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# Does the Hepatitis B Antigen HBx Promote the Appearance of Liver Cancer Stem Cells?

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## Abstract

Hepatitis B virus (HBV) is a major etiologic agent of chronic liver disease and hepatocellular carcinoma (HCC). HBV-encoded X antigen, HBx, and pathways implicated in the self-renewal of stem cells contribute to HCC, but it is not clear whether HBx expression promotes "stemness." Thus, experiments were designed to test the hypothesis that HBx triggers malignant transformation by promoting properties that are characteristic of cancer stem cells (CSC). To test this hypothesis, HepG2 cells were stably transduced with HBx and then assayed for phenotypic and molecular characteristics of "stemness." The relationship between HBx and "stemness"-associated markers was also evaluated by immunohistochemical staining of liver and tumor tissue sections from HBV-infected patients. The results showed that Oct-4, Nanog, Klf-4,  $\beta$ -catenin, and epithelial cell adhesion molecule (EpCAM) were activated by HBx *in vitro* and *in vivo*. EpCAM was detected in the nuclei of human HCC cells from infected patients. HBx promotes "stemness" by activating  $\beta$ -catenin and epigenetic upregulation of miR-181, both of which target EpCAM. HBx expression was also associated with depressed levels of E-cadherin. Moreover, HBx stimulated cell migration, growth in soft agar, and spheroid formation. This work is the first to propose that HBV promotes "stemness" in the pathogenesis of HCC. HBx-associated upregulated expression of multiple "stemness" markers supports the hypothesis that HBx contributes to hepatocarcinogenesis, at least in part, by promoting changes in gene expression that are characteristics of CSCs. *Cancer Res*; 71(10); 3701–8. ©2011 AACR.

## Introduction

Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) is among the top 5 most frequent cancers worldwide (1). About 250,000 new cases of HCC are diagnosed yearly, and most people die within a year of diagnosis. The mechanism of HBV-mediated HCC is not clear. However, HBV DNA is integrated, often highly rearranged, within host DNA in tumors and tumor-derived cell lines (2). These templates frequently produce HBx (3) that is active in transactivation assays (4). Sustained production of HBx is associated with hepatocellular transformation and represents a major contribution of HBV to HCC (5). HBx RNA and protein are highly

expressed in chronically infected livers and strongly correlate with chronic liver disease (CLD; ref. 6). HBx has transcriptional transregulatory properties that alter patterns of host gene expression (2, 6), inhibit proteasomal degradation of growth regulatory proteins (7), stimulate cellular kinases that alter signal transduction (5), and utilize epigenetic machinery (8). This suggests that HBx-mediated tumor development involves epigenetic mechanisms that become operative during CLD.

Microarray analysis of human HCC specimens, cancer cell lines, and transgenic models established the molecular similarities between cancer stem cells (CSC) and hepatic stem cells (HSC) and highlighted the importance of CSCs in the prognosis of liver cancer (9). "Stemness" transcription factors (e.g., Oct-4, Klf-4, and Nanog) reexpressed in cancer cells (10), and their reactivation contributes to tumorigenesis in somatic tissues (11). Epithelial cell adhesion molecule (EpCAM) is a marker of HSCs and CSCs (12) and acts as a mitogenic signal transducer via proteolysis and nuclear translocation of its intracellular domain, EpICD. It binds to DNA in a complex with the scaffolding protein FHL2,  $\beta$ -catenin, and lymphoid enhancer factor (LEF)-1 and regulates cell proliferation (13). EpCAM sustains "stemness" through EpICD binding to *c-Myc*, *Oct-4*, *Nanog*, and *Klf-4* promoters (14). The *EpCAM* promoter has T-cell factor (TCF)-binding sites and is a transcriptional target of  $\beta$ -catenin (15). It was also regulated by microRNA-181 (miR-181). Forced expression of miR-181 endowed EpCAM-positive HCC cells with stem cell properties, whereas

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miR-181 suppression induced differentiation (16).  $\beta$ -Catenin, like EpCAM, is important in stem cell self-renewal and in carcinogenesis (17), in part, by binding the promoters of *Oct-4* and *Nanog* (18). Aberrant activation of their self-renewal program by  $\beta$ -catenin may contribute to the appearance of CSCs (19). Importantly, HBx also transcriptionally activates  $\beta$ -catenin in up to 80% of HCCs (20). This underscores the potentially close relationship between HBx, activated  $\beta$ -catenin, and stem cell renewal.

$\beta$ -Catenin acts as a transcriptional activator in the nucleus but promotes cell adhesion when bound to E-cadherin (21), which provides a physical link between adjacent cells and is crucial for cell polarity and the structural integrity of tissue (22). Altered expression and cellular distribution of E-cadherin is frequently associated with invasiveness in human cancers including HCC (23). In this context, HBx suppresses E-cadherin expression by promoting methylation of the *E-cadherin* promoter (24). This event results in the stabilization of  $\beta$ -catenin through tyrosine phosphorylation mediated by Src, which is also activated by HBx (25). Collectively, these findings suggest that HBx promotes the development of HCC by triggering the expression of "stemness" factors in the chronically infected liver. The results of the present study support this hypothesis.

## Materials and Methods

### Cell culture

The human hepatoblastoma cell line HepG2 was stably transfected with *HBx* (HepG2X) or the control bacterial chloramphenicol acetyltransferase (*CAT*; HepG2CAT) genes as previously described (26). Cells were cultured in minimum essential medium (Invitrogen) supplemented with 100 mmol/L nonessential amino acids, 100 mmol/L sodium pyruvate, and 10% FBS (Invitrogen) at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### Patient samples

Forty-three formalin-fixed, paraffin-embedded paired tumor (HCC)/nontumor (adjacent liver) tissues were obtained from Chinese patients who underwent surgery at the Third Military Medical University, Chongqing, China. All patients were positive for hepatitis B surface antigen (HBsAg); 41 were men, the age range was from 35 to 69 years (average age = 48 years), and all were of Chinese ethnicity (Supplementary Table S1). Additional snap-frozen tumor/nontumor pairs from 20 HBV-infected patients were obtained from Queen Mary College at the University, Hong Kong, China. All patients were also positive for HBsAg; 18 were men, and their ages ranged from 33 to 71 years (average age = 50.5 years; Supplementary Table S2). All samples were used for diagnostic purposes and then used for this study. Ten uninfected human liver tissues (Abcam) were used as controls. The use of samples was approved by the Institutional Review Boards at all participating universities.

### Spheroid assay

Single-cell suspensions of HepG2X and HepG2CAT cells were plated at a density of  $1 \times 10^3$  cells in 2 mL of medium in

6-well Ultra-Low Attachment Microplates (Corning) and maintained for up to 16 days. Spheroids were observed and counted using an ECLIPSE Ti inverted microscope operated with a Nikon DS-Fi1 camera and NIS Elements software (Nikon).

### Soft agar assay

Single-cell suspensions of  $1 \times 10^4$  cells were mixed with 0.3% agar (Sigma) in complete growth medium and seeded in triplicates into 6-well plates coated with 0.5% hardened agar. Plates were incubated at 37°C for 28 days. Colonies 1 mm or more in diameter were counted under code by light microscopy.

### Cell migration assay

Single-cell suspensions of  $1.5 \times 10^5$  cells were plated in triplicates into 6-well BD BioCoat Matrigel Invasion Chambers (BD) according to enclosed instructions. Cell migration was observed after 24 hours by hematoxylin and eosin (H&E) staining.

### Tumorigenicity in nude mice

For tumorigenicity studies, 2 groups of 10 6-week-old nude mice (Charles River Laboratories) were injected subcutaneously at a single site with  $6 \times 10^6$  HepG2X or HepG2CAT cells. Tumor onset was scored visually and by palpitation independently by 2 trained laboratory personnel. Tumor sizes were determined by wet weight at the time of euthanasia (6 weeks). These experiments were approved by the Institutional Animal Care and Use Committee at Temple University.

### Protein extraction and Western blotting

Cells were lysed in cell lysis buffer with protease inhibitor cocktail (Cell Signaling). Nuclear extracts were prepared using Nuclear Extract Kit (Active Motif) according to enclosed instructions. Protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated overnight in 5% nonfat milk in Tris-buffered saline/0.1% Tween-20 with primary antibodies against HBx, Oct-4, E-cadherin,  $\beta$ -catenin, lamin A, and  $\beta$ -actin (Santa Cruz Biotechnology), Nanog (Cell Signaling), Klf-4 and EpCAM (Abcam), and Ac-Lys H3 (Active Motif). The blots were developed using the ECL Plus Detection Kit (Amersham) and exposed to Kodak imaging films (Kodak BioMax). Images were processed using ImageJ software (NIH).

### Treatment of cells with trichostatin A

Suspensions of  $3.5 \times 10^5$  cells were plated in 35-mm culture dishes with or without 350 nmol/L trichostatin A (TSA; Sigma) in 2 mL of medium. Extracts were collected at 12 and 24 hours and analyzed by Western blotting.

### PowerBlot proteomics analysis

PowerBlot analysis of HepG2X and HepG2CAT cell lysates was done by BD Transduction Labs. Briefly, 400  $\mu$ g of cell lysates was analyzed by SDS-PAGE and transferred to Immobilon P (Millipore). The membranes were blocked with 5% nonfat milk. Each membrane was cut into vertical strips, and

each strip was incubated with a complex antibody cocktail. Signals were developed by chemiluminescence and measured using PDQuest 2-D analysis software (Bio-Rad). For each of 3 experiments, 3 gels were run by using HepG2X and HepG2CAT cell lysates.

### MicroRNA analysis

Small RNAs were isolated from HepG2X and HepG2CAT cells and 20 pairs of HCC tissue samples by using Ambion mirVana miRNA isolation kit according to enclosed instructions. Microarray analysis was carried out with miRNAs isolated from HepG2X and HepG2CAT cells by LC Sciences. Differentially expressed miRNAs in cells were identified by Cy3(HepG2X)/Cy5(HepG2CAT) ratio, and those with a value of  $P < 0.01$  were considered for further characterization. Differential expression of selected miRNAs in cells and tissues was validated by (quantitative) real-time PCR [(q)RT-PCR] by using a Smart Cycler system. The  $\Delta\Delta Ct$  calculation was done as follows:  $\Delta\Delta Ct = \Delta Ct$  of miR-181a in tumor –  $\Delta Ct$  of miR-181a in nontumor tissue. Briefly, the samples with 25 ng small RNA were first denatured at 95°C for 3 minutes and then run for 40 cycles (95°C for 15 seconds and 60°C for 30 seconds). U6 was used for normalization.

### Immunohistochemistry

Tissue sections were deparaffinized, dehydrated, treated with Uni-TRIEVE antigen retrieval (Innovex), and stained using the UltraVision Detection System (Thermo Scientific) according to enclosed instructions. Antibodies used for staining were the same as those described earlier for Western blotting except for anti-HBx (anti-99 custom-made antibody; ref. 27) and anti-EpCAM (Millipore). Normal mouse or rabbit IgG (Vector Labs) were used to rule out false-positive responses. Preabsorption of primary antibodies with corresponding antigens was done on tissue sections to ensure specificity.

### Statistics

Statistical values for cell migration, growth in soft agar, and tumorigenesis were defined using an unpaired Student's  $t$  test, in which  $P < 0.05$  was considered significant. The relationship between HBx and EpCAM,  $\beta$ -catenin, and E-cadherin obtained by immunohistochemistry was determined using  $2 \times 2$  comparisons in the  $\chi^2$  test. Statistical significance was considered when  $P < 0.05$ .

## Results

### Relationship between HBx and "stemness"-associated markers

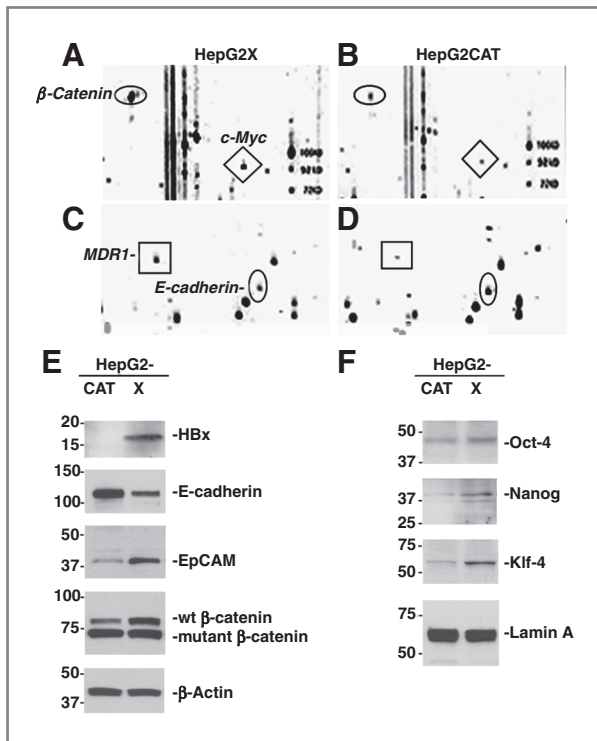
PowerBlot analysis was conducted using whole-cell lysates from HepG2X and HepG2CAT cultures. The results showed  $4.5 \pm 0.5$ -fold upregulated expression of  $\beta$ -catenin in HepG2X compared with control cells (Fig. 1A and B). Several  $\beta$ -catenin target genes were also upregulated, including MDRI ( $7.8 \pm 1.8$ -fold; Fig. 1C and D) and c-Myc ( $4.7 \pm 0.7$ -fold; Fig. 1A and B). HepG2X cells expressed  $6.7 \pm 1.2$ -fold lower levels of E-cadherin (Fig. 1C and D) than HepG2CAT cells. Selected markers were then further characterized by Western blotting

(Fig. 1E and F). In whole-cell extracts, HBx was associated with downregulation of E-cadherin (3.8-fold), and upregulation of  $\beta$ -catenin (2.5-fold) and EpCAM (3-fold). EpCAM was also upregulated in HBx-expressing Hep3B and Huh7 cells (Supplementary Fig. S1). In HepG2 cells,  $\beta$ -catenin is present as wild-type (top band) and truncated mutant (bottom band; Fig. 1E) due to partial exon 3 deletion (28). HBx upregulated wild-type  $\beta$ -catenin without modifying the levels of truncated  $\beta$ -catenin (Fig. 1E). Previous work confirmed that nuclear  $\beta$ -catenin in HBx-positive HCC is wild type by DNA sequence analysis of  $\beta$ -catenin exons obtained from paraffin blocks (29). HBx-associated upregulation of  $\beta$ -catenin has been shown in Huh7 (30, 31) and Hep3B cells (20, 31). Downregulation of E-cadherin in Hep3B cells has also been shown previously (24). Moreover, HBx-mediated repression of E-cadherin was associated with activation of  $\beta$ -catenin in Hep3B cells (24). In nuclear extracts, HBx was associated with upregulation of Oct-4 (1.6-fold), Nanog (3.4-fold), and Klf-4 (3.5-fold; Fig. 1F). These data suggest that HBx is associated with the reactivation of "stemness" transcription factors Oct-4, Nanog, and Klf-4, and "stemness"-associated EpCAM and  $\beta$ -catenin.

### Relationship between HBx, "stemness"-associated markers, and E-cadherin *in vivo*

Additional experiments were conducted to determine whether the results of PowerBlot analyses and Western blotting could be validated *in vivo*. Thus, clinical samples containing HCC and nontumor liver were stained for HBx, Oct-4, Nanog,  $\beta$ -catenin, EpCAM, and E-cadherin (Supplementary Table S1). Among 43 patients who underwent surgical resection for HBV-associated HCC, 31 had both tumor and adjacent nontumor liver, 9 had only tumor in their blocks, and 3 patients had only nontumor liver. Among these, HBx staining was observed in 26 of 40 tumors (65%) and in all 34 nontumor livers (100%). In 82% of cases with tumor and nontumor tissues, HBx staining was stronger and more widespread in liver than in tumor, as previously reported (32). Ten commercially available liver sections from uninfected individuals were uniformly negative for HBx. HBx staining was cytoplasmic in all cases (Supplementary Fig. S2), and 1 case showed nuclear staining in nontumor liver (data not shown). EpCAM was present in 26 cases in the tumor compartment (66%). Membranous EpCAM staining (Fig. 2A) was observed in the nontumor liver among 21 patients (60%; Fig. 2C), even though EpCAM is not expressed in uninfected, healthy liver (12). In tumors from 3 patients, nuclear EpCAM was also detected (Fig. 2B). With regard to  $\beta$ -catenin, staining was observed in the tumor compartment from 28 patients (74%) and in the nontumor compartment from 19 patients (61%). Although membranous  $\beta$ -catenin is characteristic of normal, uninfected liver, all cases of tumor and nontumor tissues showed both membranous and cytoplasmic staining for  $\beta$ -catenin, consistent with  $\beta$ -catenin activation, as previously shown (29).  $\beta$ -Catenin was also observed in the nuclei of 2 tumors (data not shown). In contrast, E-cadherin staining, which was exclusively membranous, was detected in only 7 tumors (18%) but was present in 20 cases with nontumor liver (74%). These results are similar to previously published results





**Figure 1.** PowerBlot analysis of HepG2X cells (A and C) and HepG2CAT cells (B and D). Selected differentially expressed genes are highlighted. Representative Western blots with HepG2X and HepG2CAT cells by using total (50 µg; E) or nuclear (100 µg; F) extracts.  $\beta$ -Actin and lamin A are loading controls. wt, wild-type.

(24). When staining was done for Nanog and Oct-4, 6 and 2 tumor samples were positive in scattered nuclei, respectively (Fig. 2D and E). Hence, "stemness" and "stemness"-like markers were observed in HBV-associated HCC.

Additional analyses were conducted to determine the relationship between HBx and these markers. In the tumor compartment, costaining between HBx and EpCAM was seen in only 16 cases (Fig. 3;  $\chi^2 = 2.03$ ;  $P > 0.1$ ), between HBx and  $\beta$ -catenin in 19 cases ( $\chi^2 = 0.23$ ;  $P > 0.5$ ) and between HBx and E-cadherin in 6 cases ( $\chi^2 = 0.88$ ;  $P > 0.3$ ). Hence, there seems to be no correlation between HBx and these markers in tumors. In contrast, in the nontumor compartment, where HBx is strongly expressed, costaining between HBx and EpCAM was seen in 21 cases ( $\chi^2 = 11.8$ ;  $P < 0.001$ ) and between HBx and  $\beta$ -catenin in 19 cases ( $\chi^2 = 5.88$ ;  $P < 0.02$ ), whereas an inverse correlation was seen between HBx and E-cadherin in 20 cases ( $\chi^2 = 6.04$ ;  $P < 0.02$ ), suggesting a strong correlation between HBx and these markers. Other blocks of chronically infected liver from patients without HCC were also evaluated, but the results obtained were the same as those from in adjacent liver tissues from HCC patients (data not shown).

Analysis of relationship between  $\beta$ -catenin and EpCAM staining and HCC grade showed an inverse correlation between EpCAM staining and HCC in grade 1 ( $\chi^2 = 10.2257$ ;  $P < 0.005$ ) and grade 2 ( $\chi^2 = 5.3831$ ;  $P < 0.025$ ) but not in grade 3 ( $\chi^2 = 1.3$ ;  $P > 0.05$ ) and grade 4 ( $\chi^2 = 1.06$ ;  $P > 0.3$ ). There was no correlation between  $\beta$ -catenin staining and tumor grade.

Experiments with a larger number of samples should be conducted to address these relationships more thoroughly.

### HBx upregulates miR-181a

MicroRNA(s) are known to contribute importantly to the pathogenesis of many tumor types (33). When miRNA microarray analysis was done on HepG2X and HepG2CAT cells, HBx was associated with upregulation of miR-181a (2-fold) and miR-181b (1.5-fold; data not shown). (q)RT-PCR was done to validate the microarray data in 20 paired HCC/nontumor cases (Supplementary Table S2). When these tissues were evaluated for HBx by staining and miR-181a by (q)RT-PCR, 11 of 15 HBx-positive nontumor liver samples had elevated miR-181a levels (73%;  $P < 0.01$ ; Fig. 4). Among the corresponding tumors, 5 were HBx positive but only 1 had higher levels of miR-181a than nontumor. These results suggest that miR-181a is an epigenetic target of HBx both in cell lines and in liver. This suggests that EpCAM may be upregulated by HBx targeting both  $\beta$ -catenin and miR-181.

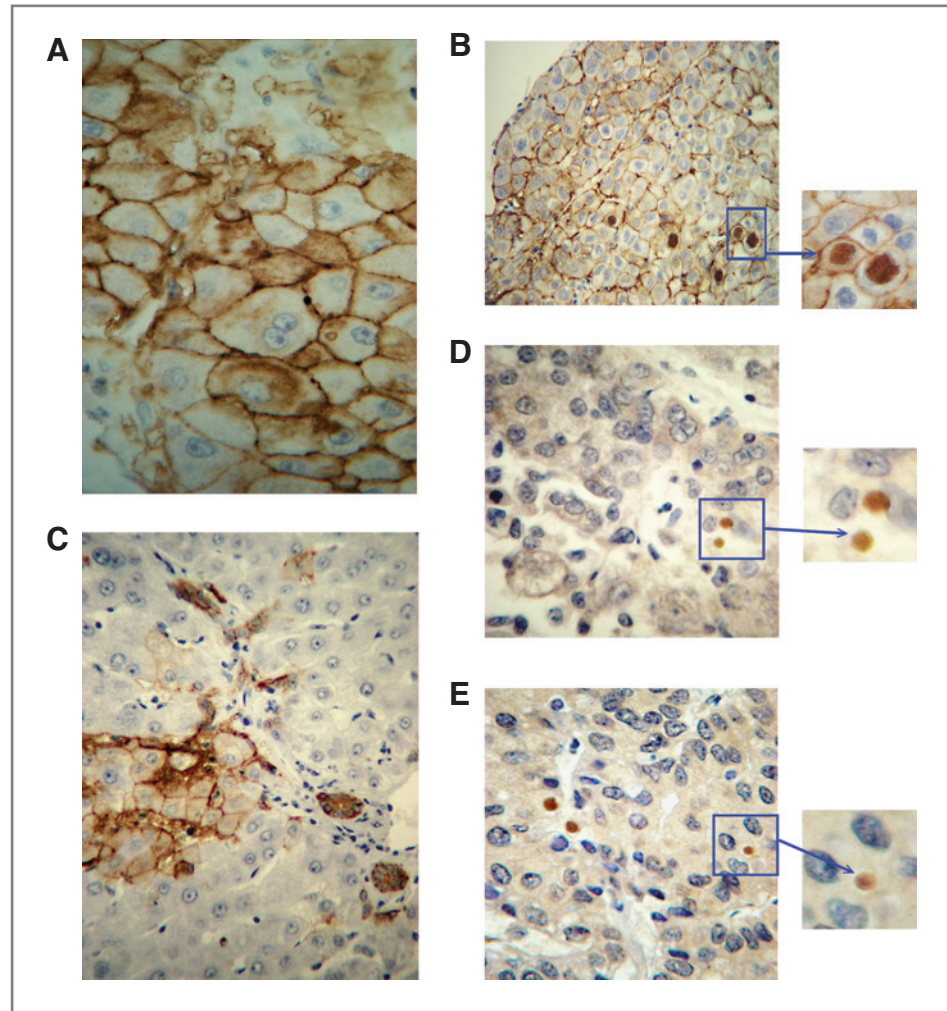
### Phenotypic characteristics of HBx-associated "stemness"

HepG2X and HepG2CAT cells were tested for their ability to form spheres in 3-dimensional culture systems (clonogenic potential), induce tumors in nude mice, and promote cell migration. It has been shown that stem/progenitor cells and CSCs can form spheroids *in vitro*, suggesting the presence of self-renewing cells (34, 35). In this work, HBx-expressing cells formed spheroids by day 3 after inoculation. About 4-fold more spheroids were derived from HepG2X than HepG2CAT cells after 16 days in culture. Some of these spheroids exceeded 100  $\mu$ m in diameter (Fig. 5A and C). In contrast, HepG2CAT cells formed mostly disorganized clusters that became adherent, with less floating spheroids (Fig. 5B). Primary spheroids enzymatically dissociated to single cells that gave rise to secondary spheroids. When anchorage-independent growth was evaluated after 28 days of culture, HepG2X cells formed  $81 \pm 20.5$  colonies whereas HepG2CAT cells produced  $37 \pm 4.9$  colonies ( $P < 0.02$ ; Fig. 5D). Cell migration was then examined by Matrigel invasion assay and H&E staining after 24 hours. About 3.5-fold more HepG2X than HepG2CAT cells migrated through Matrigel basement membrane matrix ( $P < 0.001$ ; Fig. 5E). In tumorigenicity studies, tumor onset was 30 days postinjection for HepG2X cells compared with 46 days for HepG2CAT cells ( $P < 0.01$ ). HepG2X also yielded larger tumors ( $1.6 \pm 0.3$  cm<sup>3</sup>) than HepG2CAT cells ( $0.7 \pm 0.4$  cm<sup>3</sup>;  $P < 0.01$ ). Thus, HepG2X cells showed more pronounced phenotypes consistent with stem cell behavior than HepG2CAT control cells. Hence, these phenotypic characteristics presented were consistent with the induction of "stemness" markers by HBx.

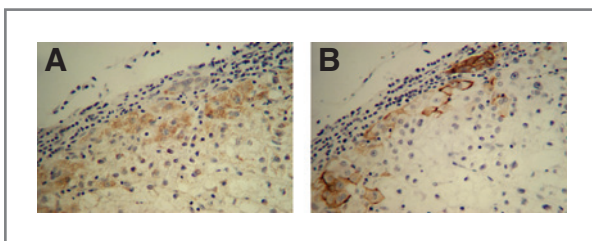
### Treatment of cells with TSA

The finding that HBx binds to histone deacetylase (HDAC1; ref. 8) and that HDAC epigenetically regulates the expression of E-cadherin (8, 24) suggests that inhibition of HDAC1 would reverse the effects of HBx on E-cadherin. To test this hypothesis, HepG2X and HepG2CAT cells were treated with the

**Figure 2.** Staining for EpCAM in tumor (A; 400 $\times$ ), tumor (B; 200 $\times$ ), and nontumor (C; 200 $\times$ ). All panels show membranous staining, and C also shows mixed membranous and nuclear localization. Detection of (D) *Oct-4* and (E) *Nanog* in human HCC tissue sections from HBV carriers (200 $\times$ ).



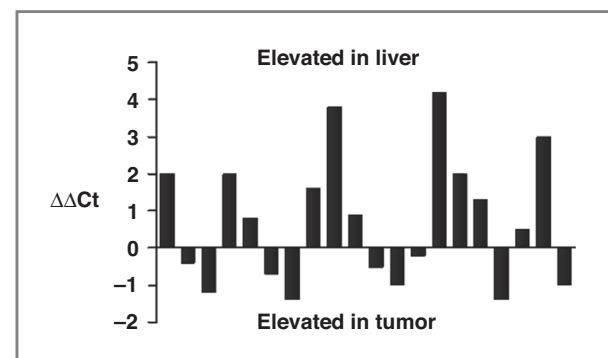
HDAC1 inhibitor TSA and the levels of E-cadherin evaluated over time. TSA treatment resulted in a time-dependent increase in H3 acetylation by 20-fold (Fig. 6). This was accompanied by increased levels of E-cadherin in HepG2X cells (7-fold) and decreased levels of wild-type  $\beta$ -catenin (5-fold) and EpCAM (9.5-fold), with little change in HepG2CAT cells. These results show that once HBx repressed E-cadherin expression is reversed by TSA, alterations in  $\beta$ -catenin and EpCAM are also observed, suggesting these events are linked.



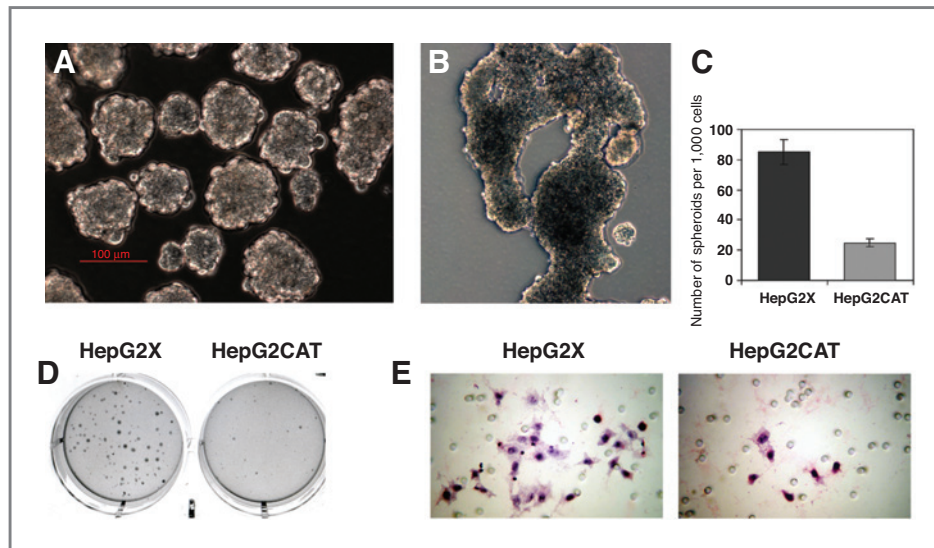
**Figure 3.** Staining for HBx (A) and EpCAM (B) in consecutive HCC sections from a representative patient (200 $\times$ ).

## Discussion

In this study, the pluripotent stem cell transcription factors *Oct-4*, *Nanog*, and *Klf-4*, and the "stemness"-associated markers



**Figure 4.** Expression of miR-181a in tumor (HCC) and nontumor liver. Negative values mean that the miRNA was expressed more in tumor than in nontumor from the same patient. Positive values mean that the miRNA was expressed more in nontumor than in tumor.



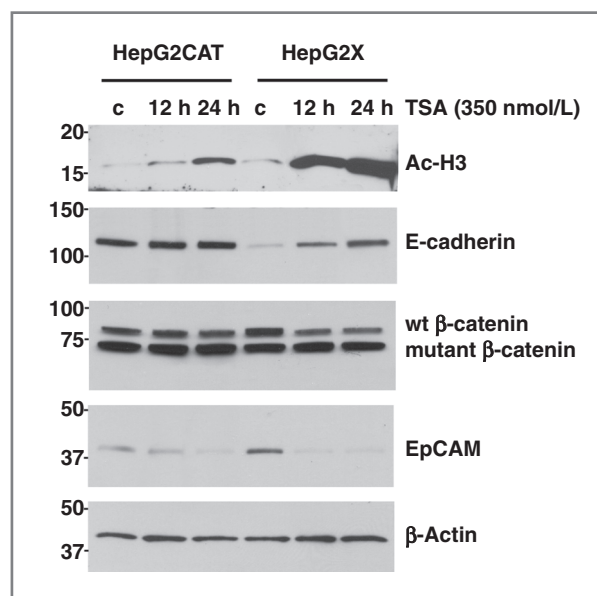
**Figure 5.** Representative images of spheroids derived from HepG2X cells (A), HepG2CAT cells (B), and relative numbers of spheroids (C) for each culture. Data are shown as mean  $\pm$  SD. D, soft agar assay. E, migration through Matrigel basement membrane. All experiments were done in triplicate.

EpCAM and  $\beta$ -catenin, were upregulated in HBx-expressing cells (Fig. 1). These observations were validated in tumor and adjacent nontumor liver from HBV-infected patients (Figs. 2 and 3). HBx-expressing cells also showed enhanced colony-forming capability and sphere-forming activity *in vitro* (Fig. 5) and were more tumorigenic *in vivo*. miRNA array analysis showed that miR-181, which targets EpCAM (16), was upregulated in HepG2X cells, and this was validated *in vivo* (Fig. 4). The tight epidemiologic association between chronic HBV infection and the development of HCC (36), combined with evidence that HBx and CSCs contribute importantly to tumor development, suggest that HBV may promote hepatocarcino-

genesis, in part, by triggering "stemness." This may be mediated by HBx-associated transcriptional *trans*-regulation and altered epigenetic regulation of host gene expression (8, 37) and activation of cellular kinases that alter signal transduction (5, 36). Together these data suggest that HBx promoted characteristics of CSCs in liver cells.

Although chronic infection often extends for decades, it is proposed that HBx promotes "stemness" most readily in liver just prior to the appearance of HCC. This is because the levels of HBx expression in the liver increase with the length of time a carrier is infected, with the highest levels of HBx expression seen in the cirrhotic liver (2, 38). As chronic infection proceeds, virus replication decreases and HBx expression from integrated templates increases (2). During this process, HBx impacts on the host pathways and gene targets mentioned earlier. In the chronically infected liver, HBx antigens expressed from integrated templates are often truncated at their C-terminal regions. Prior work has shown that these mutants share properties with full-length HBx (4, 39), suggesting that they may also promote the appearance of "stemness," although further work with a series of mutants will need to be conducted to address this point conclusively. However, the observations that HBx promotes "stemness" may help to understand the promiscuous and pleiotrophic properties of HBx because once "stemness" factors are activated, cell fate reprogramming can occur, even without the sustained expression of these markers. In this context, it is interesting that the strongest and most prevalent HBx staining was found in cirrhotic livers and relatively little or none in the majority of HCC nodules (2, 38). This is consistent with the observation that HCC arises most often from cirrhotic livers and with the notion that the HBx promotion of "stemness" in this pathologic setting is central to early-stage tumor development.

The contribution of HBx to the development of "stemness" is supported by observations that Oct-4, Nanog, Klf-4, and c-Myc are upregulated in HepG2X compared with HepG2CAT cells (Fig. 1) and Oct-4 and Nanog were detectable in several HCC nodules (Fig. 2). Independent evidence showed strongly



**Figure 6.** Western blots showing levels of acetyl-H3, E-cadherin,  $\beta$ -catenin, and EpCAM after the treatment of HepG2X and HepG2CAT cells with TSA. The blot is representative of 3 independent experiments. C, control; wt, wild-type.



positive clusters of Oct-4-positive cells in HCC (40). Importantly, CSCs comprise a minor population of cells that reestablish the phenotypic heterogeneity in the primary tumor and exhibit self-renewing capability on serial passaging (9). Oct-4, Sox-2, Nanog, and their binding partners act as key regulators of pluripotency in early mammalian development (41). These "stemness" genes are reexpressed in cancer cells, and the reactivation of these factors contributes to tumorigenesis in somatic tissues (10). For example, the expression of Oct-4 and Nanog was observed in seminoma, retinoblastoma, oral squamous cell carcinoma, bone sarcoma, and breast and colon cancer cell lines compared with their normal counterparts (42). It is proposed that some of these same pathways are turned on by HBx in hepatocarcinogenesis.

HBx contributes to the pathogenesis of HCC by several pathways, resulting in the sustained activation of EpCAM. Prior studies have shown that EpCAM is present on subsets of normal epithelia (bile duct epithelium), numerous tissue stem and progenitor cells (fetal hepatoblasts and hepatic stem cells; ref. 12), and most carcinomas including HCCs (43, 44). Although normal adult hepatocytes do not express EpCAM (12, 44), it is striking that HBV-infected hepatocytes do (Fig. 2C), implying that HBx confers "stemness" properties on at least some infected cells. The significant costaining between HBx and EpCAM in the nontumor liver is consistent with this hypothesis. EpCAM acts as a mitogenic signal transducer *in vitro* and *in vivo* via nuclear translocation of the EpICD, the latter of which was also observed in a few cases here (Fig. 2B). Importantly, the scaffolding protein FHL2, which is a coactivator of  $\beta$ -catenin, bridges EpCAM with  $\beta$ -catenin and LEF-1, thus promoting interactions of EpCAM with DNA (13). Involvement of TCF/LEF-1, a major regulator of c-Myc and cyclin D expression (45), may explain the ability of EpCAM to rapidly upregulate its expression, thereby inducing cell proliferation. Importantly, EpCAM also has the ability to sustain "stemness" through EpICD binding to Oct-4, Nanog, Sox-2, and Klf-4 promoter regions, promoting the expression of these genes in human embryonic stem cells (14). The upregulated expression of EpCAM by HBx is consistent with possibly similar events occurring in HCC.

Several studies have shown that HBx upregulates and stabilizes  $\beta$ -catenin (Fig. 1; refs. 20, 30).  $\beta$ -Catenin transcriptionally upregulates EpCAM via the presence of 2 TCF binding elements in the *EpCAM* promoter (15). Nuclear accumulation of  $\beta$ -catenin induced *EpCAM* gene expression in cultured human hepatocytes and HCC cell lines (15). In tumor xenograft transplantation models, only EpCAM-positive cells efficiently initiated the development of invasive tumors, even after serial transplantation, showing that EpCAM-positive cells display CSC-like characteristics (43). Thus, the HBx upregulation of  $\beta$ -catenin (Fig. 1) and  $\beta$ -catenin-mediated induction of EpCAM may be critical in initiating and maintaining CSCs growth.

E-cadherin, which is important for the maintenance of cell polarity and the structural integrity in tissue, is downregulated by HBx (24). Abnormalities in the expression and cellular distribution of E-cadherin are frequently associated with dedifferentiation and invasiveness in a variety of human

malignancies, including HCCs (23). HBx has been found to repress E-cadherin expression at the transcriptional level through hypermethylation of the *E-cadherin* promoter (24) and possibly by alterations in HDAC function following complex formation with HBx (8). The latter is supported by evidence that TSA, an inhibitor of HDAC1, relieves the suppression of E-cadherin by HBx (Fig. 6). Independent evidence has shown that the inhibition of HDAC by TSA could induce hyperacetylation of histones and restore the expression of E-cadherin (46). HBx-mediated repression of E-cadherin releases  $\beta$ -catenin from its role in cell adhesion. Prior work has shown that HBx-induced adherens junction disruption is Src kinase dependent and resulted in the accumulation of cytoplasmic  $\beta$ -catenin (25, 47). In the nucleus,  $\beta$ -catenin binds TCF/LEF factor and acts as a transcriptional activator of growth-regulatory genes including those involved in self-renewal of stem cells. Studies have revealed that TCF3 factor co-occupies almost all promoter regions occupied by stem cell-specific transcription factors, including Oct-4 and Nanog, and this suggests the mechanism how  $\beta$ -catenin regulates the expression of "stemness" genes (17, 18). Hence, the finding that HBx constitutively activated wild-type  $\beta$ -catenin in up to 80% of HCCs (20) underscores the potentially close relationship between HBx, activated wild-type  $\beta$ -catenin, and stem cell renewal.

Recent studies show that altered expression of specific miRNAs is involved in tumorigenesis (33). Microarray data from HBV-associated HCCs and nontumor liver tissues showed that highly conserved miR-181 family members were overexpressed in HSCs and CSCs and thus contributed to the maintenance of "stemness." Forced miR-181 expression enriched EpCAM-positive HCC cells with stem cell properties, whereas miR-181 blockage induced differentiation (16). Interestingly, data from this study showed elevated miR-181 expression in HepG2X cells compared with HepG2CAT cells and in most HBx-positive tumor and nontumor samples (Fig. 4), suggesting that miR-181 is an epigenetic target of HBx. Collectively, these data suggest that HBx may contribute to HCC by promoting the development of "stemness."

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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