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Hepatitis B Virus X Protein Inhibits Tumor Suppressor miR-205 through Inducing Hypermethylation of miR-205 Promoter to Enhance Carcinogenesis[1,2]

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Abstract

The infection of hepatitis B virus (HBV) is closely associated with the development of hepatocellular carcinoma (HCC), in which HBV X protein (HBx) plays crucial roles. MicroRNAs are involved in diverse biologic functions and in carcinogenesis by regulating gene expression. In the present study, we aim to investigate the underlying mechanism by which HBx enhances hepatocarcinogenesis. We found that miR-205 was downregulated in 33 clinical HCC tissues in comparison with adjacent noncancerous hepatic tissues. The expression levels of miR-205 were inversely correlated with those of HBx in abovementioned tissues. Then, we demonstrated that HBx was able to suppress miR-205 expression in hepatoma and liver cells. We validated that miR-205 directly targeted HBx mRNA. Ectopic expression of miR-205 downregulated HBx, whereas depletion of endogenous miR-205 upregulated HBx in hepatoma cells. Notably, our data revealed that HBx downregulated miR-205 through inducing hypermethylation of miR-205 promoter in the cells. In terms of function, the forced miR-205 expression remarkably inhibited the HBx-enhanced proliferation of hepatoma cells *in vitro* and *in vivo*, suggesting that miR-205 is a potential tumor-suppressive gene in HCC. HBx-transgenic mice showed that miR-205 was downregulated in the liver. Importantly, HBx was able to abrogate the effect of miR-205 on tumor suppression in carcinogenesis. Therefore, we conclude that HBx is able to inhibit tumor suppressor miR-205 to enhance hepatocarcinogenesis through inducing hypermethylation of miR-205 promoter during their interaction. Therapeutically, miR-205 may be useful in the treatment of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death in the world with annual death rate accounting for 700,000 [1,2]. Hepatitis B virus (HBV) is a hepatotropic noncytopathic DNA virus, which is a major cause of chronic liver disease and can lead to viral hepatitis, cirrhosis, and HCC [3–5]. As a multifunctional transactivator protein [6], the HBV X protein (HBx) plays crucial roles in HBV-associated HCC [5,7–9].

MicroRNAs (miRNAs), roughly 22-nucleotide noncoding RNAs, posttranscriptionally modulate gene expression through repression of translation and degradation of complementary target mRNAs [10], which are being increasingly recognized as a crucial determinant of tumor development and progression [11]. Several recent studies indicate that miRNAs play critical roles in variety of biologic processes, including development, differentiation, cellular proliferation, apoptosis,

Abbreviations: Aza, 5-Aza-2′-deoxycytidine; EdU, 5-ethynyl-2′-deoxyuridine; HBV, hepatitis B virus; HBx, HBV X protein; HCC, hepatocellular carcinoma; miRNA, microRNA; MSP, methylation-specific polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NC, negative control; pSi-HBx, pSilencer-X; pgRNA, pregenomic RNA; qRT-PCR, quantitative real-time reverse transcription–polymerase chain reaction; siRNA, small interfering RNA

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[2]This article refers to supplementary materials, which are designated by Table W1 and Figures W1 to W3 and are available online at www.neoplasia.com.

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metabolism, and immunity [11–13]. Growing evidence supports a role of miRNAs as both targets and effectors in aberrant mechanisms of DNA hypermethylation [14]. It has been reported that some miRNAs are inactivated in human tumors by aberrant hypermethylation of cytosine-phosphate-guanosine (CpG) islands encompassing *miRNA* genes [15,16]. Recent studies demonstrate that the expression levels of miR-205, targeting E2-promoter binding factor 1 (E2F1), human epidermal growth factor receptor 3 (HER3), and protein kinase Cε, are significantly downregulated in melanoma and breast and prostate cancers [17–19]. However, the role of miR-205 in HCC is poorly understood.

In the present study, we investigated the effect of HBx on miR-205 on hepatoma cells. Our results imply that miR-205 is a potential tumor suppressor gene. We show that HBx is able to downregulate the miR-205 during their interaction, resulting in the development of HCC. Our finding provides new insights into the mechanism of HBx-induced hepatocarcinogenesis.

Materials and Methods

Patient Samples and HBx-Transgenic Mice

Thirty-three HCC tissues and their corresponding nearby nontumorous liver tissues used in this study were obtained from Tianjin First Center Hospital (Tianjin, PR China) after surgical resection. Written consents approving the use of their tissues for research purposes were obtained from patients. The study protocol was approved by the Institute Research Ethics Committee at the Nankai University (Tianjin, PR China). HBx-transgenic mice were obtained from Genetic Laboratory of Development and Diseases, Institute of Biotechnology (Beijing, PR China) [20].

Cell Lines and Cell Culture

Hepatoma cell lines HepG2, HepG2-X (a stably HBx-transfected hepatoma HepG2 cell line), and HepG2.2.15 (a hepatoma HepG2 cell line stably transfected with HBV genome) were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) [21]. The cell lines of LO2 (a human immortalized liver cell line), LO2-X (a stably HBx-transfected LO2 cell line), and H7402-X (a stably HBx-transfected hepatoma H7402 cell line) [21] were cultured in RPMI Medium 1640 (Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO_2 at 37°C.

DNA Constructs

The 5′-flanking region (nucleotides −4178 to −2751) of miR-205 was amplified by polymerase chain reaction (PCR) from the genomic DNA of HepG2 using specific primers and was cloned into the upstream of the pGL3-Basic Vector (Promega, Madison, WI) through KpnI and XhoI sites. The resulting plasmid was sequenced and named pGL3-1428. The regions (−3429/−2751, −4178/−3416, −3888/−3416, and −3888/−3588) of miR-205 were amplified by PCR from the pGL3-1428 and were inserted into the pGL3-Basic vector to generate pGL3-679, pGL3-763, pGL3-473, and pGL3-301, respectively. All primers are listed in Table W1.

Plasmids, miRNAs, Small Interfering RNA, and DNA Transfection

The cells were cultured in a 6-well or 24-well plate for 24 hours and then were transfected with plasmid or miRNA. All transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The pSilencer-X (pSi-HBx) produces small interfering RNAs (siRNAs) targeting HBx mRNA, and pSilencer-control as negative control (NC) were used [22]. The plasmid pCH-9/3091 containing the complete HBV genome has been described previously [23]. The miR-205 mimics (miR-205), mimic NC, miR-205 inhibitor (anti–miR-205), inhibitor NC, HBx siRNA oligonucleotides [24], and NC siRNA were produced from RiboBio (Guangzhou, PR China). A ~460-bp fragment of HBx was cloned into the pGL3-Control vector (Promega) downstream of the stop codon of the luciferase gene to generate pGL3-HBx. pGL3-HBx-mut carried a substitution of five nucleotides within the core seed sequence of miR-205, which was carried out using overlapping extension PCR [25]. The sequences were all listed in Table W1.

RNA Extraction and Quantitative Real-Time Reverse Transcription–PCR

Total RNA was extracted from cells (mice or patient tissues) using TRIzol reagent (Invitrogen). For mature miR-205 detection, 20 μg of total RNA was polyadenylated by Poly(A) Polymerase (Ambion, Austin, Texas) as described previously [26]. Reverse transcription was performed using poly(A)-tailed total RNA and reverse transcription primer. Quantitative real-time reverse transcription–PCR (qRT-PCR) was performed by a Bio-Rad Laboratories (Berkeley, CA) sequence detection system according to the manufacturer's instructions using double-stranded DNA SYBR GreenPremix Ex TaqTM II Kit (Takara Bio, Tokyo, Japan). Relative transcriptional folds were calculated as $2^{-\Delta\Delta\ Ct}$ [27]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal control for normalization. All the primers used are listed in Table W1.

Luciferase Reporter Gene Assay

Luciferase reporter gene assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Cells were transferred into 24-well plates at 3×10^4 cells per well. After 24 hours, the cells were transiently cotransfected with 0.1 μg per well of pRL-TK plasmid (Promega) containing the *Renilla* luciferase gene used for internal normalization and various constructs containing different lengths of miR-205 promoter, pGL3-HBx, or pGL3-Basic. The luciferase activities were measured as previously described [21].

Western Blot Analysis

Western blot analysis was carried out as described previously [21]. The primary antibodies used were rabbit anti-E2F1 (Proteintech Group, Chicago, IL), mouse anti-HBx (Abcam, Cambridge, United Kingdom), and mouse anti–β-actin (Sigma-Aldrich, St Louis, MO).

Immunohistochemistry

The HCC tissue and normal liver tissue microarrays were obtained from the Xi'an Aomei Biotechnology Co, Ltd (Xi'an, PR China), and the primary antibody of rabbit anti-E2F1 (Proteintech Group) was used. The sections were observed under a Carl Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany) equipped with a Leica DC500 camera (Leica, Wetzlar, Germany). Categorization of immunostaining intensity was performed by three independent observers.

5-Aza-2′-Deoxycytidine Treatment and Methylation Analysis

The HepG2-X and HepG2.2.15 cells were treated with 5 μM 5-Aza-2′-deoxycytidine (Aza; DNA methylation inhibitor; Sigma-Aldrich) for 72 hours, respectively. Cells were harvested, and RNA

was extracted for qRT-PCR analysis. Genomic DNA (2 μg) of LO2, LO2-X, HepG2, and HepG2.2.15 cells and clinical HCC tissues (n = 2) was modified with sodium bisulfite using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). Methylation-specific PCR (MSP) and bisulfite-sequencing analysis were then performed as described previously [28]. Amplified bisulfite-sequencing PCR products were cloned into pEASY - T1 vector (Transgen, Beijing, PR China), and five clones from each sample were sequenced. Primer sequences are shown in Table W1.

Analysis of Cell Proliferation

HepG2-X and HepG2.2.15 cells were seeded onto 96-well plates (1000 cells per well) at 24 hours before transfection. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) and 5-ethynyl-2′-deoxyuridine (EdU; RiboBio) incorporation assays were carried out as described previously [29]. For clonogenicity analysis, at 48 hours after transfection, 1000 viable transfected cells (HepG2, HepG2-X, and HepG2.2.15) were placed in six-well plates and maintained in complete medium for 2 weeks. Colonies were fixed with methanol and stained with methylene blue.

Animal Transplantation

Nude mice were housed and treated according to guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. We conducted the animal transplantation according to the Declaration of Helsinki. Tumor transplantation in nude mice was performed as described previously [30,31]. Briefly, HepG2.2.15 (or HepG2) cells were harvested and resuspended at 1×10^6 per ml with sterile phosphate-buffered saline. Groups of 4-week-old female BALB/c athymic nude mice [Experiment Animal Center of Peking (Beijing, PR China); each group, n = 6 for HepG2.2.15, n = 5 for HepG2] were subcutaneously injected at the shoulder with 0.2 ml of the cell suspensions. The tumor size was measured daily until the tumor reached 50 mm^3. Then, 5 μg of synthetic miR-205, HBx plasmid (pCMV-X), HBx siRNA, or NC diluted in Lipofectamine (Invitrogen) solution (100-μl total volume) was intratumorally injected on days 9, 12, and 15 for a total of three injections for each tumor. Tumor volume (V) was monitored by measuring the length (L) and width (W) with calipers and calculated with the following formula: $(L \times W^2) \times 0.5$. After 27 days, mice were killed, and the tumors were excised and weighted.

Statistical Analysis

Each experiment was repeated at least three times. Statistical significance was assessed by comparing mean values (±SD) using a Student's t test for independent groups and was assumed for $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$. miR-205 expression levels in tumor tissues and matched adjacent nontumor tissues were compared using the Wilcoxon signed rank test. The correlation between miR-205 (or E2F1) and HBx mRNA levels in tumorous tissues was determined with Pearson r.

Results

HBx Is Able to Decrease miR-205 Expression in Hepatoma and Liver Cells

It has been reported that the expression levels of miR-205 are reduced in several cancers, such as melanoma and prostate and breast cancers [17,19,32]. However, it has been unclear whether miR-205 is associated with HCC. Thus, we are interested in the role of miR-205 in liver cancer. We assessed the miR-205 expression in 33 paired HCC and adjacent nontumorous liver tissues by qRT-PCR and normalized against an endogenous control (U6 RNA). Interestingly, our data indicated that the expression levels of miR-205 were reduced in clinical HBV-related HCC samples relative to their adjacent noncancerous hepatic tissues (Figure 1*A*; $P < .01$, Wilcoxon signed rank test). Next, we examined the relationships between HBx and miR-205 in HCC tissues by qRT-PCR. Intriguingly, we found that the expression levels of miR-205 were inversely correlated with those of HBx mRNA/pregenomic RNA (pgRNA; Figure 1*B*; Pearson $r = -0.551$, $P < .001$). It has been reported that the HBx sequence partially overlapped with the core sequence and completely overlapped with the pgRNA [33]. On the basis of the primer sequence, the primers would not amplify the region of core protein. It was possible that along with HBx mRNA, the primers would amplify pgRNA. Therefore, the qRT-PCR products should represent the amplified products from HBx mRNA and pgRNA. Accordingly, we proposed that HBx might downregulate miR-205 in the cells. Then, we displayed that HBx was able to decrease miR-205 expression by transient transfection of HBx plasmid (pCMV-X) in HepG2 or LO2 cells in a dose-dependent manner (Figure 1*C*). Strikingly, we observed that the knockdown of HBx by pSi-HBx resulted in the increase of miR-205 levels in HepG2-X, H7402-X, or HepG2.2.15 cells in a dose-dependent manner (Figure 1*D*), suggesting that HBx may downregulate miR-205 in the cells. The efficiency of HBx overexpression (or silencing) on miR-205 in above experiments was confirmed by Western blot analysis (Figure W1, *A* and *B*). Meanwhile, the transient transfection of full-length HBV plasmid pCH-9/3091 led to the down-regulation of miR-205 in HepG2 or LO2 cells (Figure 1*E*). To further validate the effect of HBx on miR-205, we examined whether HBx affected the expression of *E2F1*, a target gene of miR-205 [17], in hepatoma and liver cells. We found that the mRNA levels of E2F1 were positively correlated with those of HBx/pgRNA by qRT-PCR in 33 paired HCC tissues (Figure 1*F*; Pearson $r = 0.566$, $P < .001$). To demonstrate that E2F1 was upregulated in HCC tissues, we further determined the expression of E2F1 by immunohistochemistry in 110 clinical HCC tissues and 10 normal liver tissues. Our data revealed that 78.2% (86 of 110) of the HCC tissues exhibited strong nuclear E2F1 immunoreactivity. However, only 2 of 10 normal tissues displayed weak cytoplasmic and/or nuclear E2F1 immunoreactivity (Figure W1*C*). Furthermore, overexpression (or knockdown) of HBx resulted in the up-regulation (or down-regulation) of E2F1 in the cells (Figure W1*D*). Thus, we conclude that HBx is able to downregulate expression of miR-205 in hepatoma cells.

Russo's group has reported that miR-125a-5p is able to interact with HBV surface antigen and inhibits its expression [34]. In the present study, we are interested in whether miR-205 directly targets HBx mRNA. Then, the computational prediction was performed with miRanda (http://www.microrna.org, Memorial Sloan-Kettering Cancer Center, New York, NY) by scanning the genomes of HBV subtypes (*ayw*, *adw*, and *adr*) for probable target sites of the human miRNAs. As shown in Figure 2*A*, there was an miR-205-binding site in the fragment of *HBx* gene. Then, the predicted target site (or mutant target site) sequence of miR-205 was cloned into the downstream of pGL3-Control luciferase reporter gene vector (termed pGL3-HBx or pGL3-HBx-mut), respectively. When the pGL3-HBx (or pGL3-HBx-mut vector) was cotransfected with miR-205 mimics, the luciferase activities of pGL3-HBx vector were significantly decreased compared

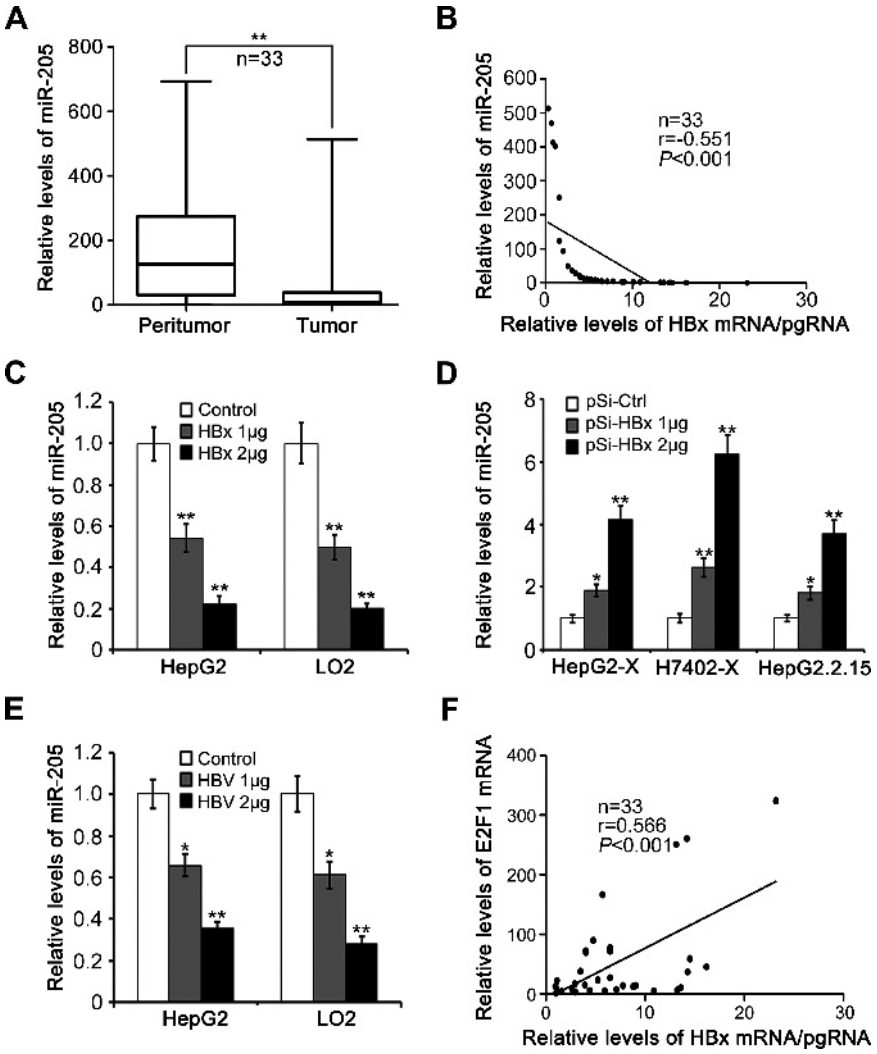

Figure 1. HBx is able to decrease miR-205 in hepatoma and liver cells. (A) miR-205 expression was quantified by qRT-PCR in HCC and peritumor tissues. (B) The correlation of HBx and miR-205 was determined by qRT-PCR in abovementioned HCC tissues. (C) miR-205 expression was examined by qRT-PCR in HepG2 hepatoma cells/LO2 human immortalized liver cells after transfection with pCMV-X (HBx plasmid). (D) miR-205 expression was detected by qRT-PCR after knockdown of HBx (pSi-HBx) in hepatoma cells. (E) miR-205 expression was examined by qRT-PCR in HepG2/LO2 cells after transfection with pCH-9/3091 (HBV plasmid). (F) The correlation of HBx and E2F1 was determined by qRT-PCR in clinical HCC tissues. Data are presented as means ± SD (*$P < .05$; **$P < .01$). We performed three separate experiments in triplicate.

with those of pGL3-HBx-mut vector in a dose-dependent manner (Figure 2*B*). However, when the pGL3-HBx (or pGL3-HBx-mut vector) was cotransfected with miR-205 inhibitors (anti–miR-205), the luciferase activities of pGL3-HBx vector were elevated compared with those of pGL3-HBx-mut vector in a dose-dependent manner (Figure 2*C*). Ectopic expression of miR-205 was able to markedly repress the expression of HBx in HepG2-X and HepG2.2.15 cells at the levels of mRNA and protein (Figure 2*D*). Conversely, miR-205 inhibitors were able to increase the HBx expression at the levels of mRNA and protein (Figure 2*E*). Therefore, we conclude that miR-205 is able to directly target HBx mRNA in the cells.

HBx Reduces miR-205 Expression through Inducing DNA Hypermethylation of miR-205 Promoter

Next, we tried to dissect the mechanism by which HBx reduced miR-205 expression. We first identified the miR-205 promoter core region. Various lengths of miR-205 5′-flanking regions, including −4178/−2751 (pGL3-1428), −3429/−2751 (pGL3-679), −4178/−3416 (pGL3-763), −3888/−3416 (pGL3-473), and −3888/−3588 (pGL3-301), were cloned and transiently transfected into the HepG2 (or hepatoma H7402) cells to determine the promoter activities, respectively. The luciferase reporter gene assays indicated that the maximum luciferase activity was exhibited by the pGL3-1428 plasmid. The pGL3-301 plasmid exhibited the maximum activity among the truncated plasmids (Figure 3*A*), indicating that the region of −3888/−3588 is the promoter core region of miR-205. It has been reported that the epigenetic mechanisms, such as DNA methylation and histone modifications, can regulate the expression of miRNAs [16]. Several miRNAs were epigenetically silenced in association with CpG island hypermethylation in many cancers [15,29]. Therefore, the miR-205 promoter region was analyzed using CpG Island Searcher (http://www.ualberta.ca/~stothard/javascript/cpg_islands.html, University of Alberta, Edmonton, Canada), and bisulfite-sequencing PCR and MSP

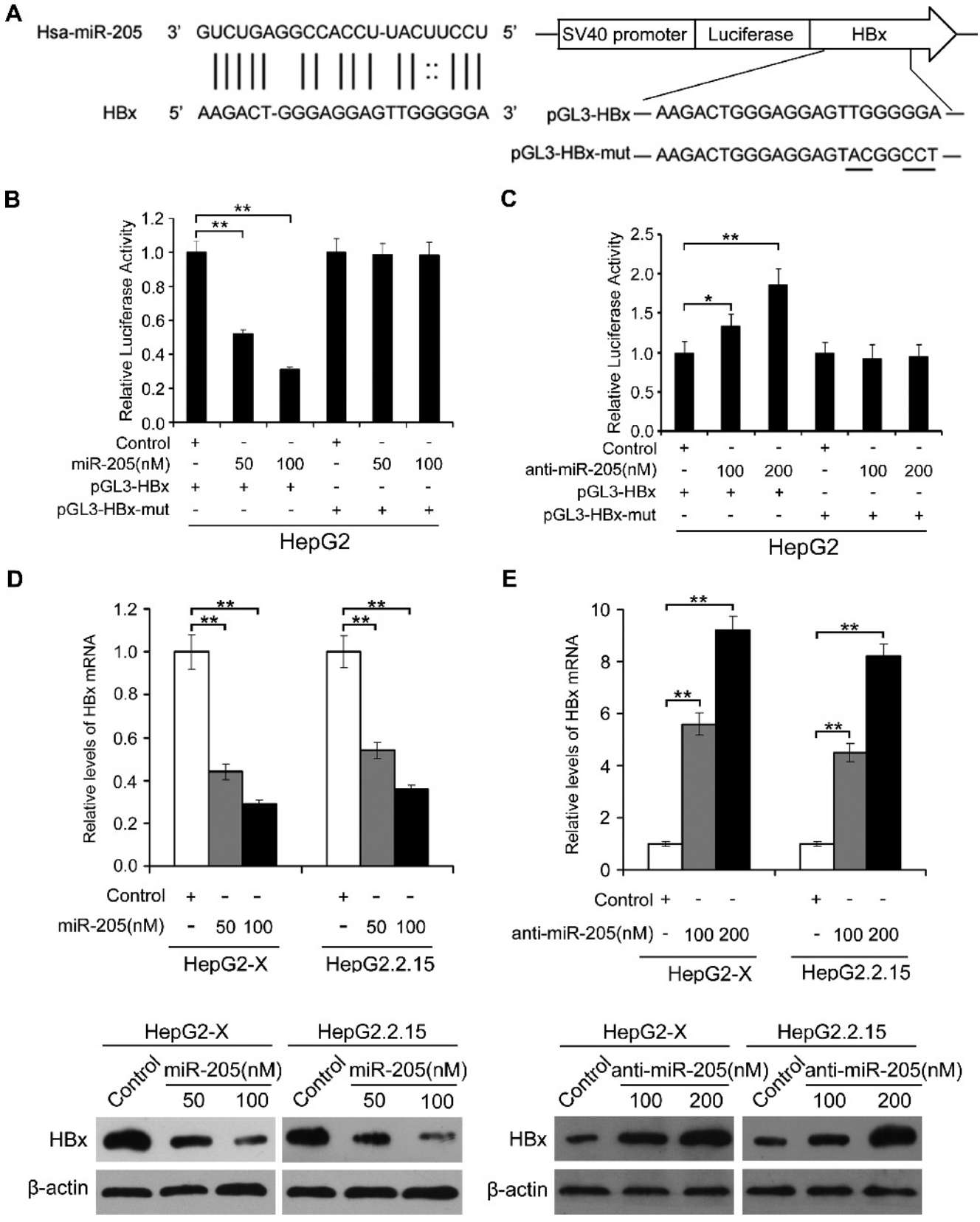

Figure 2. miR-205 directly targets HBx mRNA. (A) The predicted binding sites for miR-205 in HBx mRNA by computational methods were shown. Mutations were designed in the binding sites. (B and C) The plasmid pGL3-HBx or pGL3-HBx-mut was cotransfected with synthetic miRNA mimics (miR-205) or antagomirs (anti–miR-205) into HepG2 cells. The luciferase activities of pGL3-HBx or pGL3-HBx-mut were determined at 48 hours after transfection. (D and E) The expression of HBx was examined by qRT-PCR and immunoblot analysis, respectively, after treatment with miR-205 or anti–miR-205 in the cells. Data are presented as means ± SD (*$P < .05$; **$P < .01$). We performed three separate experiments in triplicate.

primers were designed within the identified CpG island region using the Methyl Primer Express Software version 1.0 (Life Technologies, Grand Island, NY). We noted that a typical CpG island was identified from −3825 to −3625 within the promoter region of miR-205. Then, we examined the DNA methylation pattern in the CpG island region of miR-205 promoter by bisulfite-sequencing PCR and MSP. The results showed that CpG sites were highly methylated in LO2-X and HepG2.2.15 cells (or two clinical HCC tissues) relative to LO2 and HepG2 cells (or nontumorous liver tissues; Figure 3, *B* and *C*). To detect whether HBx led to DNA hypermethylation of miR-205, we carried out qRT-PCR to assess the expression levels of miR-205 in HepG2-X or HepG2.2.15 cells treated with 5 μM Aza (DNA methylation inhibitor) for 72 hours. As shown in Figure 3*D*, miR-205 was silenced before the treatment, but it was dramatically activated by Aza treatment. Thus, we conclude that HBx downregulates miR-205 through inducing DNA hypermethylation of miR-205 promoter in hepatoma cells.

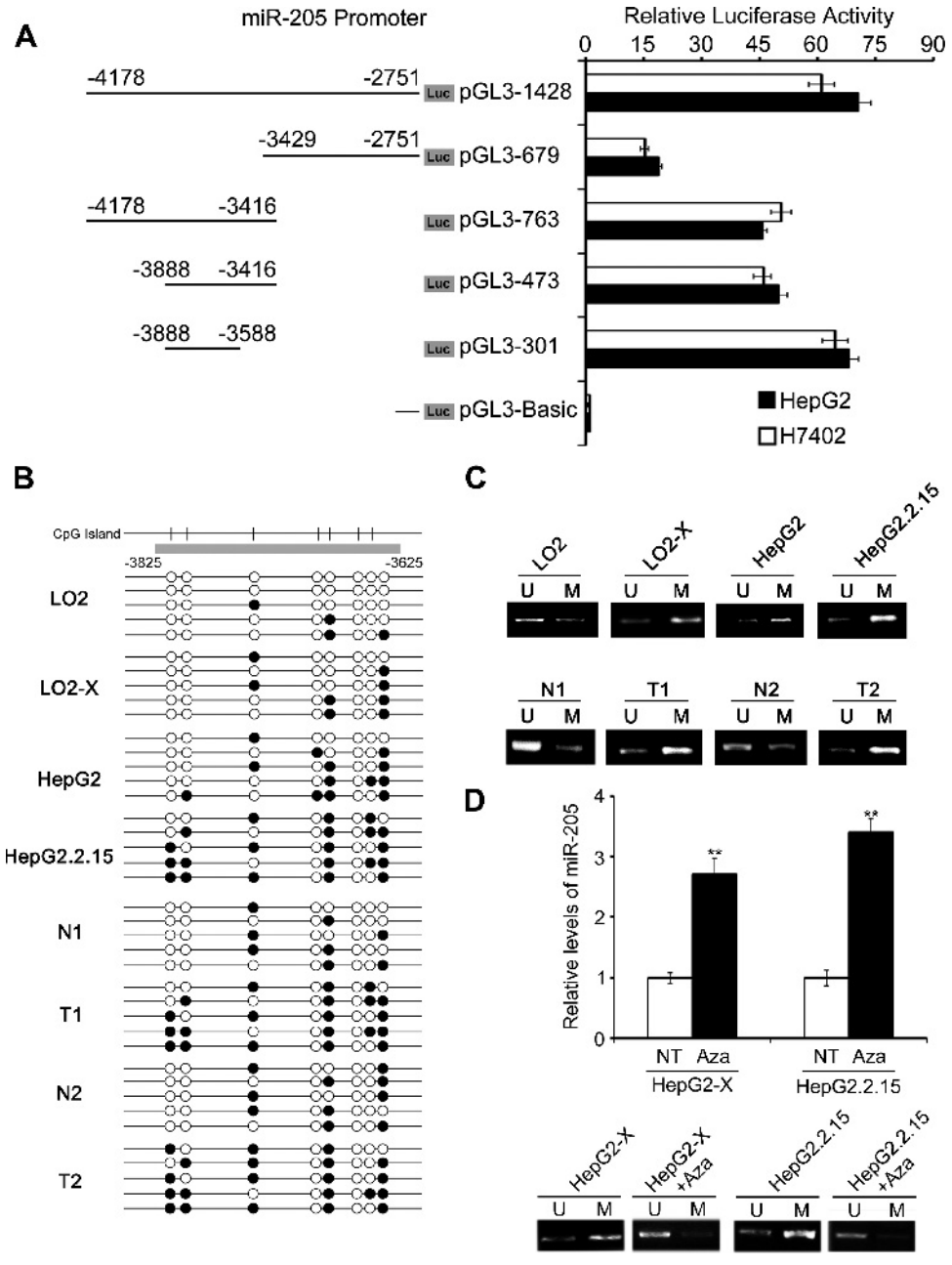

Figure 3. HBx downregulates miR-205 through inducing DNA hypermethylation of miR-205 promoter. (A) miR-205 promoter core region was identified in hepatoma cells by luciferase reporter gene assays. (B) The methylation of miR-205 CpG sites was analyzed by bisulfite-sequencing analysis in the cells, paired nontumor liver tissues (N), and HCC tissues (T). At least five independent clones were sequenced per sample. Open and filled circles represent nonmethylated and methylated CpG sites, respectively. (C) The methylation of miR-205 CpG sites was examined by MSP analysis as well. (D) miR-205 expression was measured by qRT-PCR, followed by MSP analysis in the miR-205 CpG sites in hepatoma cells treated with 5 μM Aza for 72 hours. Data are presented as means ± SD (**$P < .01$). We performed three separate experiments in triplicate.

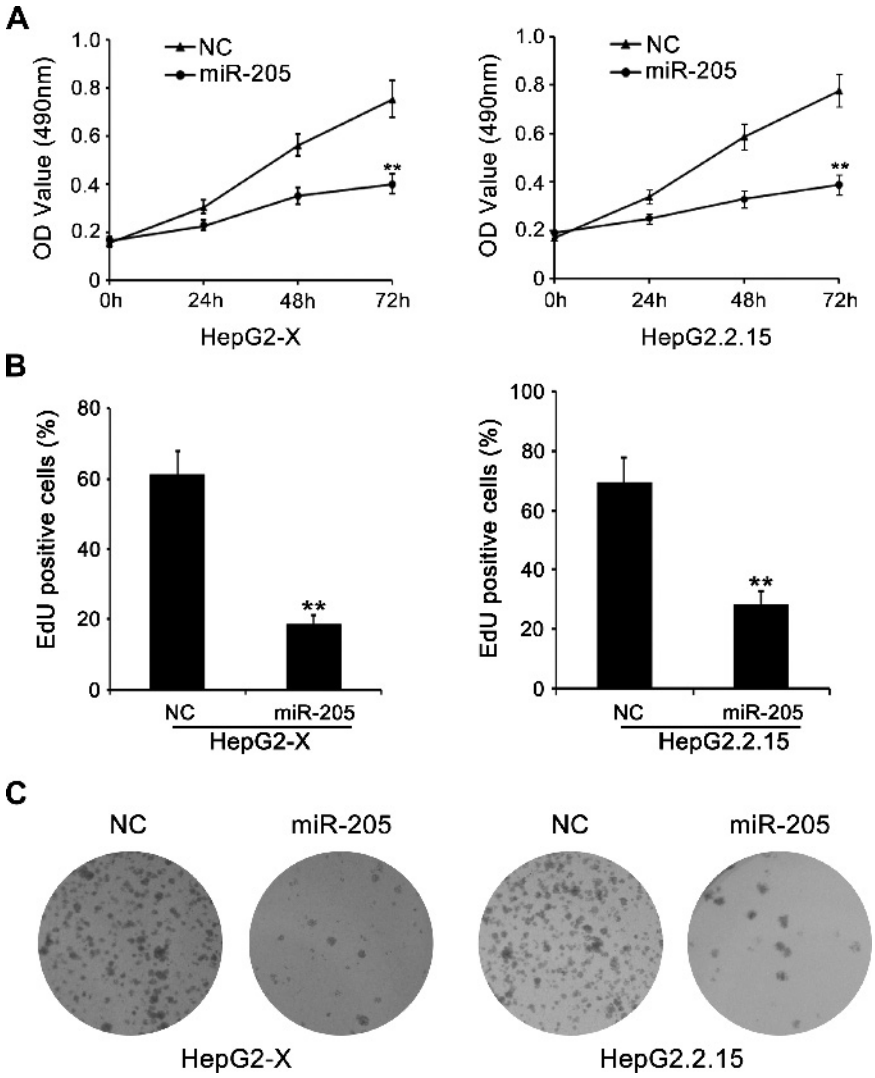

Figure 4. miR-205 inhibits the proliferation of hepatoma cells *in vitro*. (A–C) MTT assay, EdU incorporation assay, and clonogenicity analysis were carried out in the cells with transfection of 100 nM miR-205, respectively. Data are presented as means ± SD (**$P < .01$). We performed three separate experiments in triplicate.

miR-205 Is Able to Inhibit the HBx-Enhanced Proliferation of Hepatoma Cells In Vitro

Next, we are interested in the role of miR-205 in hepatocarcinogenesis mediated by HBx. We examined the effect of miR-205 on the proliferation of HBx-expressing hepatoma cells by MTT and EdU assays, respectively. Our data indicated that the overexpression of miR-205 significantly decreased the proliferation of HepG2-X (or HepG2.2.15) cells (Figure 4, *A* and *B*). Moreover, colony formation assay showed that the overexpression of miR-205 resulted in the decrease of colony numbers of HepG2-X (HepG2.2.15 or HepG2) cells (Figures 4*C* and W2*A*), suggesting that miR-205 is able to inhibit the proliferation of hepatoma cells *in vitro*.

miR-205 Is Able to Suppress the HBx-Enhanced Proliferation of Hepatoma Cells In Vivo

Furthermore, we observed that the ectopic miR-205 expression was able to strongly inhibit the proliferation of hepatoma cells (HepG2 and HepG2.2.15) in animal. Our data showed that miR-205 injection into the tumors on days 9, 12, and 15 led to a significant reduction in the size of tumor volume (Figure 5*A*). The average weight of tumor intratumorally injected with synthetic miR-205 mimics was significantly lower than that of the control injected with NC miRNA (Figures 5, *B* and *C*, and W2*B*), supporting that miR-205 is a tumor suppressor miRNA. Surprisingly, we observed that the treatment with both miR-205 and HBx was able to increase the tumor volume and average weight in mice (Figure 5, *A* and *B*). Meanwhile, the expression levels of HBx in the tumor tissues from mice were detected by immunoblot analysis, which was consistent with the different treatments (Figure 5*D*). On the basis of our findings, we conclude that miR-205 is a tumor suppressor miRNA in hepatoma cells.

HBx Promotes the Growth of Tumor through Suppressing miR-205

Our group previously reported that HBx was able to enhance the proliferation of hepatoma cells in mice [21]. Next, we tested the role of miR-205 in HBx-enhanced proliferation *in vivo*. Interestingly, our data revealed that the expression levels of miR-205 were decreased in 6- and 12-month-old HBx-transgenic mice relative to the wild-type mice (Figure 6*A*), which was consistent with the data that the expression levels of miR-205 were inversely correlated with those of HBx

in abovementioned clinical HCC tissues. Then, we found that the silencing of HBx resulted in the suppression of tumor growth in mice (Figure 6, *B–D*). As control, we found that HBx siRNA was not able to affect the growth of HepG2 cells in mice (Figure W3, *A* and *B*), suggesting that HBx siRNA has no toxicity to the cells. The efficiency of HBx RNA interference (RNAi) was shown by immunoblot analysis (Figure 6*E*). Interestingly, qRT-PCR confirmed that the expression levels of miR-205 were increased in HBx RNAi–treated tumor tissues from the mice transplanted with HepG2.2.15 cells (Figure 6*F*), suggesting that HBx RNAi releases the suppression of miR-205 mediated by HBx in the cells. In conclusion, HBx enhances the growth of hepatoma cells through suppressing miR-205.

Discussion

It has been reported that HBx plays an important role in regulating transcription, signal transduction, cell cycle progress, protein degradation pathway, apoptosis, and genetic stability by interaction with various transcription factors or components of signal transduction pathways in the development of hepatoma [35–37]. In addition, the dysregulation of miRNAs has been found in various types of human cancers including liver cancer [38–40]. However, whether HBx enhances hepatocarcinogenesis through modulation of miRNAs is poorly understood.

Growing evidence shows that the expression levels of miR-205 are downregulated in some cancers [17,18,41]. Accordingly, we proposed that the levels of miR-205 might be decreased in HCC as well. Interestingly, as expected, we observed that miR-205 expression levels were significantly decreased in 33 clinical HBV-related HCC samples compared with their adjacent noncancerous hepatic tissues. Meanwhile, we revealed that the expression levels of miR-205 were remarkably inversed to those of HBx in HCC tissues. Due to HBx sequence partially overlapped with the core sequence and completely overlapped with the pgRNA, the PCR products may contain pgRNA in Figure 1*B*. However, our data demonstrated that HBx was able to depress miR-205 and miR-205 may target HBx mRNA in the study of cell system (Figures 1 and 2), supporting that miRNA-205 is negatively associated with HBx. This finding suggests that HBx may be involved in the down-regulation of miR-205 in HCC tissues. Strikingly, we showed that the HBx was able to reduce miR-205 expression in hepatoma and liver cells. Therefore, we conclude that HBx is able to inhibit miR-205 in hepatoma and liver cells. Then, we observed that HBx was a potential target of miR-205 by target-prediction software, which was consistent with previous prediction by Wu et al. [42]. However, Wu et al. did not provide any experimental evidence to support this prediction. In this study, our data validated that miR-205 directly targeted HBx mRNA by experiment.

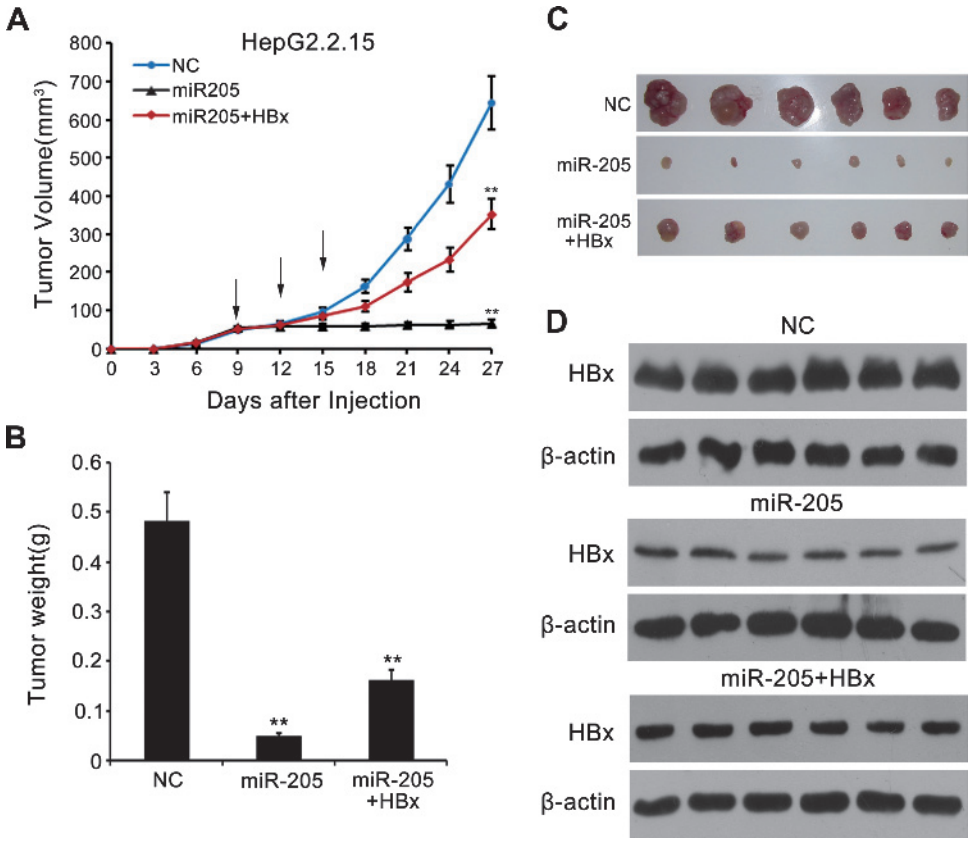

Figure 5. miR-205 inhibits the proliferation of hepatoma cells *in vivo*. (A) The nude mice ($n = 6$) were transplanted by HepG2.2.15 cells. The tumors were injected with 5 μg (100-μl total volume) of miRNA NC, miR-205, or miR-205/HBx plasmid (pCMV-X) on days 9, 12, and 15, respectively. The growth curves of the tumors in nude mice were shown. (B) The diagram showed the average weight of tumors. (C) The photos of tumors from mice transplanted with HepG2.2.15 cells were shown. (D) The expression levels of HBx were examined by Western blot analysis in the tumor tissues from mice. Data are presented as means ± SD (**$P < .01$). We performed three separate experiments in triplicate.

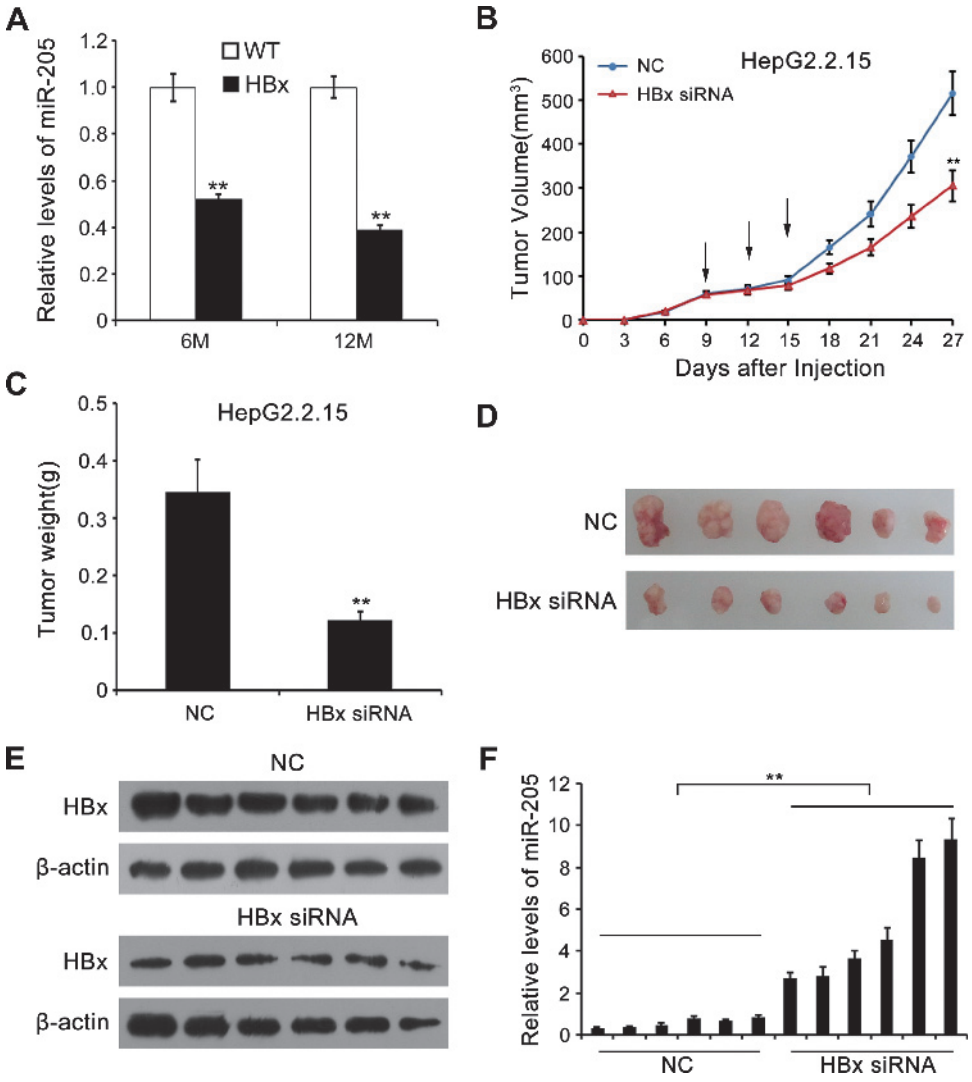

Figure 6. HBx promotes the growth of tumor through suppressing miR-205 *in vivo*. (A) miR-205 expression was examined by qRT-PCR in 6-and 12-month-old HBx-transgenic mice. (B) The nude mice ($n = 6$) were transplanted by HepG2.2.15 cells. The tumors were injected with siRNA control (NC) and HBx siRNA (5 μg/100 μl) on days 9, 12, and 15, respectively. The growth curves of the tumors in nude mice were shown. (C) The diagram showed the average weight of tumors. (D) The photos of tumors from mice were shown. (E and F) The expression levels of HBx and miR-205 were examined by Western blot analysis and qRT-PCR in the tumor tissues from mice, respectively. Data are presented as means ± SD (**$P < .01$). We performed three separate experiments in triplicate.

Moreover, we showed that miR-205 was able to inhibit the expression of HBx at the levels of mRNA and protein in hepatoma cells.

Accordingly, we puzzled over why miR-205 could not disrupt HBx in the development of HCC. We analyzed that the suppression of miR-205 by HBx should overcome the inhibition of HBx by miR-205 in the event. This intrigued us to further explore the mechanism by which HBx suppressed miR-205 in the cells. It has been reported that the expressions of miRNAs can be modulated by the epigenetic mechanisms, such as DNA methylation and histone modifications [16]. Then, we analyzed the promoter regions of miR-205 and identified a CpG island region within the promoter. We found that HBx was able to reduce miR-205 expression through inducing hypermethylation of miR-205 promoter. In function, it was observed that the expression levels of miR-205 were decreased in the liver tissues of HBx-transgenic mice (6 and 12 months old). Thus, it strongly supports that HBx enhances the hepatocarcinogenesis through suppressing miR-205. Indeed, we demonstrated that HBx contributed to the proliferation of hepatoma cells through inhibiting miR-205 *in vivo*. Therefore, we conclude that miR-205 is a tumor suppressor in hepatoma cells.

Taken together, we conclude that miR-205 acts as a tumor suppressor in HCC tissues. HBx is able to suppress miR-205 through inducing hypermethylation of miR-205 promoter. Importantly, HBx abrogates the tumor-suppressive effect of miR-205 during their interaction, resulting in the enhancement of hepatoma cell growth. Our finding provides new insights into the mechanism of HBx-induced hepatocarcinogenesis. Therapeutically, HBx may serve as a target in HBV-related HCC.

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Table W1. List of Primers Used in This Paper.

Gene	Primer	Sequence (5′-3′)
Primers for miR-205 promoter and HBx		
pGL3-1428	Forward	ACTTCAACTTCCCTTGCCCTC
	Reverse	CCTGTGCGGAACAGAAATGAC
pGL3-679	Forward	GTGGGTTCTGAGAGGTGGGAC
	Reverse	CCTGTGCGGAACAGAAATGAC
pGL3-763	Forward	ACTTCAACTTCCCTTGCCCTC
	Reverse	CTCTCAGAACCCACACCCCTG
pGL3-473	Forward	CTTCTCACAAGGGGAGCAGCA
	Reverse	CTCTCAGAACCCACACCCCTG
pGL3-301	Forward	CTTCTCACAAGGGGAGCAGCA
	Reverse	CCAGAGACCTACCTAATCGGGG
pGL3-HBx	Forward	ATGGCTGCTAGGGTGTGCT
	Reverse	CTAGGCAGAGGTGAAAAAG
pGL3-HBx-mut	Forward	AAGACTGGGAGGAGTACGGCCTGGAGATTAGG
	Reverse	CCTAATCTCCAGGCCGTACTCCTCCCAGTCTT
Primers for qRT-PCR		
E2F1	Forward	ACTGAATCTGACCACCAAGCG
	Reverse	CAGGGTCTGCAATGCTACGAA
HBx	Forward	ATGGCTGCTAGGGTGTGCT
	Reverse	TAAATCTCCTCCCCCAACTC
GAPDH	Forward	CATCACCATCTTCCAGGAGCG
	Reverse	TGACCTTGCCCACAGCCTTG
miR-205	Forward	TCCTTCATTCCACCGGAGTCTG
	Reverse	GCGAGCACAGAATTAATACGAC
U6	Forward	AGAGCCTGTGGTGTCCG
	Reverse	CATCTTCAAAGCACTTCCCT
Primers for MSP		
Methylation	Forward	AGGATAAGTTTTTGGTTGGGTTTC
	Reverse	TAATCCTCCCAAAAACCTACCTAATCG
Unmethylation	Forward	TTAGGATAAGTTTTTGGTTGGGTTTT
	Reverse	TAATCCTCCCAAAAACCTACCTAATCAAA
Bisulfite-sequencing analysis	Forward	TTAAATGTTAGGATAAGTTTTTGGTTG
	Reverse	AACCTTACACCTAAAACCTTAATCCT
siRNA duplexes		
HBx siRNA	Sense	AAGAGGACUCUUGGACUCUCAdTdT
	Antisense	UGAGAGUCCAAGAGUCCUCUUdTdT
control siRNA	Sense	UUCUCCGAACGUGUCACGUdTdT
	Antisense	ACGUGACACGUUCGGAGAAdTdT

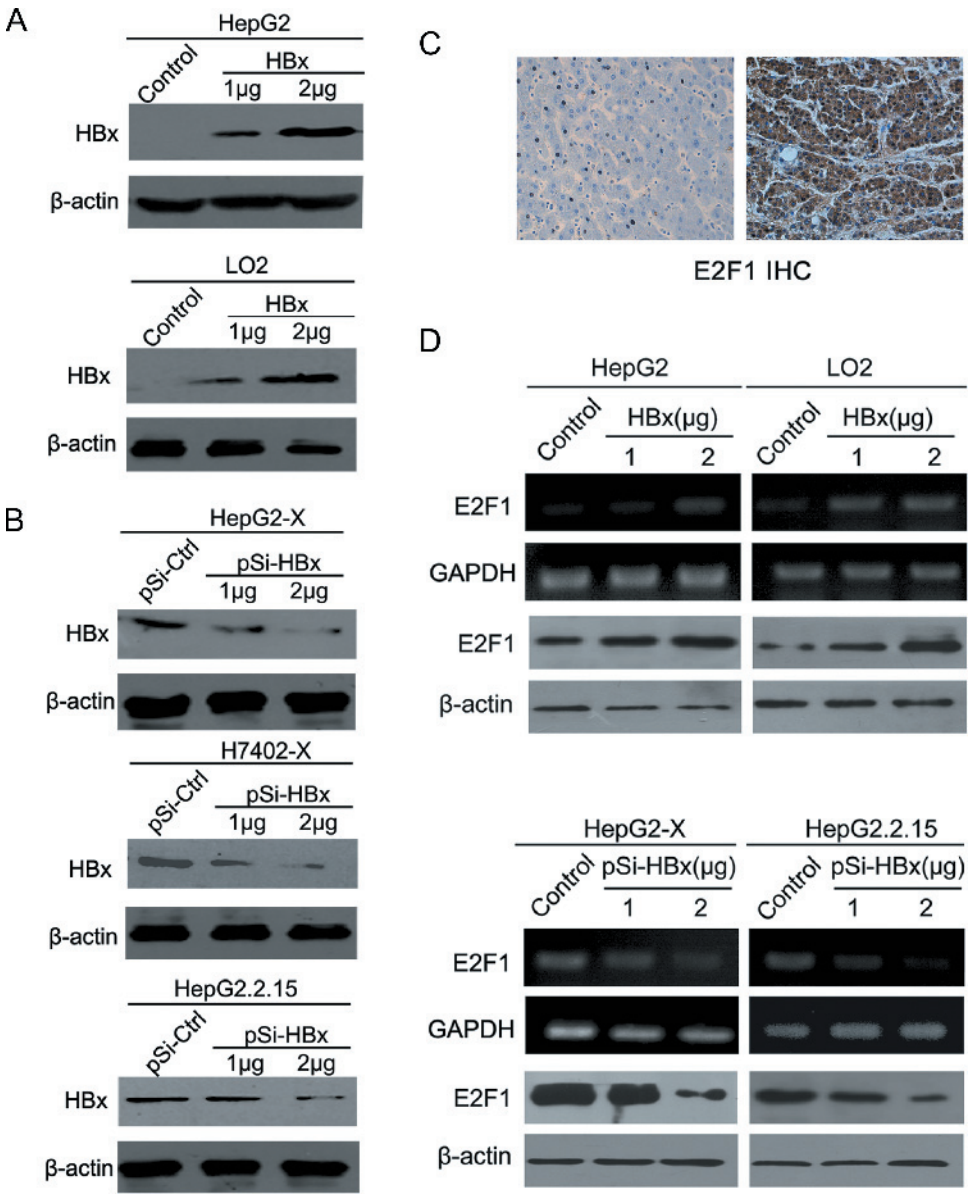

Figure W1. HBx is able to upregulate E2F1 in hepatoma and liver cells. (A and B) The expression levels of HBx were examined in the cells by Western blot analysis after transfection with pCMV-X (or pSi-HBx). (C) The expression of E2F1 was examined by immunohistochemistry in normal human liver (left) and clinical HCC (right) tissues. (D) The expression of E2F1 was examined in the cells by qRT-PCR and Western blot analysis after transfection with HBx (or pSi-HBx). We performed three separate experiments in triplicate.

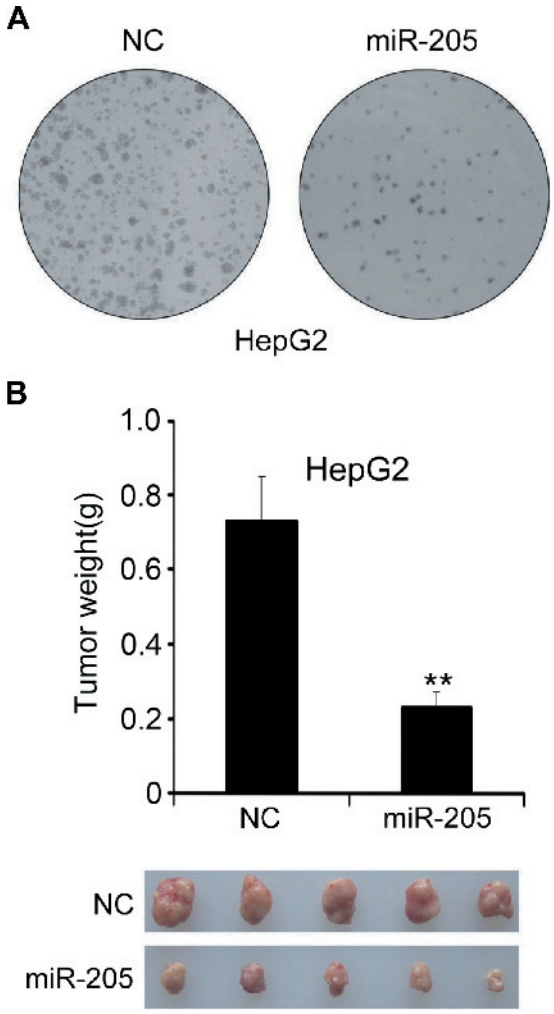

Figure W2. miR-205 inhibits the proliferation of hepatoma cells *in vitro* and *in vivo*. (A) The clonogenicity analysis was carried out in HepG2 cells transfected with miR-205 (100 nM). (B) The nude mice ($n = 5$) were transplanted with HepG2 cells. The tumors were injected with miRNA NC or miR-205 on days 9, 12, and 15, respectively. The average weight and photos of tumors were shown. Data are presented as means ± SD (**$P < .01$).

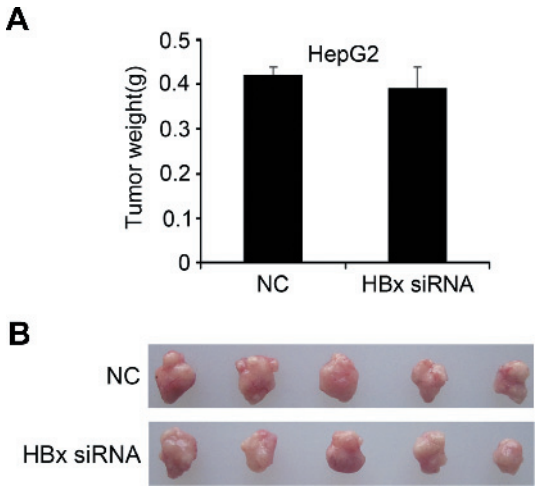

Figure W3. The treatment with HBx siRNA does not affect the proliferation of HepG2 hepatoma cells *in vivo*. (A) The nude mice ($n = 5$) were transplanted with HepG2 cells. The tumors were injected with siRNA NC or HBx siRNA on days 9, 12, and 15, respectively. The diagram showed the average weight of tumors. (B) The photos of tumors from mice transplanted with HepG2 cells were shown.