatocellular carcinoma (HCC) is a common cancer worldwide and the third leading cause of cancer-related deaths globally¹. Chronic hepatitis B virus (HBV) infection is closely related to chronic hepatitis, cirrhosis, and HCC²⁻⁴. As a functional trans-activating protein, HBV X protein (HBx) plays important roles in the development of liver cancer⁵⁻⁷. HBx can stimulate transcription, signal transduction, and cell cycle progression by binding to different transcription factors or components of various signaling pathways⁸⁻¹⁰. In addition, HBx participates in genetic and epigenetic regulation during the development of liver cancer^{11,12}. However, the underlying mechanism by which HBx modulates hepatocarcinogenesis is poorly understood.

MicroRNAs (miRNAs) are endogenous, 21–24 nucleotide RNAs that can play regulatory roles by targeting mRNAs. As a result, the targeted mRNAs are cleaved or posttranscriptionally repressed¹³⁻¹⁵. More than half of all mRNAs are regulated by miRNAs and each miRNA is estimated to regulate hundreds of mRNAs¹⁶⁻¹⁸. Studies of specific miRNAs have shown that they play distinct roles in cellular functions, such as proliferation, development, differentiation, and apoptosis¹⁹⁻²³. A previous study reported that miRNAs are critical for essential cellular processes, host-virus interactions, and virus life cycles²⁴. Many miRNAs have been reported to be involved in the host-virus interactions of more than 50 different infections, including those involving dengue virus, Ebola virus, hepatitis B virus, hepatitis C virus, and rabies virus²⁵. These findings demonstrate the important role of miRNAs in host immunity and the defense against viral infections. Immune dysregulation, instance, during chronic inflammation, can lead to chronic illness and even death²⁶⁻²⁸. Therefore, a balanced immune response is essential. MiRNAs play an important role in innate immunity

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Received August 24, 2018; accepted January 9, 2019.

Available at www.cancerbiomed.org

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by directly targeting viral transcripts²⁹⁻³¹. Various miRNAs, such as *miR-141*, *miR-501*, and *miR-125a-5p* have been implicated in the regulation of HBV replication^{32,33}.

It has been reported that *miR-18b* is able to suppress cell migration and invasiveness in melanoma³⁴. *MiR-18b* can suppress high-glucose-induced proliferation of human retinal endothelial cells (HRECs) by targeting the IGF-1/IGF1R signaling pathways³⁵. However, the role of *miR-18b* in HBV-related HCC is still unclear. Some miRNAs are decreased in human tumors due to aberrant hypermethylation of cytosine-phosphate-guanosine (CpG) islands of miRNA genes^{36,37}. Aberrant DNA methylation is also associated with the development of HCC. CpG hypermethylation serves as a mechanism for miRNA silencing, but this field remains largely unexplored. Our group reported that HBx was able to inhibit the tumor suppressor, *miR-205* and enhance hepatocarcinogenesis by inducing methylation of the *miR-205* promoter³⁸. However, the effect of HBx on *miR-18b* is undefined. Nucleolar spindle associated protein 1 (NUSAP1) is an important mitotic regulator that has been reported to be essential for many cellular processes³⁹. NUSAP1 plays crucial roles in embryogenesis and cancer⁴⁰⁻⁴² and is up-regulated in HCC when compared to nontumor liver tissues⁴³. However, the significance of NUSAP1 in HBV-related liver cancer progression remains poorly understood.

In the present study, we investigated the mechanism by which HBx modulates hepatocarcinogenesis during the host-virus interaction. Interestingly, we identified that the targeting of *NUSAP1* mRNA by the tumor suppressor *miR-18b* was controlled by HBx during the host-virus interaction, leading to hepatocarcinogenesis. Thus, our finding provides new insights into the mechanism by which HBx-mediated miRNAs modulate hepatocarcinogenesis.

Materials and methods

Patient samples

Thirty liver tissues were obtained immediately after surgical resection from randomly selected HCC patients at Tianjin First Center Hospital (Tianjin, China) and Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). Clinicopathological information for the patients was collected from hospital patient records (**Supplementary Table S1**). Written informed consent for the use of their tissues for research purposes was obtained from all patients in the study. The Institute Research Ethics Committee at the Nankai University approved the study protocol.

Cell lines and cell culture

The HepG2, HepG2-X (cell line with stable HBx transfection), HepAD38 (cell line stably producing HBV), and HepG2.2.15 (a hepatoma HepG2 cell line with integrated full-length HBV DNA), were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA). For HBV replication, HepG2.2.15 cells were seeded at optimal densities and HepAD38 cells were treated without tetracycline for 8 days. LO2 (human immortalized normal liver cell line), H7402 (hepatoma cell line), and H7402-X (cell line with stable HBx- transfection) cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin in 5% CO₂ at 37°C. HepG2-X and H7402-X cells were maintained in 200 µg/mL G418.

HBV transgenic mice and tissue analysis

BALB/c mice and HBV transgenic BALB/c mice (HBV-Tg) containing the HBV genome S, pre-S, and X domains were purchased from Vital River Laboratory Animal Technology (Beijing, China). Animals were maintained under specific pathogen-free conditions. Mice were sacrificed at the end of the experimental period and serum and liver tissues were obtained.

In vivo tumorigenicity assays

Animal transplantation experiments were performed in compliance with the principles of the Declaration of Helsinki. Nude mice were housed and treated according to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Institute Research Ethics Committee at Nankai University. Tumor transplantation experiments were performed as previously described^{44,45}. Briefly, HepG2-X cells were pretreated with control, *miR-18b*, *miR-18b*, or pcDNA3.1-NUSAP1 for 24 h. Cells were then resuspended at 2×10^7 cells/mL in sterile PBS. Four-week-old male BALB/c athymic nude mice (Experimental Animal Center of Peking, China) were grouped ($n = 6$ per group) and injected subcutaneously on the shoulder with 0.2 mL of the appropriate cell suspension. Tumor growth was measured 6 days after the injection and thereafter, every 4 days. Tumor volume was evaluated by measuring the length (L) and width (W) with calipers and was then calculated according to the formula, $(L \times W^2)/2$. On the thirtieth day, mice were sacrificed and tumors were excised and measured.

Statistical analysis

The correlation between NUSAP1 (or *miR-18b*) and HBx mRNA/pregenomic (pg) RNA levels in tumor tissues was determined by calculating the Pearson's correlation coefficient. NUSAP1 expression levels in tumor tissues and adjacent nontumorous tissues were compared using Wilcoxon's signed-rank test. Statistical significance was assessed by comparing mean values (\pm standard deviation, SD) using a Student's *t*-test for independent groups, with significance assumed at $P < 0.05$. Each experiment was repeated at least three times.

Results

HBx suppressed *miR-18b* by inducing hypermethylation of the *miR-18b* promoter

It has been reported that *miR-18b* suppresses cell migration and invasiveness in melanoma³⁴. However, the role of *miR-18b* in HBV-related liver cancer remains unclear. Therefore, we hypothesized that HBx modulates *miR-18b* during hepatocarcinogenesis. Therefore, we measured *miR-18b* expression in 30 paired HCC and adjacent nontumorous liver tissues using qRT-PCR. *miR-18b* expression was normalized to the expression of an endogenous control (*U6* RNA). Our data showed that the expression levels of *miR-18b* were significantly lower in clinical HBV-related HCC samples, relative to their adjacent noncancerous liver tissues (**Figure 1A**; $P < 0.01$, Wilcoxon's signed rank test). Next, we examined the relationship between HBx and *miR-18b* in HCC tissues using qRT-PCR. Intriguingly, we found that the expression levels of *miR-18b* were inversely correlated with those of HBx mRNA/pgRNA (**Figure 1B**; Pearson's correlation coefficient, $r = -0.5771$; $P < 0.01$). Moreover, our data showed that the expression levels of *miR-18b* were lower in the livers of HBV-transgenic mice relative to the livers of wild-type mice (**Figure 1C**), suggesting that HBx may suppress the expression of *miR-18b* during HBV infection and HCC development. The levels of HBx/pgRNA were also determined in wild-type mice and HBV-transgenic mice using qRT-PCR (**Supplementary Figure S1A**). Based on these results, we proposed that HBx directly down-regulates *miR-18b* in liver cells. Next, we transiently transfected HepG2 and H7402 cells with an HBx-expression plasmid (pcDNA3.1-HBx). The overexpression of HBx was validated in the two cell lines (**Supplementary Figure S1B**). We showed that HBx decreased *miR-18b* expression in a dose-dependent manner (**Figure 1D**), suggesting that HBx is able

to inhibit *miR-18b* in HCC cells. Given that *miR-18b* was down-regulated in clinical HCC tissues, we sought to investigate the mechanism by which HBx down-regulates *miR-18b* in hepatoma cells. It has been reported that HBx downregulates miRNA expression through methylation of the promoter region³⁸. We evaluated methylation levels in the promoter region of *miR-18b* in HCC cells and clinical HCC tissues (**Figure 1E**) using bisulfite-sequencing polymerase chain reaction (PCR). Our data showed that the methylation levels of the *miR-18b* promoter CpG islands were higher in HepG2-X, HepAD38, and HepG2.2.15 cells than in LO2 cells (**Figure 1F**), suggesting that the *miR-18b* gene promoter is hypermethylated in HBV-infected hepatoma cells relative to normal liver cells (**Supplementary Figure S1C**). *miR-18b* levels were then measured by qRT-PCR in LO2, HepG2-X, HepAD38, and HepG2.2.15 cells. Interestingly, the expression levels of *miR-18b* were negatively correlated with the methylation status of the *miR-18b* promoter in all four cell lines (**Supplementary Figure S1D**). Eight tumor tissues showed high DNA methylation levels (**Figure 1G**) and DNA methylation levels of all peritumoral tissues were low, suggesting that the *miR-18b* promoter is hypermethylated in HCC tissues relative to adjacent nontumorous liver tissues (**Supplementary Figure S1E**). Thus, we conclude that HBx down-regulates *miR-18b* by inducing the methylation of CpG islands in the *miR-18b* gene promoter.

miR-18b inhibited the expression of NUSAP1 by directly targeting the 3'UTR of its mRNA

To further elucidate the molecular mechanism whereby *miR-18b* affects the pathogenesis of HCC, we used the online miRNA target gene prediction tool, Targetscan (<http://www.targetscan.org/>) and found that NUSAP1 was a potential target gene for *miR-18b*. However, the significance of NUSAP1 in HCC is poorly understood. Therefore, we used the cancer microarray database, Oncomine (<https://www.oncomine.org/>) and Metabolic gEne Rapid Visualizer (MERAV, <http://merav.wi.mit.edu/>) to predict the expression level of NUSAP1 in HCC. These results showed that the expression levels of NUSAP1 were higher in HCC tissues relative to those in normal tissues, suggesting that NUSAP1 is a potential oncogene (**Supplementary Figure S2A** and **S2B**). Next, we analyzed the expression of NUSAP1 in clinical HCC tissues using immunohistochemical (IHC) staining of tissue arrays. In HCC tissues, NUSAP1 staining was observed in the cytoplasm and nucleus (**Figure 2A**). NUSAP1 staining was observed in 68.61% (129/188) of all

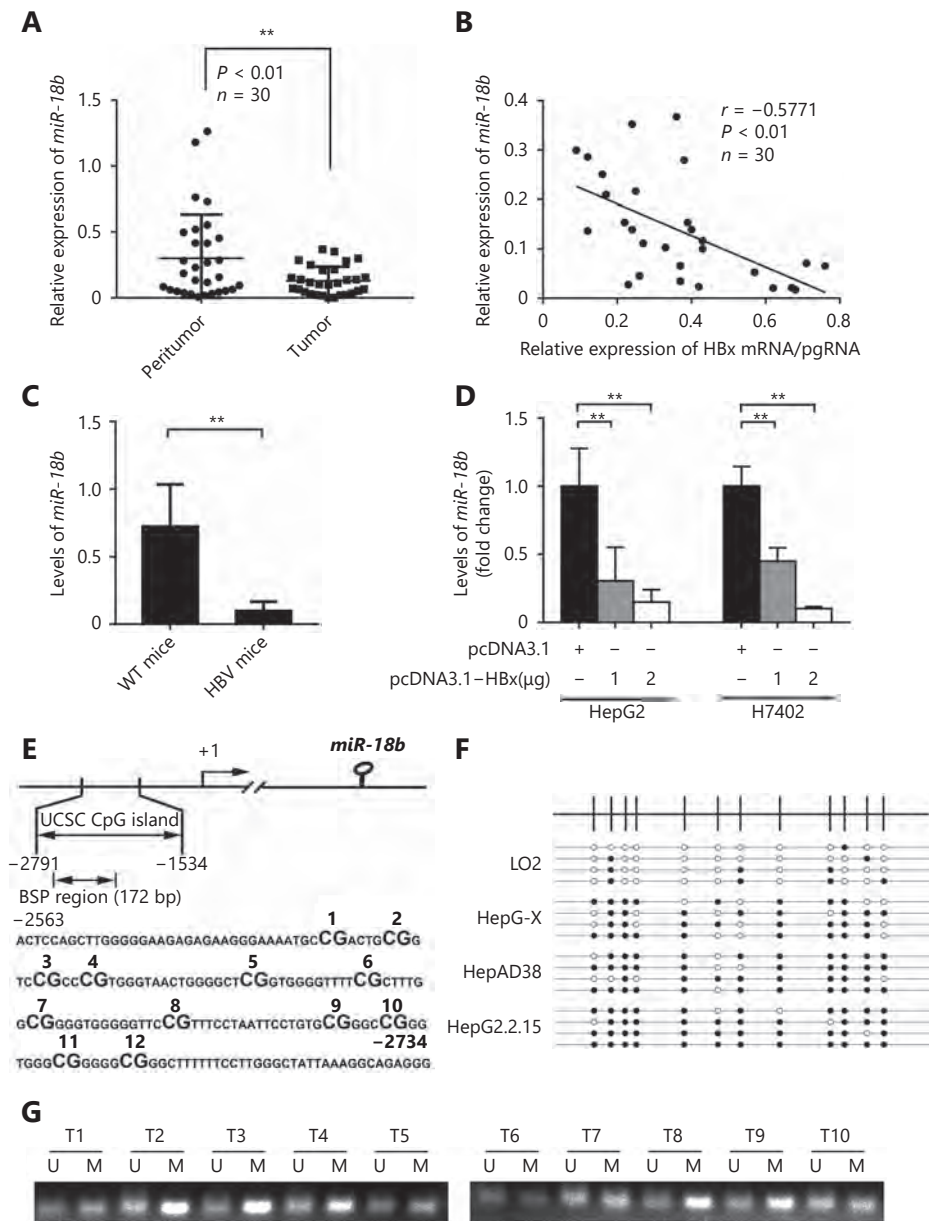


Figure 1 HBx suppresses *miR-18b* through inducing DNA hypermethylation of *miR-18b* promoter. (A) Relative mRNA levels of *miR-18b* were examined by qRT-PCR analysis in 30 pairs of HCC tissues and corresponding nontumorous tissues. $**P < 0.01$; Wilcoxon’s signed-rank test. (B) The correlation between HBx mRNA level and *miR-18b* mRNA level was analyzed by qRT-PCR analysis in 30 cases of HCC tissues ($**P < 0.01$, $r = -0.5771$; Pearson’s correlation coefficient). (C) *MiR-18b* expression was tested by qRT-PCR in HBV-transgenic mice ($n = 6$). (D) The expression of *miR-18b* was measured by qRT-PCR analysis after treatment with pcDNA3.1-HBx in HepG2 and H7402 cells. (E) Scheme depicting the genomic localization of *miR-18b*. The regions analyzed by BSP are indicated. The CpG dinucleotide within the *miR-18b* promoter regions (-2563 to -1534, 172bp) were numbered as 1-12. (F) The methylation status of the *miR-18b* promoter in LO2, HepG2-X, HepAD38 and HepG2.2.15 cell lines. The open and filled circles indicate the unmethylated and methylated CpGs, respectively. (G) The methylation status of the *miR-18b* promoter was examined by MSP analysis in HCC tissues (T).

HCC tissues and in 77.41% (24/31) of grade III HCC tissues, suggesting that NUSAP1 is closely associated with the malignancy of HCC. QPCR analysis revealed that NUSAP1

expression levels were significantly increased in 30 clinical HCC samples, relative to their adjacent nontumor tissues ($P < 0.01$, $n = 30$, Wilcoxon’s signed-rank test, **Figure 2B**).

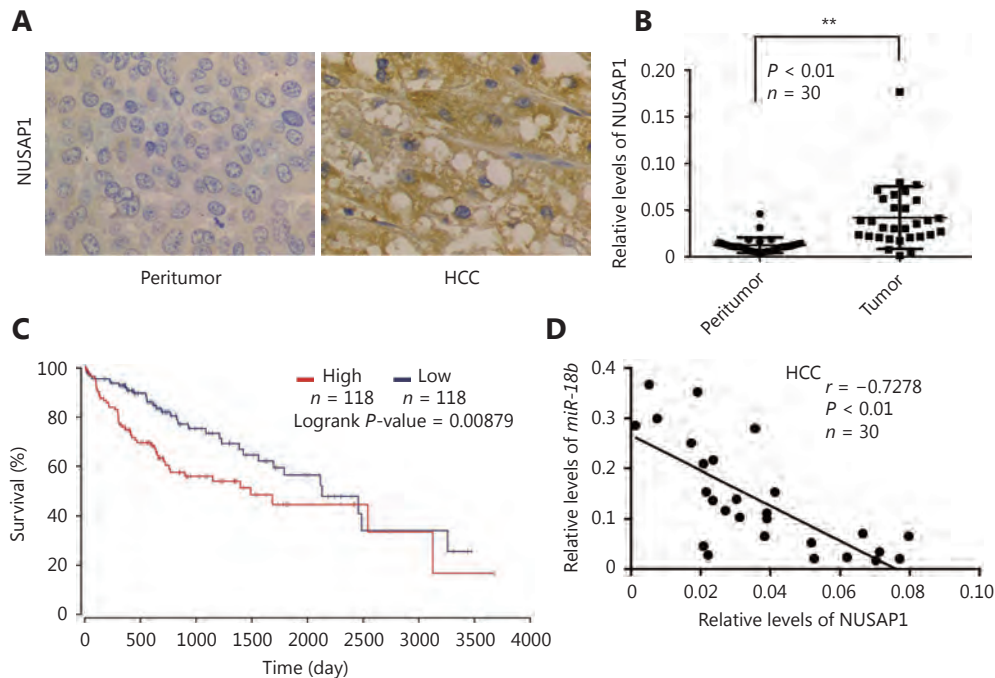


Figure 2 *MiR-18b* inhibits the expression of NUSAP1 through directly targeting its mRNA 3'UTR. (A) The expression of NUSAP1 was examined by IHC staining in normal tissues and HCC tissues using tissue array, respectively. (B) Relative mRNA levels of NUSAP1 were measured by qRT-PCR analysis in 30 pairs of HCC tissues and corresponding nontumorous tissues. ** $P < 0.01$; Wilcoxon's signed-rank test. (C) The survival rate of HCC patients analyzed by OncoLnc database (<http://www.oncolnc.org/>). (D) The correlation between NUSAP1 mRNA level and *miR-18b* mRNA level was analyzed by qRT-PCR analysis in 30 cases of HCC tissues (** $P < 0.01$, $r = -0.7278$; Pearson's correlation coefficient).

Using the OncoLnc database (<http://www.oncolnc.org/>), we found that a higher level of NUSAP1 expression was associated with a lower survival rate in HCC patients (Figure 2C). Moreover, we found that *miR-18b* expression levels were negatively correlated with NUSAP1 mRNA levels in HCC tissues (Pearson's correlation coefficient, $r = -0.7278$; $P < 0.01$; Figure 2D), suggesting that NUSAP1 may be one of the target genes of *miR-18b*. To confirm the site-specific repression of NUSAP1 by *miR-18b*, we constructed a luciferase reporter vector containing the 3'UTR of NUSAP1 mRNA (Figure 3A and 3B). Luciferase reporter gene assays showed that *miR-18b* directly targeted the NUSAP1 mRNA 3'UTR in HepG2 cells, in a dose-dependent manner, but it did not target a mutated NUSAP1 mRNA 3'UTR (Figure 3C). Conversely, an anti-*miR-18b* oligonucleotide increased the luciferase activity of pGL3-NUSAP1-3'UTR-wt in a dose-dependent manner, but had no effect on the mutant (Figure 3D), suggesting that *miR-18b* is able to directly bind to the 3'UTR of NUSAP1 mRNA. Furthermore, the over-expression of *miR-18b* suppressed the expression of NUSAP1 in HepG2 and H7402 cells, at the mRNA and protein level, in a dose-dependent manner, and the reverse effect was seen when cells

were treated with an anti-*miR-18b* oligonucleotide (Figure 3E and 3F, Supplementary Figure S2C and S2D). Our data showed that an anti-*miR-18b* oligonucleotide significantly rescued NUSAP1 levels that were decreased by *miR-18b* in HCC cells (Supplementary Figure S2E and S2F). Collectively, we conclude that *miR-18b* is able to inhibit NUSAP1 expression by directly targeting the 3'UTR of its mRNA.

HBx up-regulated NUSAP1 in hepatoma cells

Given that HBx was able to down-regulate the targeting of NUSAP1 by *miR-18b*, we investigated whether HBx could down-regulate NUSAP1 by inhibiting *miR-18b*. The mRNA levels of NUSAP1 and HBx/pgRNA were examined by qRT-PCR in 30 HCC liver tissues. Our data showed that the expression levels of HBx/pgRNA were positively correlated with those of NUSAP1 mRNA in HCC tissues (Pearson's correlation coefficient, $r = 0.7028$; $P < 0.01$; Figure 4A), suggesting that HBx is able to up-regulate NUSAP1. Moreover, our data showed that the expression levels of NUSAP1 were higher in HBV-transgenic mice relative to

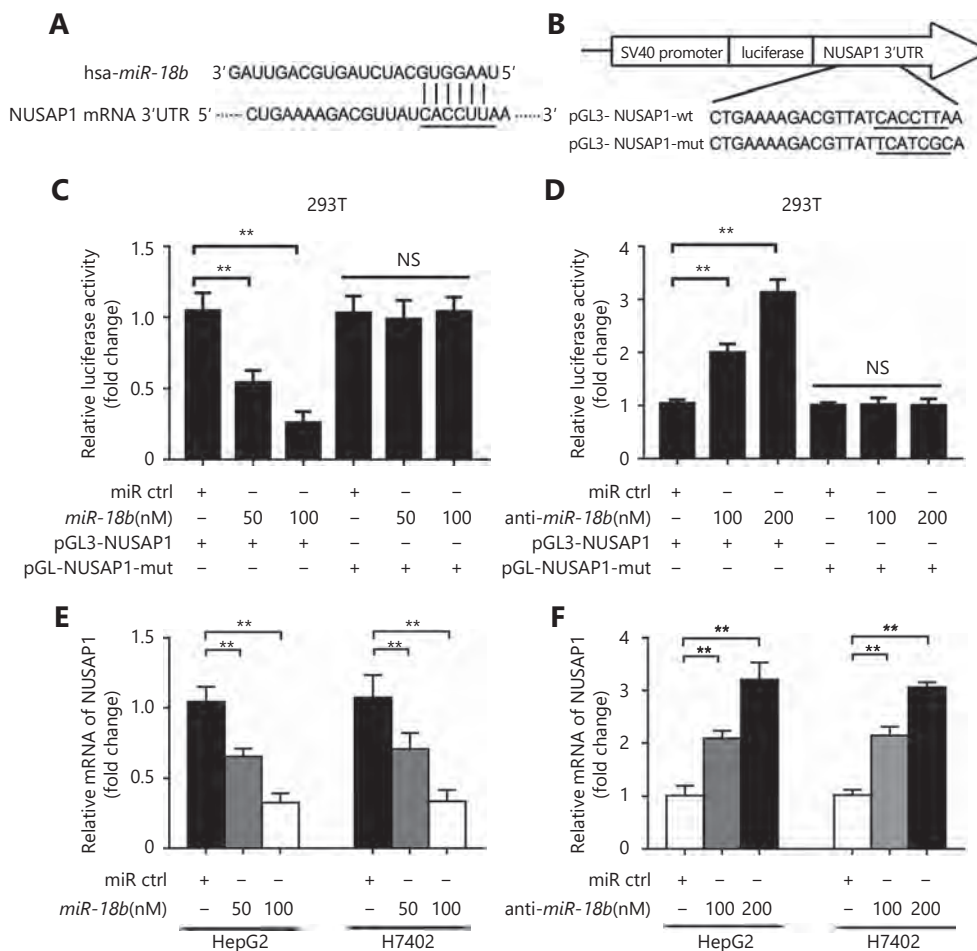


Figure 3 *MiR-18b* inhibits the expression of NUSAP1 through directly targeting its 3'UTR. (A,B) *MiR-18b* sequence and the potential *MiR-18b* -binding sites at the 3'UTR of NUSAP1 mRNA. Seed sequences and mutant are marked. (C,D) The effect of *miR-18b* (or anti-*miR-18b*) on the pGL3-NUSAP1-3'UTR-wt and pGL3-NUSAP1-3'UTR-mut reporters was measured by luciferase reporter gene assays in HepG2 cells. (E,F) The effect of *miR-18b* (or anti- *miR-18b*) on the expression of NUSAP1 was examined by qRT-PCR analysis in HepG2 and H7402 cells. Error bars indicate SD. Student's *t*-test, ***P*<0.01, NS, not significant.

wild-type mice (Figure 4B), suggesting that HBx may up-regulate the expression of NUSAP1 during HBV infection. Furthermore, we confirmed that HBx was able to up-regulate the expression of NUSAP1, in a dose-dependent manner, in HepG2 and H7402 cells transfected with HBx (Figure 4C and 4E). Conversely, HBx knockdown by si-HBx abolished the up-regulation of NUSAP1 in HepAD38 and HepG2.2.15 cells, at both the RNA and protein level, in a dose-dependent manner (Figure 4D and 4F). Thus, we conclude that HBx is able to up-regulate NUSAP1 in hepatoma cells.

HBx-induced NUSAP1 promoted the proliferation of hepatoma cells *in vitro*

NUSAP1 expression has been reported to promote cell

proliferation in renal cell carcinoma⁴⁶. We sought to determine whether NUSAP1 could also influence cell proliferation in HCC. MTT and colony-formation assays showed that NUSAP1 promoted cell proliferation in HepG2 cells (Supplementary Figure S3A and S3B). Next, we determined the effect of NUSAP1 targeting by *miR-18b* on cell proliferation using MTT assays, colony formation assays, and cell cycle analysis in HepG2, HepG2-X, H7402, and H7402-X cells. We found that the proliferation of HepG2-X and H7402-X cells was significantly reduced by *miR-18b*. However, the over-expression of NUSAP1 could rescue the proliferation of these cells (Figure 5A–5C and Supplementary Figure S4A–4D), suggesting that *miR-18b* targeting of NUSAP1 is able to suppress the HBx-induced proliferation of hepatoma cells. Taken together, we conclude

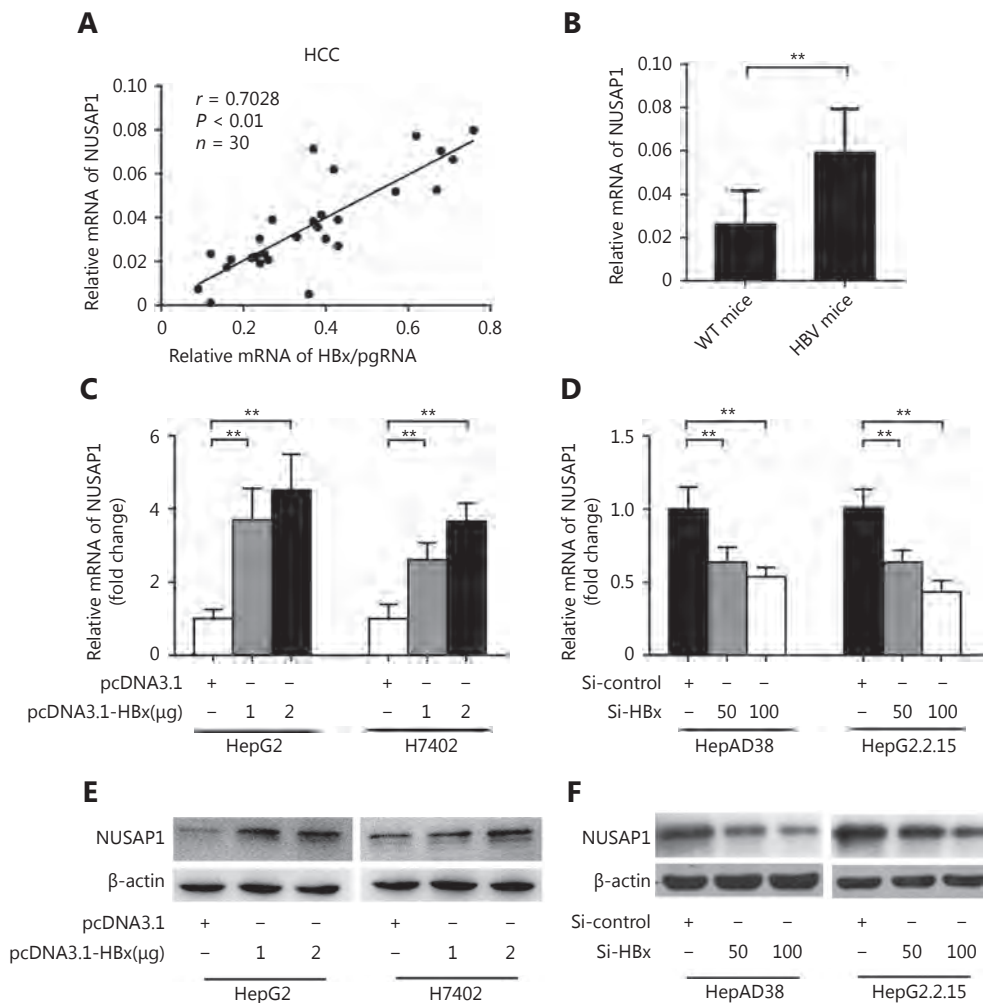


Figure 4 HBx is able to up-regulate NUSAP1 in hepatoma cells. (A) The correlation between NUSAP1 mRNA level and HBx mRNA level was examined by qRT-PCR analysis in 30 cases of HCC tissues (** $P < 0.01$, $r = 0.7028$; Pearson's correlation coefficient). (B) *MiR-18b* expression was measured by qRT-PCR in HBV-transgenic mice ($n = 6$). (C-F) The expression of NUSAP1 was analyzed by qRT-PCR and Western blot analysis in the cells transfected with pcDNA3.1-HBx plasmid (or si-HBx). Error bars indicate SD. Student's t -test. ** $P < 0.01$.

that HBx-induced NUSAP1 increases the proliferation of hepatoma cells *in vitro*.

HBx-induced NUSAP1 facilitated the growth of liver cancer *in vivo*

To further evaluate the effect of NUSAP1 on tumor growth, we subcutaneously injected pretreated cells into 4-week-old BALB/c athymic nude mice. Interestingly, tumor weight and volume in mice injected with *miR-18b*-transfected HepG2-X cells were significantly lower than in mice injected with negative control-transfected HepG2-X cells. Over-expression of NUSAP1 rescued the decrease in tumor size in mice injected with *miR-18b*-transfected HepG2-X cells (Figure

6A–6C), suggesting that the targeting of NUSAP1 by *miR-18b* is able to suppress the HBx-induced growth of hepatoma cells. In addition, IHC staining was used to determine the expression levels of Ki-67, a marker of proliferation, in tumor tissues from athymic nude mice. Ki-67 expression results were consistent with the results of tumor growth analysis (Figure 6D). Thus, we conclude that HBx-induced NUSAP1 facilitates the growth of liver cancer *in vivo*.

Discussion

HBV is a widespread virus and chronic HBV infection is a major risk factor for HCC^{47,48}. HBx is a key factor involved in hepatocarcinogenesis. Host miRNAs play an important role

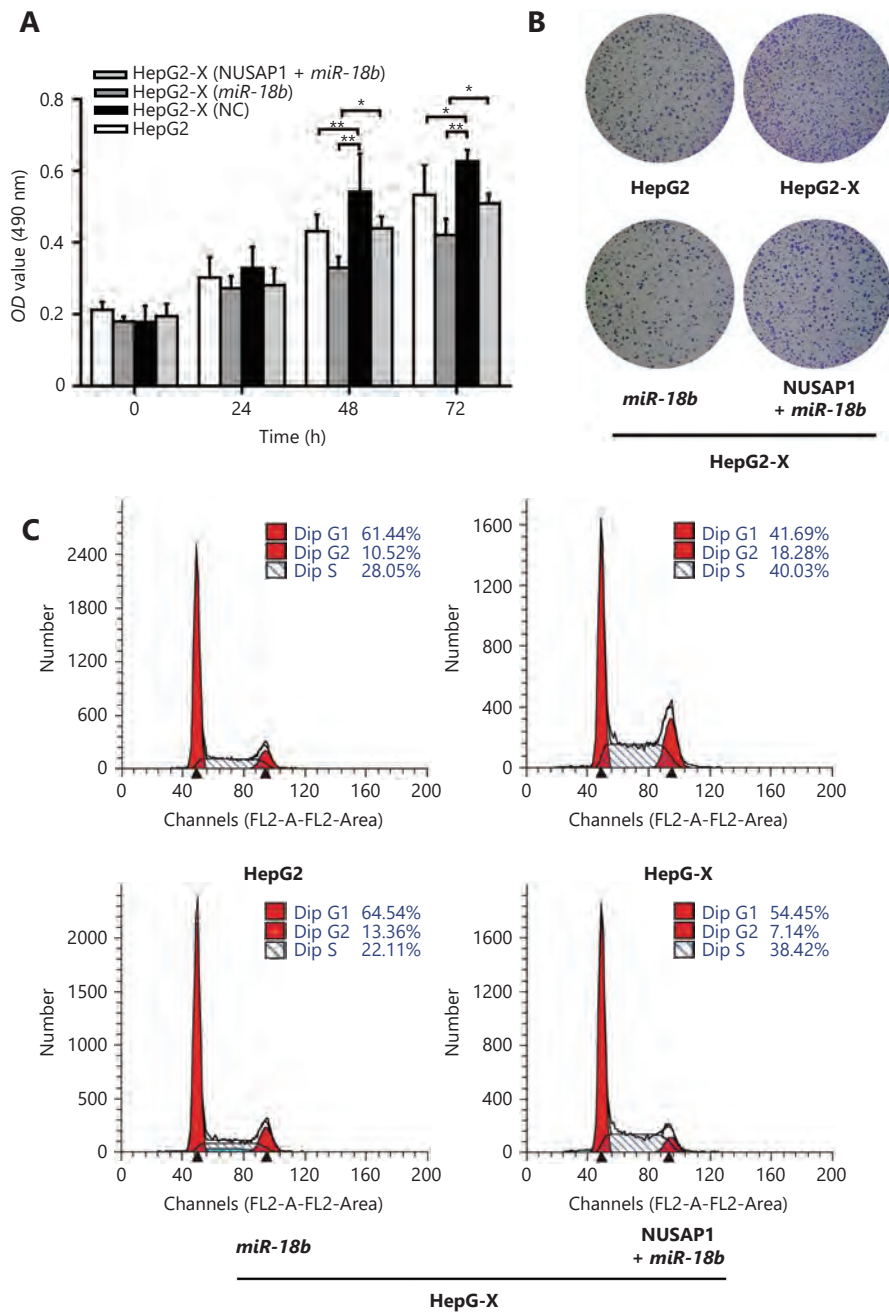


Figure 5 HBx-elevated NUSAP1 promotes the proliferation of hepatoma cells *in vitro*. (A) The effect of *miR-18b* and NUSAP1 on HBx-induced cell proliferation was determined by MTT assays in HepG2-X cells. (B) The effect of *miR-18b* and NUSAP1 on HBx-induced cell proliferation was measured by colony formation assays in HepG2-X cells. (C) The effect of *miR-18b* and NUSAP1 on HBx-induced cell proliferation was examined by cell cycle assays in HepG2-X cells. Error bars indicate SD. Student's *t*-test, **P*<0.05, ***P*<0.01, NS, not significant.

in the development of liver cancer associated with HBV infection⁴⁹. However, the underlying mechanism by which HBx-modulated miRNAs promote liver cancer during the host-virus interaction is still unclear. In this study, we investigated the mechanism by which HBx modulates

hepatocarcinogenesis.

In the early stage of HBV infection, the host can use its own miRNA as a defense against HBV. Some miRNAs, including *hsa-miR-210*, *hsa-miR-199-3p*, *hsa-miR-125a-5p*, and *hsa-miR-151-5p*, have been shown to affect HBV gene

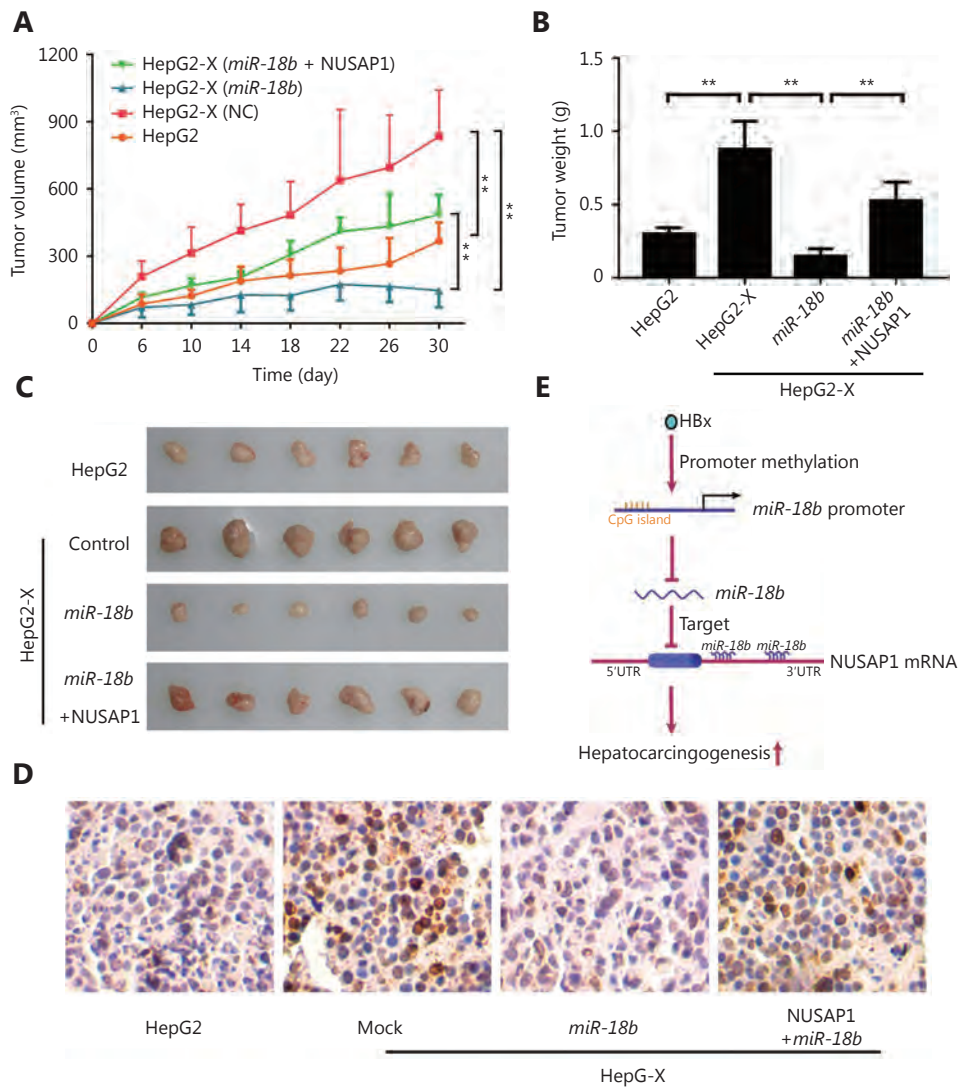


Figure 6 HBx-elevated NUSAP1 facilitates the growth of liver cancer *in vivo*. (A) Growth curve of tumors transplanted with HepG2 and HepG2-X cells pretreated with *miR-18b* or co-transfected *miR-18b* and NUSAP1 in nude mice. (B) Diagram of average weight of tumors. (C) The photographs of dissected tumors from nude mice were shown. (D) Ki-67, a biomarker of proliferation, was examined by IHC staining in tumor tissues from nude mice, respectively (scale bar: 50 μ m). (E) A model shows that HBx enhances hepatocarcinogenesis through depressing *miR-18b* targeting NUSAP1 mRNA.

expression in cultured cells by direct binding to viral transcripts⁵⁰⁻⁵². However, the role of *miR-18b* in hepatocarcinogenesis remains unclear. It has been reported that *miR-18b* is down-regulated in HCC tissues compared to normal liver tissue⁵³. In addition, *miR-18b* expression levels decrease during chronic HBV infection⁵⁴. However, we observed that *miR-18b* expression levels were low in clinical HBV-related HCC samples, relative to their adjacent noncancerous hepatic tissues and in HBV-transgenic mice, relative to wild-type mice. This implies that HBV may suppress the expression of *miR-18b* during HBV infection

and the subsequent development of HCC.

Our group previously reported that HBx can inhibit the tumor suppressor, *miR-205*, by inducing methylation of the *miR-205* gene promoter³⁸. Accordingly, we hypothesized that HBx may be a key factor in overcoming the host defense against HBV. As expected, HBx was shown to down-regulate the expression of *miR-18b* by inducing the hypermethylation of the *miR-18b* gene promoter in hepatoma cells. Therefore, we conclude that HBx can inhibit *miR-18b*. Next, we evaluated the significance of HBx-inhibited *miR-18b* in hepatocarcinogenesis. Using a miRNA target gene prediction

website, we identified *NUSAP1* as a potential target gene of *miR-18b*. Using the gene prediction database, we found that *NUSAP1* was expressed at high levels in liver cancer. It has been reported that *NUSAP1* is a microtubule- and chromatin-binding protein. The physiological function of *NUSAP1* is to stabilize microtubules, maintain spindle integrity, and further format spindle networks⁵⁵. *NUSAP1* can promote the invasion and metastasis of prostate cancer and increase the aggressiveness of astrocytoma through the Hedgehog signaling pathway^{56,57}. It has been previously reported that *NUSAP1* plays crucial roles in various types of cancer⁴⁰⁻⁴². *NUSAP1* is up-regulated in HCCs, when compared to nontumor liver tissues⁴³. Our findings in the present study are consistent with these previous reports. It has been reported that *miR-18b* can modulate many biological functions by targeting different genes. Interestingly, in this study we demonstrated that *miR-18b* was capable of targeting the 3'UTR of *NUSAP1* mRNA, resulting in the down-regulation of *NUSAP1* in HCC cells. Therefore, we hypothesized that HBx may up-regulate *NUSAP1* expression by down-regulating *miR-18b*, to promote the development of liver cancer. qRT-PCR analysis showed that the level of *NUSAP1* expression was higher in HBV-transgenic mice than in wild-type mice. Functionally, we found that the overexpression of *miR-18b* significantly inhibited the growth of HepG2-X and H7402-X cells *in vitro* and *in vivo*. Thus, *miR-18b* can down-regulate *NUSAP1* in hepatoma cells and HBx can promote the proliferation of hepatoma cells by inhibiting *miR-18b*. Thereby, HBx can promote the development of liver cancer by also inhibiting other target genes of *miR-18b*.

Conclusions

We conclude that the targeting of *NUSAP1* mRNA by the tumor suppressor *miR-18b* is controlled by HBx-modulated promoter methylation during the host-virus interaction, leading to hepatocarcinogenesis (**Figure 6E**).

Acknowledgements

This work was supported in part by grants from National Basic Research Program of China (973 Program, Grant No. 2015CB553703), National Natural Science Foundation of China (Grant No. 31670769 and 31470756). All authors would like to acknowledge the First Center Hospital (Tianjin, China) and Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) for providing the patient samples.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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- Cite this article as:** Yang Z, Li J, Feng G, Wang Y, Yang G, Liu Y, et al. Hepatitis B virus X protein enhances hepatocarcinogenesis by depressing the targeting of *NUSAP1* mRNA by *miR-18b*. *Cancer Biol Med.* 2019; 16: 276-87. doi: 10.20892/j.issn.2095-3941.2018.0283

Supplementary materials

Materials and methods

Plasmid construction and treatment

The CDS sequence of NUSAP1 was obtained by PCR from the cDNA of HepG2 using specific primers and was cloned into the pcDNA3.1 vector. The plasmid was named pcDNA3.1-NUSAP1. To construct luciferase reporter plasmids of NUSAP1, the wild type or mutant of NUSAP1 mRNA 3'UTR, which were targeted by *miR-18b*, were amplified by PCR and were inserted into the pGL3-Control vector to generate pGL3-NUSAP1-wt or pGL3-NUSAP1-mut, respectively. The *miR-18b* mimics and anti-*miR-18b* were purchased from RIBOBIO (Guang Zhou, China).

RNA extraction, reverse-transcription, and quantitative real-time polymerase chain reaction (qRT-PCR)

Using Trizol reagent (Invitrogen, Carlsbad, CA), we got total RNAs from cells (or liver tissues from mice and clinical HCC patient tissues). First-strand cDNA was synthesized by using first strand cDNA synthesis kit (Thermo fisher, Shanghai, China), according to the manufacturer's instructions. To analyze *miR-18b* expression, total RNA were polyadenylated by poly (A) polymerase (Ambion, Austin, TX, USA). Reverse transcription was performed using poly (A)-tailed total RNA and reverse transcription primer with ImPro-II Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. QRT-PCR was performed by a Bio-Rad sequence detection system according to the manufacturer's instructions. Relative transcriptional folds were calculated as $2^{-\Delta\Delta C_t}$. Experiments were performed in three independent assays. β -actin was used as an internal control for normalization. U6 was used as an internal control to normalize *miR-18b* levels. All primers used in this paper were listed in **Supplementary Table S2**.

Cell proliferation assay

HepG2/H7402, HepG2-X/H7402-X cells were plated into 96 well plates (1000 cells/well) for 24 hours before transfection and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell proliferation.

Luciferase reporter gene assay

The Dual-Luciferase Reporter Assay System (Promega, USA) was used to perform the luciferase reporter gene assay according to the manufacturer's instructions. Cells were plated into 24 well plates at 3×10^4 cells per well. After 24 hours, the cells were transiently co-transfected with 50 ng/well of pRL-TK plasmid (Promega, USA) containing the Renilla luciferase gene used for internal normalization, pGL3-NUSAP1-wt or pGL3-NUSAP1-mut (100 ng/well). All experiments were performed at least three times.

Western blot analysis

Cells were disrupted in cell lysis buffer. Proteins in the same amount were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After incubation with specific antibodies for NUSAP1 (Proteintech, USA), HBx (Santa Cruz Biotechnology, Santa Cruz, CA) and β -actin (Sigma, USA), the blots were incubated with goat anti-rabbit or anti-mouse secondary antibody (Sigma, USA) and visualized with enhanced chemiluminescence. The results were obtained by using Luminescent Imaging Workstation (Tanon, Shanghai, China).

Genomic DNA extraction and bisulfite treatment

The Axygen genomic DNA purification kit (Axygen Biotechnology, Hangzhou, China) was used to extract genomic DNA was from cells. Treat Genomic DNA (0.5 μ g) with sodium bisulfite with the DNA methylation quantification kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions.

Bisulfite genomic sequencing

Treated DNA, which extracted from LO2, HepG2-X, HepAD38 and HepG2.2.15 cells with bisulfite. Then the DNA was amplified with specific primers to the gene promoters by PCR. The genomic PCR products were then gel-extracted, cloned into pEASY-T vectors (TransGen, Beijing, China), transformed into *Escherichia coli* for sequencing. The sequence results were shown in **Supplementary Table S3**.

Colony formation

48 hours after transfection, about 1,000 viable transfected cells were placed into 6-well plates and grew in complete medium for 2 weeks. Colonies were fixed with methanol for 20 minutes and then stained with methylene blue.

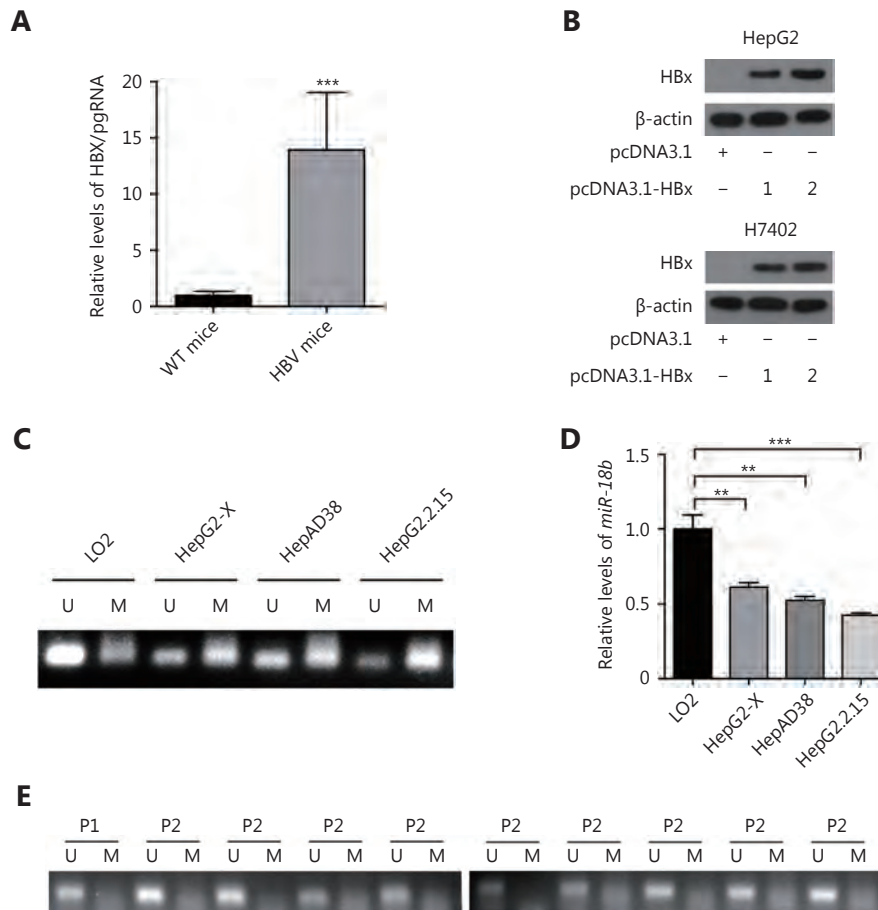


Figure S1 HBx suppresses *miR-18b* through inducing DNA hypermethylation of *miR-18b* promoter. (A) The levels of HBx/pgRNA were tested by qRT-PCR in HBV-transgenic mice ($n=6$). (B) The levels of HBx were examined by Western blot analysis in HepG2 and H7402 cells transfected with pcDNA3.1 or pcDNA3.1-HBx. (C) The methylation status of the *miR-18b* promoter was examined by MSP analysis in LO2, HepG2-X, HepAD38 and HepG2.2.15 cells after treatment with DZNeP (10 μ M) for 72 h. (D) The levels of *miR-18b* were determined by qRT-PCR in the cell lines of LO2, HepG2-X, HepAD38 and HepG2.2.15. (E) The methylation status of the *miR-18b* promoter was measured by MSP analysis in HCC peritumor tissues (P). Error bars indicate SD. Student's *t*-test, ** $P < 0.01$, *** $P < 0.001$.

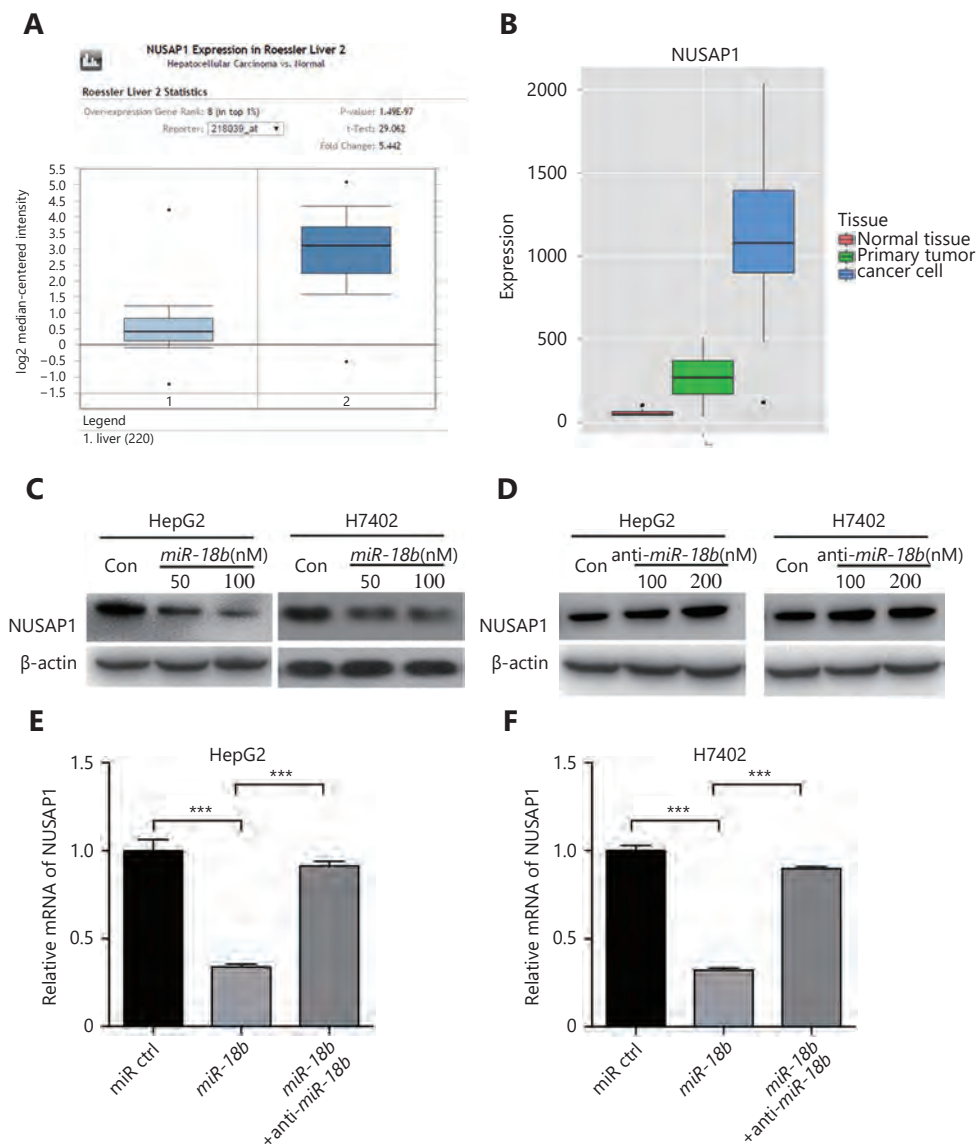


Figure S2 *MiR-18b* inhibits the expression of NUSAP1 through directly targeting its 3'UTR. (A and B) the expression level of NUSAP1 was predicted in cancer microarray Database OncoPrint (<https://www.oncoPrint.org/>) and Metabolic gEnE RApid Visualizer (MERAV) (<http://merav.wi.mit.edu/>). (C and D) The effect of *miR-18b* (or anti-*miR-18b*) on the expression of NUSAP1 was measured by Western blot analysis in HepG2 and H7402 cells. (E and F) The expression of NUSAP1 mRNA was examined by qRT-PCR in HepG2 and H7402 cells transfected with *miR-18b* (100 nM) or co-transfected with both *miR-18b* (100 nM) and anti-*miR-18b* (100 nM). Error bars indicate SD. Student's *t*-test, ***P* < 0.01, ****P* < 0.001.

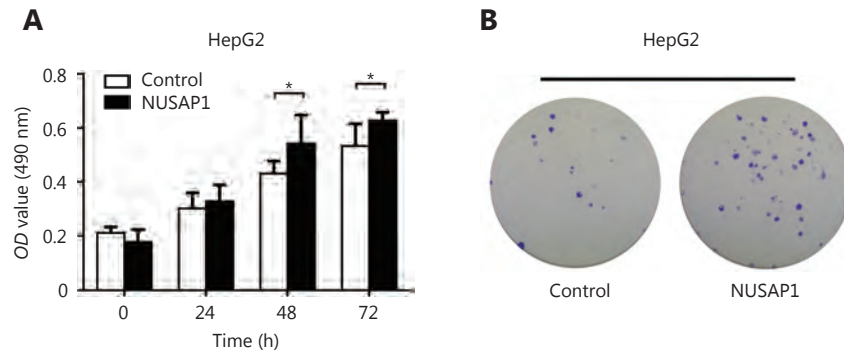


Figure S3 HBx-elevated NUSAP1 targeted by *miR-18b* promotes the proliferation of hepatoma cells *in vitro*. (A) The effect of NUSAP1 on cell proliferation was determined by MTT assays in HepG2 cells. (B) The effect of NUSAP1 on cell proliferation was determined by colony formation assays in HepG2 cells. Error bars indicate SD. Student's *t*-test, * $P < 0.05$, ** $P < 0.01$, NS, not significant.

Table S1 The characteristics of patients

No.	Age	Gender	Organ	HBV
1	59	F	Liver	+
2	60	M	Liver	+
3	65	M	Liver	+
4	43	M	Liver	+
5	60	F	Liver	+
6	41	M	Liver	+
7	45	M	Liver	+
8	56	M	Liver	+
9	70	M	Liver	+
10	67	M	Liver	+
11	59	M	Liver	+
12	57	M	Liver	+
13	61	F	Liver	+
14	51	F	Liver	+
15	56	M	Liver	+
16	60	M	Liver	+
17	54	M	Liver	+
18	36	M	Liver	+
19	46	M	Liver	+
20	60	M	Liver	+
21	59	M	Liver	+
22	57	F	Liver	+
23	51	M	Liver	+
24	38	M	Liver	+
25	56	M	Liver	+
26	49	M	Liver	+
27	58	F	Liver	+
28	46	M	Liver	+
29	56	M	Liver	+
30	54	M	Liver	+

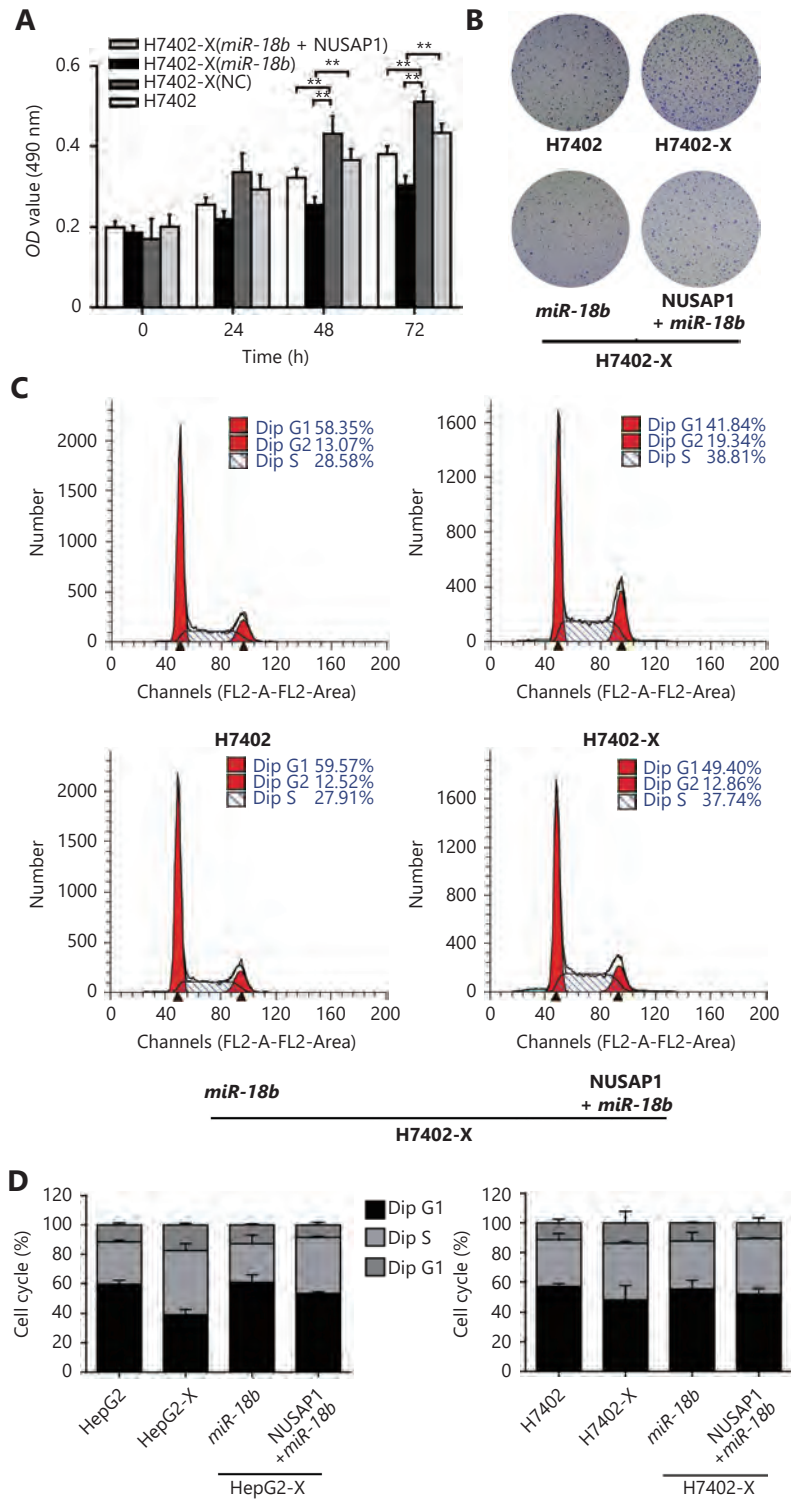


Figure S4 HBx-elevated NUSAP1 targeted by *miR-18b* promotes the proliferation of hepatoma cells *in vitro*. (A) The effect of *miR-18b* and NUSAP1 on HBx-induced cell proliferation was determined by MTT assays in H7402-X cells. (B) The effect of *miR-18b* and NUSAP1 on HBx-induced cell proliferation was determined by colony formation assays in H7402-X cells. (C) The effect of *miR-18b* and NUSAP1 on HBx-induced cell proliferation was determined by cell cycle assays in H7402-X cells. (D) The statistical results of cell cycle assays from Figure 5C and Figure S4C were shown. Error bars indicate SD. Student's *t*-test **P* < 0.05, ***P* < 0.01, NS, not significant.

Table S2 List of primers used in this paper

Primer	Forward primer (5'-3')	Reverse primer (5'-3')
Primers for NUSAP1 3'UTR		
pGL3-NUSAP1-3'UTR-wt	GCTCTAGAGCACATCTTGAAATATTCCTG	GGGGGCCGGCCAACAGAATTTTAGGATGGGT
pGL3-NUSAP1-3'UTR-mut	CTCTGAAAAGACGTTATTCATCGCAAGCTCAAATCTTTG	CAAAGAATTTGAGCTTGCGATGAATAACGTCTTTCAGAG
pGL3-HBx-wt	GCTCTAGAGCATGGCTGCTAGGCTGTGCT	GGGGGCCGGCCTTAGGCAGAGGTGAAAAAG
pGL3-HBx-mut	CGTCCGACCACGGGGCTAGTTGCTCTTTACGCGGTCTCCC	GGGAGACCGCGTAAAGAGCAACTAGCCCCGTGGTCCGACG
Primers for CDS cloning		
NUSAP1	CGGGATCCCGATGATCATCCCCTCTCTAGAGG	CCGCTCGAGCGGTTAATCTTCAGCCAAAATGAGG
Primers for RT-PCR and qRT-PCR		
NUSAP1	AACTTACAAACAACCCCATCTCC	GTTTCTTCGGTTGCTCTTCCTTT
mNUSAP1	ACCTTAGACTCGGAGGACCC	GTCCAATGAAGACGCAGGGA
mGAPDH	AGAGGGATGCTGCCCTTACC	ATCCGTTACACCCGACCTTC
HBx	ATGGCTGCTAGGGTGTGCT	TAAATCTCCTCCCCAACTC
<i>miR-18b</i>	TAAGGTGCATCTAGTGCAATTAG	GCGAGCACAGAATTAATACGAC
mmu- <i>miR-18b</i>	TAAGGTGCATCTAGTGCTGTTAG	GCGAGCACAGAATTAATACGAC
U6	CTCGCTTCGGCAGCACA	AACGCTTACGAATTTGCGT
GAPDH	ATCACCATCTCCAGGAGCGA	CCTTCTCCATGGTGGTGAAGAC
Primers for <i>miR-18b</i> promoter methylation		
<i>miR-18b</i> BSP	ATTTTAGTTTGGGGGAAGAGAGA	CCCTCTACCTTTAATAACCCAAAA
<i>miR-18b</i> M	AGCGCGGGGATTTTCGTAC	GACCCCGAAACGCTCG
<i>miR-18b</i> U	TGGAGAGTGTGGGGATTTTGTATG	CACCCAACCCCAAACACTCA
siRNAs sequences		
siHBx	AAUGUCAACGACCGACCUUGA	
siNUSAP1	CAGCCAACGACGCUCGCAA	

Table S3 BSP methylation analysis of *miR-18b* promoter CpG islands sequences

>Genome

ACTCCAGCTTGGGGGAAGAGAGAAGGGAAAATGCCGACTGCGGTCCGCCCGTGGGTAAGTGGGGCTCGGTGGGGTTTTCGCTTTGGCGGGGTGGG
GGTTCGTTTTCTAATTCCTGTGCGGGCCGGGTGGGCGGGGCGGGCTTTTTCTTGGGCTATTAAAGGCAGAGGG

>LO2-1

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTGTGGGTAATTGGGGTTTGGTGGGGTTTTGTTTTGGCGGGGTGGGGGT
TTGTTTTTAATTTTGTGCGGGTGGGTGGGTGGGGTGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>LO2-2

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTGTGGGTAATTGGGGTTTGGTGGGGTTTTGTTTTGGCGGGGTGGGGGT
TTGTTTTTAATTTTGTGCGGGTGGGTGGGTGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>LO2-3

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTGTGGGTAATTGGGGTTTGGTGGGGTTTTGTTTTGGTGGGGTGGGGGT
TTGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGTTGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>LO2-4

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTGTGGGTAATTGGGGTTTGGTGGGGTTTTGTTTTGGTGGGGTGGGGGT
TTGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGTTGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepG2-X-1

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTCGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTGGTGGGGTTTTCGTTTTGGTGGGGTGGGGGT
TTCGTTTTTAATTTTGTGCGGGTGGGTGGGTGGGGTGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepG2-X-2

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTCGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTCGTTGGGGTTTTGTTTTGGTGGGGTGGGGGT
TTCGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGTTGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepG2-X-3

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTCGTTGGGGTTTTCGTTTTGGTGGGGTGGGGGT
TTCGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGTTGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepG2-X-4

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTCGTTGGGGTTTTGTTTTGGCGGGGTGGGGGT
TTCGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepAD38-1

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTCGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTCGTTGGGGTTTTGTTTTGGCGGGGTGGGGG
TTTCGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepAD38-2

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTCGTTGGGGTTTTCGTTTTGGCGGGGTGGGGGT
TTCGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGTTGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepAD38-3

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTCGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTCGTTGGGGTTTTGTTTTGGTGGGGTGGGGGT
TTCGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepAD38-4

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTCGTTGGGGTTTTGTTTTGGCGGGGTGGGGGT
TTCGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepG2.2.15-1

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTCGATTGCGGTTTCGTTCTGGGTAATTGGGGTTTCGTTGGGGTTTTCGTTTTGGCGGGGTGGGGG
TTTCGTTTTTAATTTTGTGCGGGTGGGTGGGCGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepG2.2.15-2

Continued

Continued

>Genome

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTTGATTGCGGTTTCGTTTCGTGGGTAATTGGGGTTCGGTGGGGTTTTTCGTTTTGGCGGGGTGGGGG
TTTCGTTTTTAATTTTGTGTGGGTTCGGTGGGCGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepG2.2.15-3

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTCGATTGCGGTTTCGTTTCGTGGGTAATTGGGGTTCGGTGGGGTTTTTCGTTTTGGCGGGGTGGGGG
TTTCGTTTTTAATTTTGTGCGGGTTCGGTGGGCGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG

>HepG2.2.15-4

ATTTTAGTTTGGGGGAAGAGAGAAGGGAAAATGTCGATTGCGGTTTCGTTTCGTGGGTAATTGGGGTTCGGTGGGGTTTTGTTTTGGCGGGGTGGGGG
TTTTGTTTTTAATTTTGTGCGGGTTCGGTGGGCGGGGCGGGTTTTTTTTTTGGGTTATTAAAGGTAGAGGG
