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Epigenetic repression of E-cadherin expression by Hepatitis B virus x Antigen (HBx) in liver cancer

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Abstract

Loss of E-cadherin is associated with acquisition of metastatic capacity. Numerous studies suggest histone deacetylation and/or hypermethylation of CpG islands in E-cadherin gene (*CDH1*) are major mechanisms responsible for E-cadherin silencing in different tumors and cancer cell lines. The Hepatitis B virus (HBV) encoded X antigen, HBx, contributes importantly to the development of hepatocellular carcinoma (HCC) using multiple mechanisms. Experiments were designed to test if in addition to *CDH1* hypermethylation HBx promotes epigenetic modulation of E-cadherin transcriptional activity through histone deacetylation and miR-373. The relationships between HBx, E-cadherin, mSin3A, Snail-1 and miR-373 were evaluated in HBx expressing (HepG2X) and control (HepG2CAT) cells by western blotting, immunoprecipitation, chromatin immunoprecipitation as well as by immunohistochemical staining of liver and tumor tissue sections from HBV infected patients. In HepG2X cells, decreased levels of E-cadherin and elevated levels of mSin3A and Snail-1 were detected. Reciprocal immunoprecipitation with anti-HBx and anti-mSin3A demonstrated mutual binding. Further, HBx-mSin3A co-localization was detected by immunofluorescent staining. HBx down-regulated E-cadherin expression by the recruitment of the mSin3A/HDAC complex to the Snail-binding sites in human *CDH1*. Histone deacetylation inhibition by Trichostatin A treatment restored E-cadherin expression. Mir-373, a positive regulator of E-cadherin expression, was down-regulated by HBx in HepG2X cells and tissue sections from HBV infected patients. Thus, histone deacetylation of *CDH1* and down-regulation of miR-373, together with the previously demonstrated hyper-methylation of *CDH1* by HBx, may be important for the understanding of HBV-related carcinogenesis.

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Conflict of interests

The authors declare that no conflict of interest exists and have no other disclosures to make.

Keywords

Hepatocellular carcinoma; mSin3A; Snail-1; HDAC; miR-373

Introduction

The transmembrane glycoprotein E-cadherin provides a physical link between adjacent cells and is crucial for cell polarity and the structural integrity of tissue (van Roy *et al*., 2008). In addition, it is a component of the cellular signaling network (Takeichi, 1991). Altered expression and cellular distribution of E-cadherin is frequently associated with dedifferentiation and invasiveness in human cancers, including HCC (Hirohashi *et al*., 2003; Endo *et al*., 2000). Loss of E-cadherin is also a critical feature of intrahepatic metastasis in HCC (Osada *et al*., 1996). Frequent (67%) CpG methylation of *CDH1* correlated with reduced E-cadherin expression in both primary HCCs and liver tissues of patients with chronic liver disease (CLD) (Kanai *et al*., 1997).

HBV is a major etiologic agent of CLD and HCC. The virally encoded X antigen (HBx) contributes importantly to the development of HCC through multiple mechanisms in diverse subcellular compartments, ranging from the nucleus (Weil *et al*., 1999) to the cytoplasm (Sirma *et al*., 1998) and mitochondria (Waris *et al*., 2001). Cytoplasmic HBx may influence the regulation of gene expression through different signal transduction pathways (Kekule *et al*., 1993), while nuclear HBx directly affects transcriptional machinery (Nomura *et at*., 1999), including epigenetic modulation (Park *et al*., 2007). In this context, HBx has been shown to suppress E-cadherin at both the mRNA and protein levels (Liu *et al*., 2006) by inducing the activities of the DNA-methyltransferases DNMT1 and DNMT3A and by promoting methylation of *CDH1* (Park *et al*., 2007; Lee *et al*., 2005).

Recently, another epigenetic mechanism was described that represses transcription of the E-cadherin gene; the zinc finger transcription factor Snail binds to the E-boxes of the *CDH1* promoter (Giroldi *et al*., 1997) and recruits a transcriptional repressor complex containing mSin3A/HDAC (Peinado *et al*., 2004). Inhibition of Snail in epithelial cancer cell lines lacking E-cadherin restored the expression of *CDH1* (Batlle et al., 2000). Moreover, there is a strong inverse correlation between the Snail and E-cadherin in various carcinomas such as breast, pancreas, colon and HCC (Jiao *et al*., 2002; Sugimachi *et al*., 2003). Snail has been found over-expressed in the cells located at the invasive front of tumors (Guarino *et al*., 2007), promoting tumor invasiveness. Snail genes are activated by most pathways triggering epithelial-to-mesenchymal transition (EMT) (Xu *et al*., 2009). Such pathological activation of Snail and repression of E-cadherin were observed during fibrosis and tumour progression (Moustakas *et al*., 2007). HCC is characterized by high levels of HDAC1 (Rikimaru *et al*., 2007). HBx was shown to up-regulate HDAC1, resulting in increased angiogenesis and inhibition of apoptosis (Yoo *et al*., 2008). In addition, HBx recruitment of HDAC1 repressed insulin-like growth factor binding protein 3 transcription (Shon *et al*., 2009). Since HBx-HDAC1 (Shon *et al*., 2009; Zheng *et al*., 2009) and Snail-HDAC1/2-mSin3A (Peinado et al., 2004) interactions have been demonstrated, it is possible that HBx may also interact with mSin3A, and recruit this complex to *CDH1* to repress E-cadherin. This may provide an

additional epigenetic mechanism of E-cadherin repression by which HBx contributes to pathogenesis of CLD and HCC.

Prior work has shown that altered expression of specific miRNAs is involved in tumorigenesis (Agami, 2010). miRNA(s) regulate gene expression by repressing translation or directing sequence-specific degradation of mRNA. However, RNA duplexes mediate transcriptional activation through the targeting of complementary motifs in gene promoters, such as those of E-cadherin, $p21^{WAF1/CIP1}$ and VEGF (Li *et al*., 2008, Janowski *et al*., 2007). In this regard, transfection of miR-373 and its precursor hairpin RNA (pre-miR-373) into PC-3 cells induced E-cadherin expression (Place et al., 2008). The roles of miR-373, as well as Snail-HDAC1/2-mSin3A, in the regulation of *CDH1* expression in pathogenesis of HBx associated hepatocarcinogenesis, have not been explored. The results from this study show that these molecules may also be operative, underscoring the importance of suppressed *CDH1* expression in liver cancer, and that HBx is central to triggering these changes.

Results

Relationships between HBx, E-cadherin, mSin3A and Snail-1 in vitro

Initial experiments were performed to determine whether HBx altered the expression of E-cadherin, mSin3A and/or Snail-1 in cultured liver cells. Accordingly, whole cell lysates from HepG2X and HepG2CAT cells were analyzed by western blotting. There was a 3.8 ± 0.3-fold decrease in the levels of E-cadherin in HepG2X compared to control cells (Fig. 1A), as shown previously (Liu *et al*., 2006). Similar results were observed in Hep3BX compared to Hep3BCAT cells (Liu *et al*., 2006). In HepG2X nuclear cell extracts, there was up-regulation of mSin3A (2.5 ± 1.1-fold) and Snail-1 (2.0 ± 0.7-fold) (Fig. 1B). When HBx was introduced into the primary human hepatocytes, E-cadherin was down-regulated 1.1 ± 0.5-fold or 24% ($P < 0.03$) four days later (Fig. 1C and D). These data show that HBx suppresses E-cadherin and stimulates the expression of mSin3A and Snail-1.

Relationship between HBx, E-cadherin, mSin3A and Snail-1 in vivo

Clinical samples containing HCC and nontumor liver were stained for HBx, E-cadherin, mSin3A and Snail-1. Among 42 patients who underwent surgical resection for HBV associated HCC, 30 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 27 of 39 tumors (69%) and in all 33 nontumor livers (100%) (Suppl. Table 1). In 82% of cases with tumor and nontumor tissues, HBx staining was stronger and more widespread in liver compared to tumor, as previously reported (Lian *et al*., 2006). Commercially available liver sections from 10 uninfected individuals were negative for HBx. HBx staining was cytoplasmic in all cases (Fig. 2A) (Wang *et al*., 1991a). Nuclear HBx staining was also observed (Suppl. Fig. S1), as shown earlier (Wang et al., 1991b). For E-cadherin, membranous staining was detected in 10 of 39 tumors (26%), in 21 of 33 cases where nontumor liver was available (64%) (Suppl. Table 1) and in uninfected controls (Suppl. Fig. S2). These results are similar to those previously published (Liu *et al*., 2006). Nuclear mSin3A staining was observed in the nontumor liver from 19 of 33 patients (58%) (Suppl. Table 1, Fig. 2A), and in 20 of 39 tumors (51%) (Fig. 2B, Supp. Fig. S3). mSin3A and

Snail-1 staining in uninfected liver was undetectable (Figs. 2B and C). With regard to Snail-1, staining was observed in the nontumor compartment from 17 of 33 patients (52%) (Fig. 2A), and in the tumor compartment from 22 of 39 patients (56%) (Suppl. Table 1, Fig. 2C, and Suppl. Fig. S3). Hence, mSin3A and Snail up-regulation was observed in HBV associated HCC.

Additional analyses were conducted to determine the relationship between HBx and these markers. An inverse correlation was seen between HBx and E-cadherin in 21 cases of non-tumor ($P < 0.025$). As expected, there was no correlation between HBx and E-cadherin in the tumor compartment, with co-staining in only 7 cases ($P > 0.95$). HBx and mSin3A were significantly associated in nontumor liver ($P < 0.005$) but not in HCC ($P > 0.75$). Likewise, HBx and Snail-1 were significantly associated in nontumor liver ($P < 0.005$) but not in tumor ($P > 0.7$). This is not surprising in light of the fact that HBx staining was stronger and widespread in liver tissue compared to most HCC nodules in the same patients (Wang et al., 1991b). Therefore, if HBx promotes up-regulation of mSin3A and Snail-1, this would be observed most readily in liver tissue surrounding tumor nodules. In addition, up-regulated mSin3A and Snail-1 were observed in both nontumor liver ($P < 0.001$) and in tumor ($P < 0.001$). This is expected, since it is known that Snail-1 binds to mSin3A (MacPherson *et al*., 2010). Given that Snail and mSin3A make up part of a complex that represses E-cadherin expression (Peinado *et al*., 2004), the finding of no significant relationships between the expression of E-cadherin with mSin3A or Snail-1 is consistent with their expected roles in the suppression of E-cadherin *in vivo*.

Interaction between HBx and mSin3A

The tissue staining results above suggest that HBx may suppress E-cadherin expression, in part, by altering the E-cadherin suppressor complex containing mSin3A. To determine whether HBx physically interacts with mSin3A, reciprocal immunoprecipitation experiments were performed with HepG2X and HepG2CAT cell lysates. Prior work showed that HBx is present in both the cell cytoplasm (Sirma *et al*., 1998) and nucleus (Weil *et al*., 1999). Since mSin3A is a nuclear protein, it was first important to verify that HBx was detectable in nuclear extracts. The latter was shown by western blotting with anti-HBx (Fig. 3A, lane 1). Immunoprecipitation with anti-mSin3A showed HBx in cell lysates from HepG2X but not HepG2CAT cells (Fig. 3A, lanes 2 and 3, respectively). Reciprocal immunoprecipitation with anti-HBx, followed by western blotting with anti-mSin3A showed strong reactivity with mSin3A in HepG2X but not HepG2CAT cells (Fig. 3B, lanes 1 and 2), suggesting physical association between mSin3A and HBx. To confirm the subcellular localization where this association occurs, HepG2X cells were stained with anti-HBx (red staining in Fig. 3C), anti-mSin3A (green staining in Fig. 3D), and DAPI (blue staining in Fig. 3E). When the images were merged, HBx-mSin3A co-localization was observed in the nuclei of HepG2X cells as small orange regions (Fig. 3F). These staining results were analogous to those observed in tissue sections from infected patients (e.g., in Fig. 2) and support the hypothesis of HBx-mSin3A nuclear co-localization.

HBx recruits mSin3A to the E-cadherin promoter

HBx was shown to recruit DMNTs to suppress E-cadherin expression (Park *et al*., 2007; Zheng *et al*., 2009). Thus, ChIP assays were performed to determine if HBx recruits the mSin3A/HDAC complex, which is known to suppress E-cadherin (Peinado *et al*., 2004). In this context, the human *CDH1* contains three regulatory elements consisting of a CANNTG (called "E-boxes") located in the proximal promoter and in exon 1 (Giroldi *et al*., 1997). Since Snail mediates E-cadherin repression by the recruitment of the mSin3A/HDAC1 complex to the E-boxes (Peinado *et al*., 2004), experiments were designed to target this fragment (which included all three E-boxes) for PCR amplification following ChIP (Fig. 4A). ChIP assays showed increased levels of mSin3A and HDAC1, but decreased levels of Acetyl-H3 and RNA Pol II at the regions of the Snail binding sites in HBx-expressing cells compared to control (Fig. 4B). To verify the functional importance of this finding, HepG2X and HepG2CAT cells were treated with the HDAC inhibitor, TSA. HDAC inhibition by TSA treatment restored H3 acetylation and E-cadherin expression (Fig. 4C). Since HBx can directly interact with HDAC (Shon *et al*., 2009; Zheng *et al*., 2009) and with mSin3A (Fig. 3), these results confirm that HBx mediates *CDH1* deacetylation by recruiting the mSin3A/HDAC complex.

HBx down-regulates miR-373

miRNAs contribute importantly to the pathogenesis of many tumor types (Agami, 2010). When miRNA array analysis was performed on HepG2X and HepG2CAT cells, miR-373 was down-regulated 5.09-fold in HepG2X compared to control cells ($P < 0.01$) (data not shown). RT/PCR analysis using miRNAs extracted from these cells showed that miR-373 was suppressed to a similar extent (5.8-fold, $P < 0.001$) (Suppl. Fig. S4) in HepG2X compared to HepG2CAT cells. Validation of miRNA expression profiles was conducted using snap frozen tumor and adjacent nontumor tissues from 19 Chinese patients. The results were compared to HBx staining in samples from these same cases. HBx staining was present in the nontumor compartment from 18 (95%) of these patients. HBx was also detectable in HCCs from 6 patients. When these tissues were evaluated for miR-373 by real-time PCR, the ΔΔCt values showed that 14 of 19 HBx positive nontumor liver samples had decreased miR-373 (74%) relative to corresponding tumor from the same patient (Fig. 5A, Suppl. Table 2) ($P < 0.005$), as in HepG2X compared to HepG2CAT cells. ΔΔCt values also showed that miR-373 was elevated in HCC from 5 of 19 cases (26%) (Suppl. Table 2). When the ΔΔCt values were converted to "fold change" in miR-373 expression, there was an average 95-fold increase in miR-373 levels in tumor compared to nontumor among the 14 patients with negative miR-373 values (Fig. 5A and Suppl. Table 2), and an average of 4.5-fold change in the remaining 5 patients. HBx suppressed miR-373 in nontumor compared to tumor by an average of 21-fold.

When looking at the relationships between HBx, miR-373, and histopathology, there was a strong correlation of HBx with chronic hepatitis ($P < 0.02$) and cirrhosis ($P < 0.01$), as expected (Wang et al., 1991a, b). HBx expression in liver inversely correlated with the levels of miR-373 in the same compartment ($P < 0.005$), and the same relationship was also seen in HCC ($P < 0.05$). Importantly, suppressed miR-373 in nontumor correlated with

Edmondson grade III–IV ($P < 0.005$) and with venous invasion ($P < 0.001$), suggesting that suppressed miR-373 in liver was associated with more aggressive tumors.

To further validate that E-cadherin is targeted by miR-373, HepG2X cells were transiently transfected with hsa-miR-373 precursor and levels of E-cadherin were measured by western blotting (Fig. 5B). As expected, in the presence of miR-373, E-cadherin was up-regulated (3-fold, $P < 0.015$). Together, these results suggest that miR-373 may be a target of HBx both in human liver and cell lines.

Discussion

There are roughly 350 million carriers of HBV worldwide at risk for CLD and HCC. Chronic HBV infection underlies up to 80% of HCC cases (Park *et al*., 2006). The HBV encoded HBx protein contributes importantly to HCC, although the associated mechanisms are not clear. HBx is localized in the cytoplasm (Sirma *et al*., 1998), mitochondria (Waris *et al*, 2001), and the nucleus (Nomura *et al*., 1999). Thus, HBx may exert effects upon pathogenesis in different subcellular compartments (Keasler *at al*., 2009). For example, cytoplasmic HBx may regulate gene expression through signal transduction pathways that affect the control of the cell cycle, proliferation or apoptosis (Lupberger *et al*., 2007). An association of HBx with the outer membrane of mitochondria induces oxidative stress (Waris *et al*, 2001), while nuclear HBx may exert transcription factor-like functions (Haviv *et al*., 1996). Epigenetic modulation of transcriptional activity of the target genes could be an important mechanism for HBx-mediated transformation since HBx does not bind DNA. In agreement with this suggestion, HBx was shown to elevate the overall intracellular activities of DNMT1, DNMT3A1, and DNMT3A2, selectively promote hypermethylation of tumor suppressor genes (such as *GSTP1* and *CDKN2B*) (Park et al., 2007), and, importantly, *CDH1* (Lee *at al*., 2005; Zheng *et al*., 2009). Previous work showed E-cadherin suppression in HBx-positive cells and human liver tissue sections at both the protein and mRNA levels. This was associated with hypermethylation of CpG islands in *CDH1* (Liu *et al*, 2006). Moreover, depressed E-cadherin correlated with HBx *trans*-activation, and expression of E-cadherin was restored by treatment with the DNMT inhibitor 5′-Aza-2′dC (Lee *et al*, 2005). In this study, HBx was also shown to suppress E-cadherin expression via recruitment of the mSin3A/HDAC complex to *CDH1* (Figs. 3 and 4) and by suppression of miR-373 (Fig. 5, Suppl. Table 2), indicating that additional mechanisms target suppression of E-cadherin in chronic hepatitis B.

The main adhesion molecule of epithelia, E-cadherin, maintains cells in a stationary, non-motile state (van Roy *et al*, 2008). Abnormalities in the expression and cellular distribution of E-cadherin are frequently associated with de-differentiation, invasiveness and poor prognosis in a variety of human malignancies, including HCC (Endo et al., 2000). Re-establishing the functional E-cadherin complex in tumour cell lines resulted in a reversion from an invasive to a benign epithelial phenotype (Perl *et al*., 1998). Down-regulation of E-cadherin induces EMT that is characterized by acquisition of a motile mesenchymal phenotype. As a result, cells detach and disseminate to distant sites (Iwatsuki *et al*., 2009). The finding that HBx promotes the migration of liver cells, and that this is associated with

down-regulated expression of E-cadherin (Liu et al., 2006), underscores the importance of suppressed CDH1 to HBV associated HCC.

Another cellular mechanism that triggers chromatin remodeling is histone deacetylation, which yields a more compact chromatin structure, and represses gene transcription by limiting the accessibility of transcription factors to target genes (Ropero *et al*., 2007). The transcription factor Snail-1 has been described as a direct repressor of E-cadherin through interaction with the *CDH1* promoter region and recruitment of mSin3A/HDAC deacetylase activity (Peinado *et al*., 2004). The analyses of biopsies obtained from HCC confirmed that Snail-1 expression correlates with the decreased levels of E-cadherin and with dedifferentiation and invasiveness (Sugimachi *et al*., 2003). Recent studies revealed HDAC1 as a direct HBx-interacting partner (Shon *et al*., 2009; Zheng *et al*., 2009), thus raising the question as to whether HBx is associated with other members of this epigenetic complex. The finding that HBx interacts with mSin3A *in vitro* (Fig. 3), and correlates with both mSin3A and Snail-1 *in vivo* (Fig. 2, Suppl. Table 1), supports this hypothesis. An important characteristic of the interaction between DNA methylation and HDAC activity, particularly in maintaining the aberrant silencing of hyper-methylated genes in cancer, is that the methylation seems to function as the dominant event that seals transcriptional repression: histone deacetylation is secondary to DNA methylation (Cameron *et al*., 1999). Interestingly, the inhibition of histone deacetylation by the HDAC inhibitor TSA could induce hyperacetylation of histones and restore the expression of methylated E-cadherin (Ou *et al*., 2007). Thus, methylation and deacetylation could act together to potentiate the repressed state of E-cadherin. In fact, it was shown that methyl-CpG binding protein 2 (MeCP2) recruits mSin3a/HDAC to the methylated *CDH1* promoter, leading to histone-3 deacetylation (Takeno et al., 2004). In this context, it is documented that HBx promotes both hypermethylation of CDH1 (Liu *et al*., 2006) and histone deacetylation through recruitment of the mSin3A/HDAC complex to *CDH1* (Fig. 4). Further, it is expected that these mechanisms are also operative with regard to other HBx target genes in hepatocarcinogenesis.

Cancer development has also been linked to alterations in miRNA expression through the silencing of target genes (Agami, 2010; He *et al*., 2004). However, miRNAs may function as transcription factors targeting complementary motifs in promoter regions to positively regulate gene expression (Place *et al*., 2008). In this regard, miR-373 was shown to induce E-cadherin expression by binding to a target site in the *CDH1* promoter. Transfection of miR-373 and its precursor hairpin RNA (pre-miR-373) into PC-3 cells induced E-cadherin expression (Place et al., 2008). In the present work, there was an inverse relationship between HBx and miR-373 both *in vitro* and *in vivo* (Figs. 1 and 5, Suppl. Table 2), suggesting that miRNA epigenetic control of *CDH1* (and perhaps other) gene expression is also regulated by HBx. Depressed miR-373 in nontumor cells also correlated with the presence of undifferentiated tumors with venous invasion, which is characteristic of aggressive HCC. The latter may indicate that in tumor cells, which often lack HBx, restoration of miR-373 expression may promote tumorigenesis. In addition to HBx, restoration of miR-373 in tumors may be due to other factors that remain to be deciphered.

In addition to the mechanisms above, HBx may also suppress *CDH1* by activation of TGFβ. TGFβ activates Snail expression through the ERK and PI3K pathways and/or through phosphorylation of SMADs that, upon nuclear translocation, trigger Snail expression (Peinado *et al*., 2003). Interestingly, ERK, PI3K and selected SMADs are also activated by HBx (Chung *et al*., 2004; Lee *et al*, 2001). HBx, in turn, directly promotes TGFβ expression through *trans*-activation (Yoo *et al*., 2001), by stimulating Smad4 (which mediates TGFβ signaling) (Lee *et al*, 2001) and by reducing expression of the major TGFβ inhibitor alpha2-macroglobulin (α2-M) (Pan *et al*., 2004). Thus, HBx, through activation of TGFβ, may potentially up-regulate Snail and down-regulate E-cadherin. These pathways will be explored in the future. Together, the findings herein emphasize the role of the HBx as a central player in HBV-induced epigenetic alterations that contribute to the pathogenesis of HCC. E-cadherin suppression would provide an example of how HBx promotes the development of epigenetic changes that ultimately give rise to HCC.

Materials and methods

Cell culture

The human hepatoblastoma cell line, HepG2, was stably transfected with HBx (HepG2X) or the bacterial chloramphenicol acetyltransferase (control) gene (HepG2CAT) and maintained in culture as previously described (Lian et al., 1999). Primary human hepatocytes were purchased from Zen-Bio (Research Triangle Park, NC) and cultured in medium provided by the manufacturer.

Patient samples

Formalin fixed, paraffin embedded paired tumor (HCC)/nontumor (adjacent liver) tissues were obtained from 42 Chinese patients who underwent surgery at the Third Military Medical University, Chongqing, China. All were hepatitis B surface antigen (HBsAg) positive in blood, 40 were males, the age range was from 36–69 (average: 48), and all were of Chinese ethnicity. Additional snap frozen tumor/nontumor pairs from 19 HBV infected Chinese patients were obtained from Queen Mary College, University of Hong Kong, China. All patients were positive for HBsAg in blood, 18 were male, and their ages ranged from 33–71 years (average: 51). Ten uninfected human liver tissues (Abcam, Cambridge, MA) were used as controls. All samples were used for diagnostic purposes, and then for this study. The use of these samples was approved by the Institutional Review Boards at all participating universities.

Protein extraction and western blotting

Cells were lysed in Cell Lysis Buffer with protease inhibitor cocktail (Cell Signaling, Danvers, MA). Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to enclosed instructions. Protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Sanford, ME). The membranes were incubated overnight in 5% nonfat milk in Tris Buffered Saline/0.1% Tween-20 with primary antibodies against HBx, E-cadherin, Snail-1, lamin A, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), mSin3a (Abcam Cambridge, MA), and Ac-Lys H3 (Active Motif). The blots were developed using the ECL plus detection kit (Amersham,

Piscataway, NJ) and exposed to Kodak imaging films (Rochester, NY). Signal intensities were quantified using ImageJ software (NIH).

Introduction of HBx into human hepatocytes

Five million primary human hepatocytes were treated with 8 μg/ml of cell penetrating recombinant protein, 11R-HBx (LD BioPharma, San Diego, CA), and 2.5 μM of fusogenic peptide TAT-HA2 to stabilize 11R-HBx from macropinocytosis (Wadia *et al*., 2004) twice per day for four days. 11R- based recombinant peptides were previously used in a similar protocol for the generation of induced pluripotent stem cells (Zhou *et al*., 2009). TAT-H2 was synthesized at the Peptide Synthesis core facility, Temple University. Cells were lysed and analyzed by western blotting. This experiment was reproduced with three different batches of human hepatocytes.

Treatment of Cells with Trichostatin A (TSA)

Suspensions of 3.5×10^5 cells were plated in 35-mm culture dishes with or without 350 nM TSA (Sigma) in 2 ml of medium. Extracts were collected at 12 and 24 hours and analyzed by western blotting.

Immunohistochemistry

Tissue sections were deparaffinized, dehydrated, treated with Uni-TRIEVE antigen retrieval (Innovex, Richmond, CA) and stained using the UltraVision Detection System (Thermo Scientific, Rockford, IL) according to enclosed instructions. Antibodies used for staining were the same as those described for western blotting except for anti-HBx (anti-99 peptide antibody) (Feitelson *et al*., 1988) and anti-mSin3A (Santa Cruz Biotechnology). Normal mouse or rabbit IgG (Vector Labs, Burlingame, CA) were used to rule out false-positive responses. Pre-absorption of primary antibodies with corresponding antigens, and staining liver sections from uninfected individuals, were performed on tissue sections to insure specificity.

Immunoprecipitation (IP)

Protein extracts (500 μg) were incubated with anti-HBx (Santa Cruz) or anti-mSin3a (Abcam) and protein G sepharose beads (GE Healthcare, Uppsala, Sweden), in HNTG buffer (20mM Hepes pH 7.5, 150mM NaCl, 0.1% Triton X-100, 10% glycerol, and protease inhibitor cocktail [Cell Signaling, Danvers, MA]) overnight at 4°C. The immunoprecipitates were washed with HNTG buffer, resolved by SDS-PAGE and visualized by immunoblotting with antibodies against HBx or mSin3a, as described in Western Blotting protocol.

Chromatin immunoprecipitation (ChIP) assay

HepG2X and HepG2CAT cells were cross-linked with 1% formaldehyde for 20 min. at room temperature. Prior to formaldehyde cross-linking, cells were treated for 20 min. with 10 mM dimethyl adipimidate (DMA) (Sigma), a protein-to-protein cross-linking agent for the proteins that bind indirectly to DNA (Zeng *et al*., 2006). The cross-linking reaction was quenched with 50 mM glycine-PBS for 10 min. Cells were washed with PBS, dounce homogenized in hypotonic buffer (Active Motif) and centrifuged 10 min. at 5000 rpm. The

pellet was resuspended in a buffer consisting of 50mM HEPES, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, and protease inhibitor cocktail. Sonication was done in a Branson SonifierR SLP (Branson Ultrasonic Corporation, Danbury CT) at 80% power. The sheared samples were centrifuged for 15 minutes at 13,000 rpm. Anti-mSin3A (Abcam), anti-Ac-H3, anti-HDAC1, RNA Pol II (all from Millipore, Billerica, MA), IgG (negative control) and protein G Sepharose beads (GE Healthcare) were used for IP. The samples were washed, resuspended in the elution buffer (1% SDS, 50 mM $NaHCO_3$), and reverse cross-linked. DNA was extracted with phenol-chloroform. PCR amplification was carried out with PCR Master Mix (Promega, Madison, WI) for 25–33 cycles consisting of 30 sec at 95°C, 30 sec at 58°C, and 60 sec at 72°C. The primers for the *CDH1* were: (F) 5′-TAGAGGGTCACCGCGTCTAT-3′ and (R) 5′-GGGCTGGAGTCTGAACTGA -3′. Ready positive control primers (Ambion, Austin, TX) amplify a 361 bp fragment, which is a highly conserved region of a constitutively expressed "housekeeping" gene, rig/S15. The synthesized fragments were separated on 2% agarose gel and visualized with ethidium bromide.

Immunofluorescence microscopy

HepG2X and HepG2CAT cells were fixed with ice-cold ethanol/acetic acid (95:5, v/v) and incubated with anti-HBx and anti-mSin3a (Santa Cruz). FITC- and Rhodamine-conjugated secondary antibodies were from Santa Cruz. Sections were washed, mounted in Vectashield aqueous mounting medium with DAPI (Vector Labs, Burlingame, CA), and analyzed using an ECLIPSE Ti inverted microscope, fitted with a Nikon DS-Fi1 camera and containing NIS Elements software (Nikon, Melville, NY).

MicroRNA analysis

Small RNAs were isolated from HepG2X and HepG2CAT cells and 19 pairs of HCC tissue samples using Ambion mirVana miRNA isolation kit (Austin, TX) according to enclosed instructions. MicroRNA array analysis was performed by LC Sciences (Houston, TX). Differentially expressed miRNAs were identified by Cy3(HepG2X)/Cy5(HepG2CAT) ratio, and those with $P < 0.01$ were considered for further characterization. Differential expression of selected miRNAs was validated by qRT-PCR using miScript SYBR Green PCR Kit (Qiagen, Valencia, CA) and a Mastercycler realplex system (Eppendorf, Humburg, Germany). The ΔΔCt calculation was done as follows: ΔΔCt = ΔCt of miR-373 in tumor – ΔCt of miR-373 in non-tumor tissue. Briefly, samples were first reverse-transcribed, and qPCR was performed using miScript PCR Kit RNA as follows: 15 min at 95 °C, and 40 cycles (94°C for 15 sec and 70 °C for 30 sec). U6 was used for normalization. hsa-miR-373 and U6 were from Qiagen.

Transient transfection of HepG2X cells with 100nm hsa-miR-373 precursor was performed using DharmaFECT 1 (Dharmacon, Lafayette, CO) according to enclosed instructions. Experiments were done in triplicate.

Statistics

The relationships between HBx and E-cadherin, mSin3A and Snail-1 obtained by immunohistochemistry, as well as relationships between HBx, miR-373 and tissue

histopathology, were determined using 2 × 2 comparisons in the Chi square (χ^2) test. Statistical significance was considered when $p < 0.05$. Student's t-test was performed to compare paired samples from western blotting and PCR.

Supplementary Material

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Abbreviations

HBx	hepatitis B x antigen
CDH1	E-cadherin gene
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
EMT	epithelial-mesenchymal transition
HDAC1	histone deacetylase 1
RNA Pol II	RNA Polymerase II
DNMT	DNA-methyltransferase
TSA	Trichostatin A
IP	immunoprecipitation
ChIP	chromatin immunoprecipitation
MeCP2	methyl-CpG-binding protein 2
CLD	chronic liver disease

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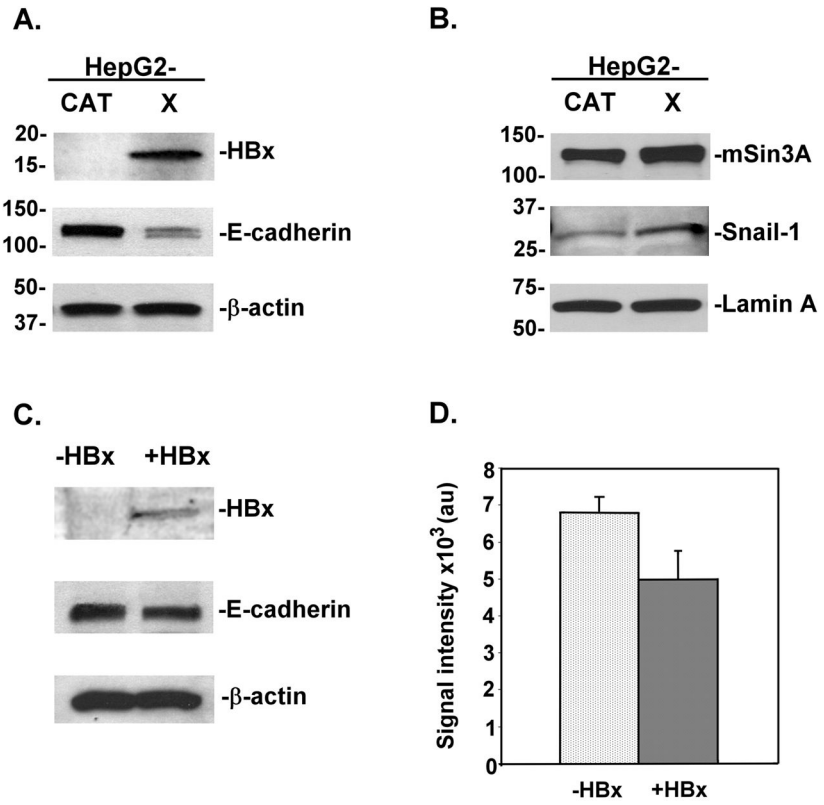


Fig. 1.

Representative western blots with 50 μg of (**A**) total or (**B**) nuclear extracts of HepG2CAT (CAT) and HepG2X (X) cells. β-actin and lamin A are loading controls. Western blot analysis (**C**) and quantification (**D**) of protein extracts (50 μg) prepared from human hepatocytes after the treatment with transducible recombinant peptide 11R-HBx (+HBx) and control hepatocytes (-HBx). au = arbitrary units. This result was reproduced in each of three independent experiments with different batches of commercially available cells.

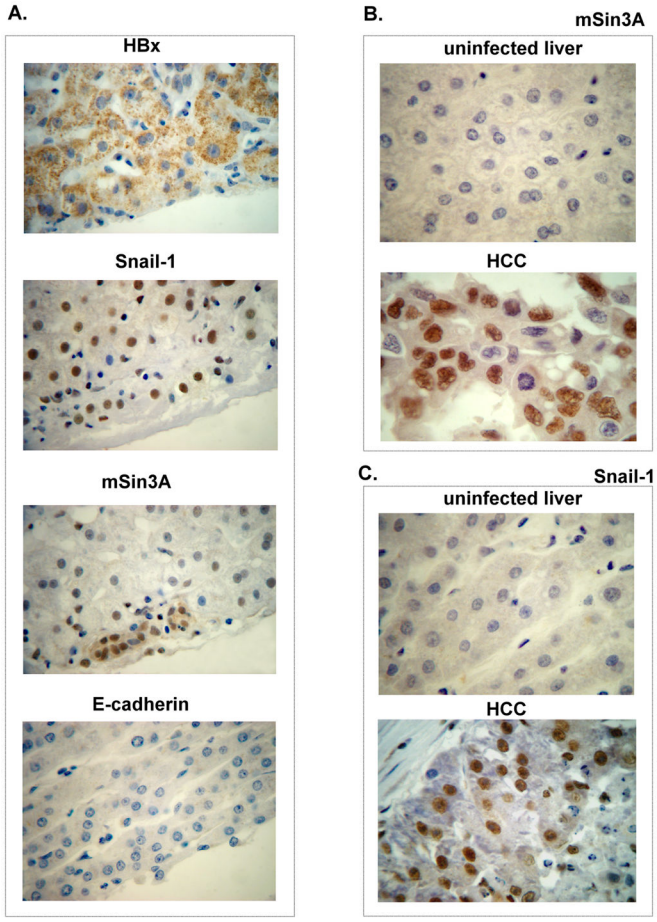


Fig. 2.

Representative staining for (**A**) HBx, Snail-1, mSin3A, and E-cadherin in consecutive sections from a block of a patient with HBV infected liver (x400). Representative staining for mSin3A (**B**) and Snail-1 (**C**) from uninfected human liver (top photo in **B** and **C**) and from a block of HCC (bottom photo in **B** and **C**) (x400).

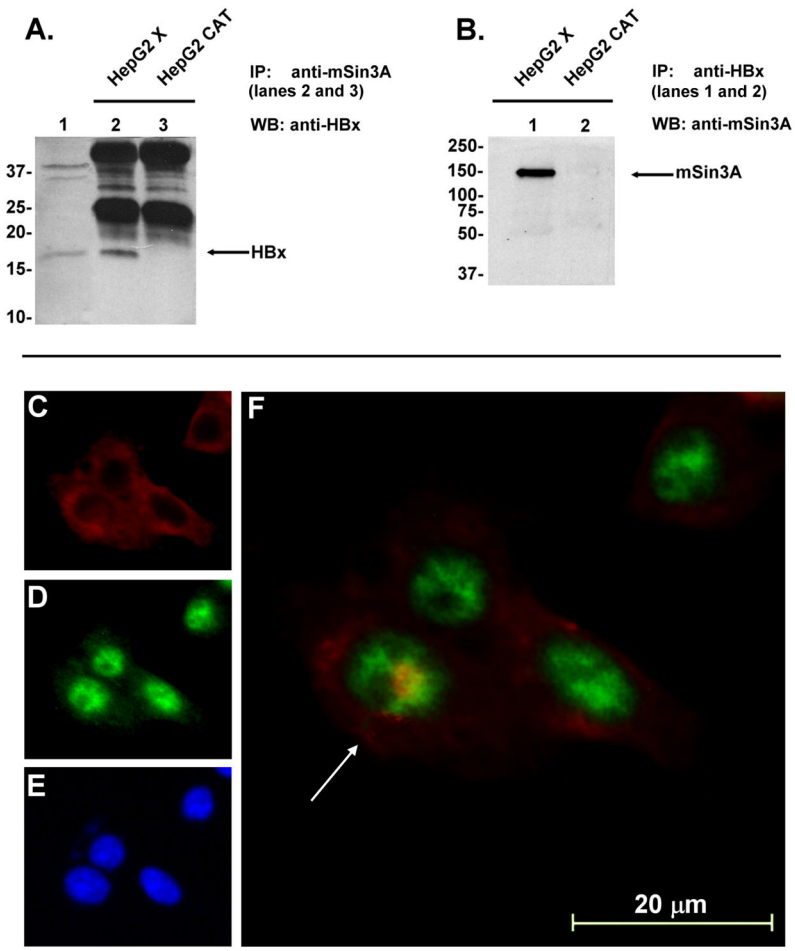


Fig. 3.

Reciprocal immunoprecipitation of HepG2X and HepG2CAT protein extracts (**A**) with anti-mSin3A and western blot detection of HBx and (**B**) with anti-HBx and western blot detection of mSin3A. Lane 1 in panel A is a nuclear extract of HepG2X cells (70 μg). Immunofluorescent staining of HepG2X cells with anti-HBx (**C**), mSin3A (**D**), and DAPI (**E**). (**F**) is a merged image of HBx and mSin3A staining showing co-localization in the nucleus (arrow).

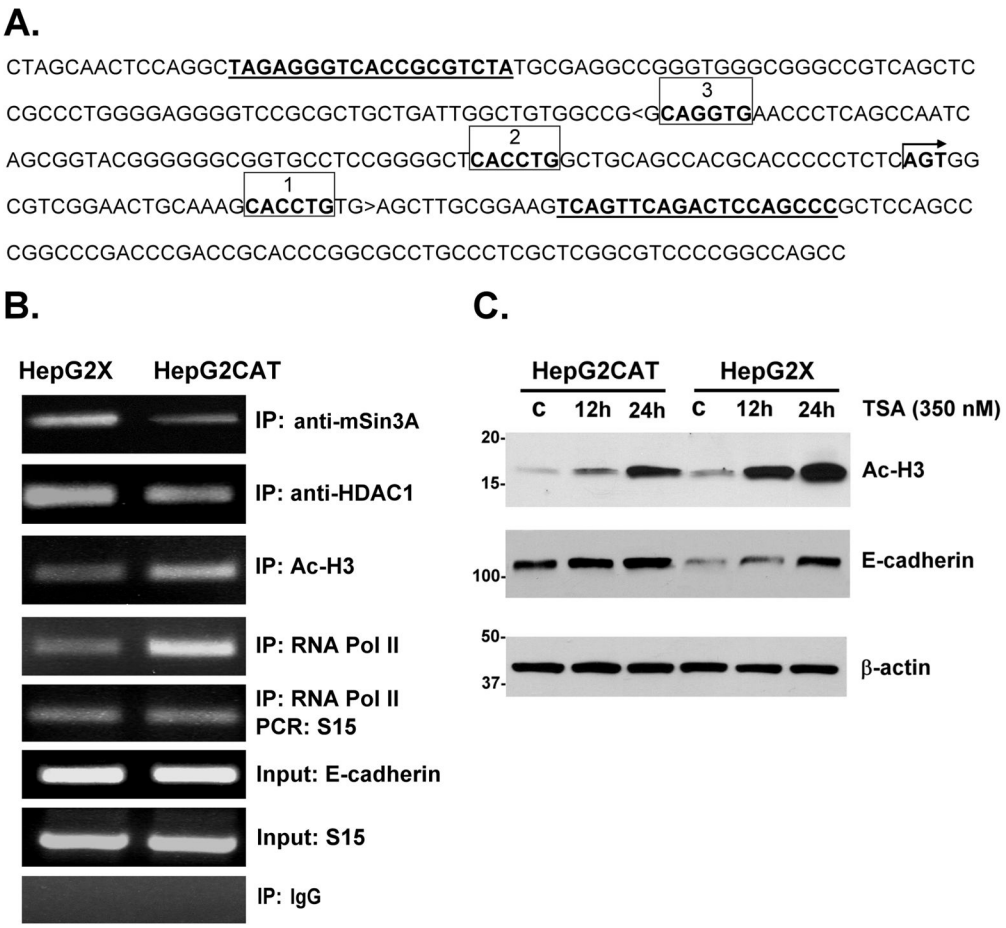
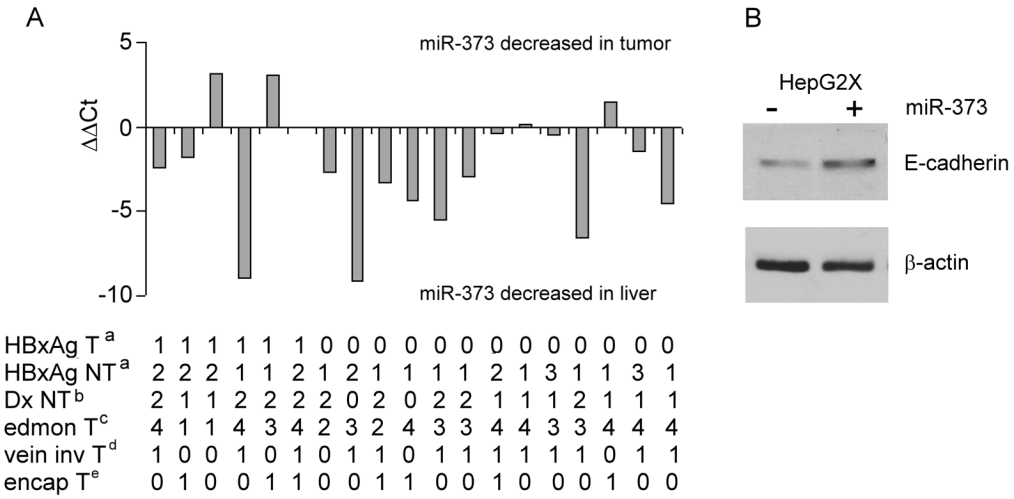


Fig. 4.

(**A**) Presented sequence for the human *CDH1* promoter region was used for the design of primers and verified at AceView (NCBI). Primer binding sites for the target fragment (231 bp) are underlined, and the target fragment included the three E-boxes (1, 2, 3) that are Snail binding sites (Batlle et al., 2000). (**B**) ChIP assays of the E-cadherin promoter occupancy by mSin3A, HDAC1, Acetyl-H3 (Ac-H3), and RNA Polymerase II (RNA Pol II) in HepG2X and HepG2CAT cells. IP with IgG was used as a control. Primers for S15 (housekeeping gene) amplify a 361 bp fragment which was used as a control for ChIP with RNA Pol II. (**C**) Representative western blots showing levels of acetyl-H3 and E-cadherin after treatment of HepG2X and HepG2CAT cells with TSA.



HBxAg T[a]	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
HBxAg NT[a]	2	2	2	1	1	2	1	2	1	1	1	1	2	1	3	1	1	3	1
Dx NT[b]	2	1	1	2	2	2	2	0	2	0	2	2	1	1	1	2	1	1	1
edmon T[c]	4	1	1	4	3	4	2	3	2	4	3	3	4	4	3	3	4	4	4
vein inv T[d]	1	0	0	1	0	1	0	1	1	0	1	1	1	1	1	1	0	1	1
encap T[e]	0	1	0	0	1	1	0	0	1	1	0	0	1	0	0	0	1	0	0

Fig. 5.

(**A**) Expression of miR-373 in tumor and non-tumor liver tissues. Each bar represents the data collected from one patient. The difference in miRNA expression between tumor (T) and non-tumor (NT) was determined by qRT-PCR and determination of ΔΔCt, where ΔΔCt = ΔCt of miR-373 in tumor – ΔCt of miR-373 in non-tumor. U6 was used for normalization. Negative values indicate that miR-373 levels were higher in tumor compared to adjacent non-tumor. Positive values indicate that miR-373 levels were higher in NT (liver) compared to T. **HBxAg NT[a]** and **HBxAg T[a]**: HBxAg staining in tumor (T) and nontumor (NT) is scored as follows: 0 = negative, 1 = up to 25% of cells stained positive, 2 = 25–50% of cells stained positive, 3 = > 50% cells stained positive. **Dx NT[b]** refers to diagnosis of lesions in nontumor liver. These are as follows: 0 = no significant lesions, 1 = chronic hepatitis, 2 = cirrhosis. **edmon T[c]** refers to the Edmondson classification of cellular differentiation within tumor nodules. They are as follows: 1 = Edmondson I–II, 2 = Edmondson II, 3 = Edmondson II–III, 4 = Edmondson III. **Vein inv T[d]** refers to venous invasion of the tumor nodule, where 0 = no evidence for invasion, and 1 = presence of venous invasion. **Encap T[e]** refers to tumor encapsulation, where 0 = none and 1 = encapsulation. (**B**) Western blot detection of E-cadherin with 50 μg of protein extracts from HepG2X cells (−) and from HepG2X cells transiently transfected with miR-373 precursor (+).