HEPATITIS B X ANTIGEN PROMOTES “STEMNESS” IN THE PATHOGENESIS OF HEPATOCELLULAR CARCINOMA

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ABSTRACT

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Hepatitis B virus (HBV) is a major etiologic agent of chronic liver disease (CLD) and hepatocellular carcinoma (HCC). The virally encoded X antigen, HBx, contributes importantly to the development of HCC through its trans-activating role in various signal transduction pathways. Pathways implicated in stem cell self-renewal also contribute to carcinogenesis. Thus, experiments were designed to test if HBx triggers malignant transformation by promoting properties that are characteristic of cancer stem cells (CSCs). To test this hypothesis, HBx expressing (HepG2X) and control (HepG2CAT) human cell lines were assayed for phenotypic and molecular characteristics of “stemness.” Western blotting of protein extracts from HepG2X and HepG2CAT cells as well as immunohistochemical staining of HCC and adjacent liver tissue sections from HBV infected patients showed up-regulation of “stemness”-associated (EpCAM and β-catenin) and “stemness” (Oct-4, Nanog, Klf-4) markers by HBx. Moreover, HBx stimulated cell migration and spheroid formation. HBx expression was also associated with depressed levels of E-cadherin and subsequent activation of β-catenin and EpCAM. Results from ChIP-chip data performed previously in this lab suggest an associative link between HBx and the expression of epigenetic co-repressor, mSin3A, which is known to repress E-cadherin when complexed.
with histone deacetylases. Thus, experiments were also designed to test if HBx represses the E-cadherin gene (CDH1) through histone deacetylation by the mSin3A/HDAC complex. In HepG2X cells, decreased levels of E-cadherin and elevated levels of mSin3A were detected. Reciprocal immunoprecipitation with anti-HBx and anti-mSin3A demonstrated mutual binding. Further, HBx-mSin3A co-localization was showed by immunofluorescent staining. Chromatin immunoprecipitation revealed that HBx mediated the recruitment of the mSin3A/HDAC complex to the CDH1 promoter. HDAC inhibition by Trichostatin A treatment restored E-cadherin expression. Thus, HBx-associated epigenetic repression of E-cadherin and up-regulated expression of multiple “stemness” markers support the hypothesis that HBx contributes to hepatocarcinogenesis, at least in part, by promoting changes in gene expression that are characteristic of CSCs. This work is the first to propose that HBV promotes “stemness” in the pathogenesis of HCC.
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I dedicate this work to my future children.

“The reward of a thing well done, is to have done it.”
- Ralph Waldo Emerson
TABLE OF CONTENTS

PAGE

ABSTRACT ................................................................................................................................. i

ACKNOWLEDGMENTS ........................................................................................................ iii

DEDICATION .......................................................................................................................... iv

LIST OF FIGURES ................................................................................................................ viii

LIST OF TABLES .................................................................................................................. x

CHAPTER

1. INTRODUCTION ................................................................................................................ 1

1.1 Hepatitis B Virus and Hepatocellular Carcinoma ......................................................... 1

1.2 Hepatitis B Virus Genome ............................................................................................ 2

1.3 Hepatitis B x Antigen .................................................................................................. 5

1.4 Cancer Stem Cells ....................................................................................................... 8

1.5 Epithelial Cell Adhesion Molecule ............................................................................ 12

1.6 Wnt/β-catenin .............................................................................................................. 15

1.7 E-cadherin ................................................................................................................... 21

1.8 mSin3A/HDAC ........................................................................................................... 25

1.9 Overview and Specific Aims ....................................................................................... 29

2. METHODS ........................................................................................................................ 33

2.1 Cell Culture .................................................................................................................. 33

2.2 Patient Samples ............................................................................................................ 33

2.3 Protein Extraction and Western Blotting ................................................................... 34

2.4 Immunohistochemistry ............................................................................................... 34
2.5 Statistics ..............................................................................................35
2.6 Immunoprecipitation (IP) Assay ..........................................................35
2.7 Chromatin Immunoprecipitation (ChIP) Assay ....................................35
2.8 Treatment of Cells with Trichostatin A (TSA) .......................................37
2.9 Immunofluorescence Microscopy ..........................................................37
2.10 Spheriod Assay ..................................................................................38
2.11 Cell Migration Assay ..........................................................................38

3. RESULTS ...............................................................................................39

3.1 Analysis of EpCAM and β-catenin expression in the presence of HBx ..........................................................39
3.1.1 HBx up-regulates EpCAM and β-catenin in vitro ...............................39
3.1.2 Relationships between HBx, EpCAM and β-catenin in clinical samples ..................................................................................41
3.2 Evaluation of the mechanism of HBx-mediated mSin3A/HDAC repression of E-cadherin expression ........................................47
3.2.1 Relationships between HBx, E-cadherin, mSin3A and Snail-1 in vitro ..................................................................................47
3.2.2 Interaction between HBx and mSin3A in vitro ....................................48
3.2.3 HBx promotes recruitment of mSin3A to the E-cadherin promoter ..................................................................................51
3.2.4 HDAC inhibition reveals relationship between all markers ..........53
3.2.5 Relationships between HBx, E-cadherin, mSin3A, and Snail-1 in clinical samples ..........................................................55
3.3 Exploration of the ability of HBx to promote properties that are characteristic of cancer stem cells ........................................60
3.3.1 HBx up-regulates “stemness” markers in vitro ..................................60
3.3.2 Relationships between HBx and “stemness” markers in clinical samples ..................................................................................61
3.3.3 HBx-expressing cells exhibit clonogenic potential ............................62
3.3.4 HBx-expressing cells exhibit invasive potential ...............................64

4. DISCUSSION....................................................................................................65

REFERENCES ........................................................................................................73
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Schematic organization of the hepatitis B virus genome</td>
</tr>
<tr>
<td>2.</td>
<td>Putative signal transduction pathways and cellular factors affected by HBx</td>
</tr>
<tr>
<td>3.</td>
<td>Activation of EpCAM</td>
</tr>
<tr>
<td>4.</td>
<td>Role of β-catenin in Wnt signaling and the cadherin complex</td>
</tr>
<tr>
<td>5.</td>
<td>The mSin3A/HDAC complex</td>
</tr>
<tr>
<td>6.</td>
<td>Hypothesized convergence of relevant signaling pathways in the presence of HBx</td>
</tr>
<tr>
<td>7.</td>
<td>Model outlining the putative relationship between HBx and “stemness” in the pathogenesis of HBV associated HCC</td>
</tr>
<tr>
<td>8.</td>
<td>Upregulation of “stemness”-associated markers in the presence of HBx</td>
</tr>
<tr>
<td>9.</td>
<td>Immunohistochemical detection of HBx in HBV-mediated HCC clinical samples</td>
</tr>
<tr>
<td>10.</td>
<td>Immunohistochemical detection of EpCAM in HBV-mediated HCC clinical samples</td>
</tr>
<tr>
<td>11.</td>
<td>Immunohistochemical detection and correlation of HBx and EpCAM in consecutive HBV-mediated HCC clinical samples</td>
</tr>
<tr>
<td>12.</td>
<td>Expression of HBx, E-cadherin, mSin3A and Snail-1 in the presence of HBx</td>
</tr>
<tr>
<td>13.</td>
<td>HBx and mSin3a interact</td>
</tr>
<tr>
<td>14.</td>
<td>mSin3A/HDAC occupies the E-cadherin promoter in the presence of HBx</td>
</tr>
<tr>
<td>15.</td>
<td>HDAC inhibition reveals relationship between all markers</td>
</tr>
<tr>
<td>16.</td>
<td>Immunohistochemical detection of E-cadherin in uninfected hepatocytes</td>
</tr>
<tr>
<td>17.</td>
<td>Immunohistochemical detection of HBx, Snail-1, mSin3a, and E-cadherin in clinical samples</td>
</tr>
</tbody>
</table>
18. Upregulation of “stemness” markers in the presence of HBx ......................60
19. Immunohistochemical detection of “stemness” markers in HBV-mediated HCC clinical samples.......................................................61
20. HBx-expressing cells exhibit clonogenic potential...........................................63
21. HBx-expressing cells exhibit invasive potential.............................................64
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Correlation between HBx and “stemness”-associated markers in HBV-mediated HCC</td>
<td>42</td>
</tr>
<tr>
<td>2. Correlation between HBx and E-cadherin, mSin3A and Snail-1 in HBV-mediated HCC</td>
<td>56</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Hepatitis B Virus and Hepatocellular Carcinoma

Primary liver cancer is the fifth most common cancer type and the third leading cause of cancer mortalities in the world, resulting in approximately 600,000 deaths each year (WHO, 2009). According to the World Health Organization, chronic hepatitis B virus (HBV) infections are the cause of at least 80% of these deaths (WHO, 2009). Epidemiologic evidence for the correlation between chronic HBV infection and hepatocellular carcinoma (HCC) is greatly supported by the detection of integrated HBV DNA sequences in HBV-related HCC (Feitelson, 1986; Shafritz & Rogler, 1984). An estimated 350 million people worldwide are chronically infected with HBV, and are at risk for the development of cirrhosis, and HCC (WHO, 2009). Of those who develop HCC, life expectancy from the time of diagnosis is only about 6 months, with a mean survival rate of only 3% over 5 years (Feitelson et al., 2002). Treatment options for HCC include surgical resection or liver transplantation but less than 10% of patients are deemed suitable for treatments (Lai et al., 1995). In most cases early HCC is asymptomatic, and late diagnoses result in inoperable advanced tumors (Khatri & Schneider, 2004). Attempts to prevent progression of HBV-mediated HCC through antiviral therapy have only been transiently effective in a small proportion of patients (Sorrell et al., 2009). Furthermore, identification of suitable candidates is complicated by the lack of identified early markers of HCC.
development. The complication is a result of little understanding of the molecular mechanisms by which HBV contributes to the pathogenesis of HCC. Until we can fully elucidate the mechanism that drives the development and progression of HCC in HBV infected patients, therapeutically targeting the appropriate pathways is futile.

Over the last three decades, many studies have contributed to the understanding that HBV is an important etiologic agent of HCC. It is well documented that HBV DNA is integrated, although often highly rearranged, within host DNA in tumors and tumor-derived cell lines (Feitelson, 1986; Shafritz & Rogler, 1984). HBV DNA integrates into many sites within multiple chromosomes (Matsubara & Tokino, 1990), and appears to promote loss of heterozygosity (LOH) (Hino et al., 1989), which is common in HCC (Ding & Habib 1995). Furthermore, although no cellular oncogenes are consistently activated (Shiozawa et al., 1988; Gu et al., 1988), much evidence exists for the involvement of viral antigens in trans-activation pathways (Kekule et al., 1990).

1.2 Hepatitis B Virus Genome

HBV is a reverse transcribing dsDNA virus of the Hepadnaviridae family that naturally infects human hepatocytes. The circular, partially double-stranded genome encodes four families of proteins, known as the surface/envelope (pre-S/S), core (C), X, and polymerase (P) polypeptides that are targeted by the immune system upon infection (Feitelson, 1989; Fig. 1). The pre-S/S open reading frame encodes the large (L), middle (M), and small (S) surface
glycoproteins and collectively make up the hepatitis B s antigen (HBsAg). The pre-core/core open reading frame is translated into a pre-core polypeptide, which is modified into a soluble protein, hepatitis B e antigen (HBeAg) and the nucleocapsid protein, hepatitis B core antigen (HBcAg) (Feitelson, 1992). The polymerase protein functions as a reverse transcriptase as well as a DNA polymerase. The X protein is the hepatitis B x antigen (HBxAg or simply HBx) and is strongly implicated as the major genetic contributor to HBV-mediated HCC (Feitelson and Duan, 1997).

Figure 1. Schematic organization of the hepatitis B virus genome. The HBV genome is a relaxed circular, partially double stranded DNA of about 3200 base pairs. There are four partially overlapping open reading frames encoding the envelope (pre-S/S), core (precore/core), polymerase (P), and X proteins (X) (Figure from Park et al., 2006).
Upon HBV infection, viral DNA is transported to the nucleus, where it is converted into covalently closed, circular, double-stranded DNA (cccDNA). The cccDNA is the template for all viral transcription but is not used for viral replication (Summer et al., 1975). Instead, host enzymes transcribe the cccDNA into the four viral mRNAs and the large pregenomic RNA (pgRNA) that serves as the template for viral replication. The pgRNA becomes encapsidated within newly assembled viral capsids composed of core protein in the cytoplasm, together with viral polymerase. Similar to retrovirus replication, the viral polymerase reverse transcribes the pgRNA to synthesize new viral DNA (Summers and Mason, 1982). As the virus replicates, it buds into the endoplasmic reticulum by envelopment within the viral HBsAg proteins and is eventually secreted from the infected cell as mature virions (Ganem and Schneider, 2001), while a small portion of core particles is transported back to the nucleus to maintain a stable pool of cccDNA. Unlike retroviruses, HBV does not encode an integrase protein since integration into the host genome is not required for replication. However, integration events do occur, although often highly mutated and rearranged. Presumably, these integration events are responsible for persistent expression of viral proteins, like HBx, even after viral infection is cleared (Diamantis et al., 1992). It has already been well established that persistent expression of HBx correlates with the transformation of hepatocytes (Henkler & Koshy, 1996), but the exact mechanisms of hepatocarcinogenesis are unclear.
1.3 **Hepatitis B x Antigen**

HBx is the most prevalent virus antigen in the livers and tumors of HBV carriers, and strongly correlates with chronic liver disease (CLD) (Diamantis et al., 1992). Accordingly, HCC is shown to develop in HBx transgenic mice with sustained, high levels of HBx (Kim et al., 1991; Koike et al., 1994), but when HBx expression is low or absent, HCC does not develop (Lee et al., 1990). HBx has transcriptional trans-regulatory properties that alter patterns of host gene expression (Feitelson and Duan, 1997; Diamantis et al., 1992), inhibits proteasomal degradation of growth regulatory proteins (Huang et al., 1996), stimulates cellular kinases that alter signal transduction (Henkler and Koshy, 1996), and utilizes epigenetic machinery (Zheng et al., 2009). These mechanisms provide some insight to how HBx contributes to the development of HCC, but the overall specific mechanisms leading to hepatocyte transformation have yet to be elucidated.

HBx has a molecular mass of approximately 17.5 kDa and it is 154 amino acids in size. The three-dimensional structure of HBx is poorly characterized since HBx eludes high-resolution crystallization and nuclear magnetic resonance. However, it is suspected that HBx amino acids 52 to 148 are essential for its various reported activities (Yen, 1996). Furthermore, deletion of its N-terminal amino acids 1 to 50 was shown to regulate the transcriptional functions of HBx, suggesting that this region may act as a negative regulatory element (Murakami et al., 1994). Higher-order structures of HBx are also poorly characterized, but evidence of HBx-dimerization exists (Ganem and Schneider, 2001).
HBx is reported to interact with almost all components of regulation within the cell, including components of basal transcription machinery, various transcription factors, cell cycle regulatory proteins, kinases, mitochondrial membrane proteins, and subunits of the proteosome (Ganem and Schneider, 2001). The cellular location of HBx may govern its role in these interactions. For example, HBx has been reported to be localized primarily in the cytoplasm but also detectable in the nucleus (Sirma et al., 1998; Weil et al., 1999). Although HBx was thought to be small enough to diffuse passively through the nuclear pore (Haviv et al., 1998), it has also been determined that HBx can be introduced into nuclei by protein-protein interaction (Nomura et al. 1999; Weil et al., 1999). However, more recent observations of the HBx sequence revealed a short, hydrophobic, leucine-rich nuclear export signal motif (NES) located in the center region of HBx (residues 89–100) (Forgues et al., 2001). Other studies indicate that HBx is also found in the mitochondria where it is able to associate with the outer membrane of mitochondria and induce oxidative stress (Waris et al., 2001; Kim et al., 2007; Takada et al., 1999).

Thus, HBx appears to exert effects upon pathogenesis in most of the subcellular compartments (Keasler et 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 and Hildt, 2007; Fig. 2). Several studies have demonstrated that HBx can interact with and/or stimulate many kinases. Some of these include Src, PKC, Jak1, IKK, PI3K, Akt/PKB, ERKs, and SAPK/JNKs (Benn et al., 1996; Tarn et al., 1999; Tarn et
al., 2001; Lara-Pezzi et al., 2001; Tarn et al., 2002). For example, HBx was shown to constitutively activate Src kinases (Klein et al., 1999; Klein et al., 1997), which promotes alteration of cellular adherens junctions (Lara-Pezzi et al., 2001). Other studies have shown that HBx is able to stimulate Phosphatidylinositol 3 kinase (PI3K) and its downstream target, protein kinase B (PKB)/Akt, inhibiting apoptotic death through an HBx-PI3K-Akt-Bad-dependent pathway (Lee et al., 2001). HBx has also been shown to activate the MAPK signal cascade (Chirillo et al., 1996; Menzo et al., 1993; Wang et al., 1997; Henkler et al., 1998; Benn and Schneider, 1995). HBx-mediated activation of the RAS-RAF-MAPK pathway is associated with accelerating entry of cells into S phase (Mansour et al., 1994), detrimentally affecting the cell cycle.

HBx affects the regulation of apoptosis and promotion of survival through several other mechanisms, including interaction with caspases, transcription factors, and survivin (Han et al., 2000; Gottlob et al., 1998). HBx has been reported to block cell death mediated by TNF-α, Fas, p53, or TGFβ (Elmore et al., 1997, Pan et al., 2001; Shih et al., 2000). It has been reported that HBx is an activator of transcription factor NFκB, which was one of the first HBx-responsive motifs to be identified (Seto et al., 1990; Lucito et al., 1992). NFκB stimulated by HBx promotes the survival of liver cells against Fas-mediated apoptosis (Pan et al., 2001). Furthermore, nuclear HBx can interact with numerous other transcription factors (p53, HIF-1α, E4F, SMAD4, among many others) and components of the basal transcription machinery (RPB5, TFIIB, TBP, and TFIIH, among others) (Ganem and Schneider, 2001) altering expression of various host
genes. Hence, HBx is the viral contribution to HCC and may contribute to hepatocarcinogenesis through various mechanisms (Fig. 2).

Figure 2. Putative signal transduction pathways and cellular factors affected by HBx. HBx may cause increased proliferation, cell differentiation, apoptosis and malignant transformation by either activating or inhibiting various signal transduction pathways and/or by interaction with transcription factors (Figure from Zhang et al., 2006).

1.4 Cancer Stem Cells

Somatic stem cells are capable of self-renewal and differentiation into one or more mature cell types to replace cell loss due to senescence or damage (Wu, 2008). A small amount of adult stem cells are present in many tissues, and may persist throughout the entire human lifespan (Pardal et al., 2003). These cells possess unique characteristics, such as motility, enhanced resistance to
apoptosis, and an ability to interact with appropriate factors at a secondary location to ensure their continued survival and proliferation. These features make them particularly suitable targets for carcinogens (Croker and Allan, 2008). Within established tumors, the great majority of cancer cells cannot sustain the tumor mass, nor metastasize. Instead, only a minority of cancer cells appear to be tumor initiating and possess the metastatic phenotype (Piscaglia, 2008). These cells are capable of self-renewal, prolonged survival, and the ability to differentiate into any cell within the tumor population. Given the similarities between tumor-initiating cells and normal stem cells, tumor-initiating cells have been termed cancer stem cells (CSC).

Since normal stem cells and cancer stem cells must renew themselves, pathways and factors implicated in embryonic stem cell (ES) self-renewal may also play a role in carcinogenesis (Lobo et al., 2007; Stewart et al., 2006). These "stemness" factors (such as Pou5f1 encoded transcription factor Oct-4, Sry-related HMG box transcription factor Sox2, and the homeobox transcription factor Nanog) and their binding partners act as key regulators of pluripotency in early mammalian development and form a regulatory feedback circuit that maintains pluripotency (Rodda et al., 2005; Niwa, 2007; Loh et al., 2006; Chen et al., 2008; Pan et al., 2007; Nichols et al., 1998; Niwa et al., 2000). Another important factor associated with "stemness" is Krüppel-like factor 4 (Klf-4) based on its use in reprogramming differentiated somatic cells into inducible pluripotent stem cells (Takahashi and Yamanaka, 2006; Evans and Liu, 2008). Furthermore key signals provided by components of the Wnt pathway, LIF/STAT3, c-Myc, EpCAM
and EMT are also implicated in the achievement of pluripotent phenotype (Jaenisch and Young, 2008; Munz et al., 2009; van Zijl et al., 2009).

Although the mechanisms inducing pluripotency remain elusive (Wang et al., 2006), these “stemness” genes and pathways are re-expressed in cancer cells (Monk et al., 2001), and the reactivation of these pluripotency associated factors contributes to tumorigenesis in somatic tissues (Hochedlinger et al., 2005). For example, Oct-4 and Nanog expression was observed in seminoma, retinoblastoma, oral squamous cell carcinoma, bone sarcoma, breast and a colon cancer cell lines compared to their normal counterparts (Jin et al., 1999; Uche et al., 2005; Hart et al., 2005; Chiou et al., 2008; Gibbs et al., 2005). Furthermore, strongly positive clusters of Oct-4-positive cells were observed in HCC (Tang et al., 2008). Overexpression of Sox2 has also been observed in brain, colon, and prostate cancers, in lung adenocarcinoma, and in HCC (Schoenhals et al., 2009; Dong et al., 2004). In other tumor types, such as breast and squamous cell carcinomas, Klf-4 expression is also shown to be elevated (Schoenhals et al., 2009; Pandya et al., 2004; Huang et al., 2005).

It is not yet clear as to whether CSCs originate from adult stem cells that have lost the ability to regulate their proliferation or from differentiated cells that acquire the ability to self-renew as a result of oncogenic mutations (Sell et al., 2008). It is proposed that cell proliferation at the time of carcinogen exposure is pivotal for “fixation” of genotoxic injury into a heritable form. Therefore, any proliferative cell in an organ, such as the liver, can be susceptible to neoplastic transformation (Alison, 2005). In normal adult liver, mature hepatocytes are
Hepatocytes are highly differentiated cells with a long lifespan, which can reenter the cell cycle and restore the liver mass in response to parenchymal loss (Piscaglia, 2007). This is an efficient system, so that after 2/3 partial hepatectomy in rats, proliferation of hepatocytes and cholangiocytes can restore the original liver mass in < 2 weeks, suggesting that they may act as functional liver “stem” cells, with high clonogenic potential, as shown in serial transplantation experiments (Roskams, 2006; Piscaglia, 2007).

In HBV associated CLD, 0.3% - 3% of all hepatocytes are killed daily and replaced to maintain a stable liver cell mass (Nowak et al., 1996). By the time cirrhosis develops, hepatocyte proliferation rates fall, and regeneration can be achieved by the activation and differentiation of liver stem cells into the hepatic and/or biliary lineages. This “ductular” reaction in human liver is equivalent to the oval cell reaction seen in many rodent models of HCC (Alison, 2005; Marshall et al., 2005; Roskams et al., 2003). Importantly, hepatic progenitor cells are activated in the majority of liver diseases, and the extent of activation is correlated with disease severity. In CLD, both progenitor cell activation and HBx expression levels correlate with the severity of inflammation (Piscaglia, 2007; Libbrecht and Roskams, 2002; Wang et al., 1991; Jin et al., 2001). Most HCCs arise in a cirrhotic liver, i.e., characterized by long-standing hepatocyte damage and chronic inflammation leading to fibrosis. HBx expression also closely correlates with the development of fibrosis and cirrhosis (Norton et al., 2004).

A minor population of cells with CSC properties has been detected in a number of established hepatocellular carcinoma (HCC) cell lines (Chiba, 2009).
Recent microarray and genomic analyses of HCC have disseminated important molecular similarities between CSCs and liver stem cells that also reveal several key stemness and oncogenic pathways dysregulated in hepatocarcinogenesis (Marquardt, 2010; Lobo et al., 2007; Stewart et al., 2006). Independent observations suggest that HCC arises from dysfunctional liver stem cells (Libbrecht, 2006; Sun et al., 2008), which raises the question as to whether HBx contributes to liver stem cell dysfunction.

1.5 Epithelial Cell Adhesion Molecule

EpCAM (Epithelial Cell Adhesion Molecule) is a 39-42-kDa transmembrane glycoprotein that mediates calcium-independent homotypic adhesion. It is not structurally related to any of the major families of the adhesion molecules (Balzar et al., 1999). The EpCAM protein is composed of a large extracellular domain (EpEX) with EGF-like repeats responsible for the formation of inter- and intracellular adhesions (Fornaro et al., 1995), a transmembrane part and a short cytoplasmic domain (EpICD) of 26 amino acids (Balzar et al., 1999).

EpCAM is detected on subsets of normal epithelia (bile duct epithelium), numerous tissue stem and progenitor cells (fetal hepatoblasts and hepatic stem cells), in cancer-initiating cells, and most carcinomas including HCC (Gastl et al., 2000, Schmelzer et al., 2006; Dan et al., 2006; Dalerba et al., 2007; Yamashita et al., 2009). EpCAM was shown to be a reliable diagnostic and prognostic marker for carcinomas (Gastl et al., 2000), and is a target in tumor therapy trials (Riethmüller et al., 1994). Adult hepatocytes do not express EpCAM (Breuhahn
et al., 2006). Moreover, EpCAM is strongly associated with the maintenance of the undifferentiated state of human embryonic stem cells (hESCs); therefore, it is considered as a new surface marker of undifferentiated hESCs (Ng et al., 2010; Lu et al., 2010).

EpCAM has recently been shown to act as a mitogenic signal transducer in vitro and in vivo via nuclear translocation of the EpICD, in association with FHL2, β-catenin, and Lef-1, after intramembrane proteolysis of the EpCAM catalyzed by TACE (TNF-α converting enzyme) and PS-2 (presenilin-2) (Maetzel et al., 2009; Munz et al., 2004) (Figure 3). FHL2 (Four and a Half LIM-2) participates in various transcription and signal transduction pathways (Johannessen et al., 2004). Through its function as a co-activator of β-catenin (Wei et al., 2003), FHL2 serves as a scaffolding protein, bridging together EpCAM with transcriptional regulators β-catenin and Lef-1 and thus providing interaction of EpCAM with DNA. A role of FHL2 in EpCAM signaling was also confirmed by reports of the interaction of FHL2 with TACE and PS-2 (Canault et al., 2006; Kang et al., 2005). Involvement of TCF/Lef-1, a major regulator of c-Myc and cyclin E expression (Shtutman et al., 1999), explains the ability of EpCAM to rapidly upregulate expression of c-Myc, cyclin E and induce cell proliferation. Importantly, EpCAM has also ability to sustain “stemness” through EpICD binding to c-Myc, Oct4, Nanog, Sox2, and Klf4 promoter regions, promoting the expression of these genes in human ES cells (Lu et al., 2010). Hence, full-length EpCAM should be viewed as a precursor for its mitogenic signaling moiety EpICD (Munz et al., 2009).
Figure 3. Activation of EpCAM. Upon cleavage, EpICD translocates into the nucleus in a multiprotein complex. Together with FHL2, β-catenin, and Lef-1, EpICD binds DNA at TCF/Lef-1 consensus sites (Modified from Lu et al., 2010)

Recent studies demonstrated that altered expression of specific miRNAs is involved in tumorigenesis (Croce et al., 2005). Moreover, a distinct miRNA subset is specifically expressed in pluripotent embryonic stem cells but not in adult tissues (Suh et al., 2004). Array data from 148 hepatitis B virus (HBV)-positive samples and their paired non-HCC tissues were used to search for HCC-associated miRNAs (Ji et al., 2009). It was shown that highly conserved miR-181 family members are overexpressed in both hepatic normal stem and tumor initiating cells and thus contribute to the maintenance of “stemness.” Forced miR-181 expression enriched EpCAM-positive HCC cells with stem cell properties, whereas inhibition of miR-181 induced differentiation (Ji et al., 2009).
Expression of EpCAM has been linked to the formation of metastases due to negative modulation of E-cadherin-mediated cell adhesion by disruption of the link between α-catenin and F-actin. EpCAM not only weakens/abrogates the cadherin-mediated adhesions, but replaces them with EpCAM adhesions becoming predominant to some extent (Litvinov et al., 1997) and in this way regulates proliferation and tissue maintenance (Winter et al., 2003). These findings were confirmed by the fact that elevated EpCAM expression was linked to an increase in lymph node metastases (Seligson et al., 2004). Furthermore, cell migration did not occur in renal and breast carcinoma cell lines after EpCAM was down-regulated using siRNA (Osta et al., 2004). Thus, EpCAM is not only a marker of adult stem cells and carcinomas that can activate pluripotent markers, but also functions as a negative regulator of cell adhesion.

1.6 Wnt/β-catenin

Beta-catenin (β-catenin) is a 92kDa protein encoded by the CTNNB1 human gene (Kraus et al., 1994). The catenin family of proteins are specialized for protein-protein binding since they contain multiple copies of the armadillo repeat domain (Huber et al., 1997). An important ability of β-catenin is the mutually exclusive binding among various complexes present in different cell compartments. The specific sub-cellular localization of β-catenin and the precision of its concentration in each compartment contribute to the multiple functions of this single molecule. In addition, the binding abilities and various functions of β-catenin are in part regulated by its phosphorylation (Müller et al.,
β-catenin is found in three cell compartments: the plasma membrane, the cytoplasm and the nucleus. In general, β-catenin exists either in complex with E-cadherin regulating adhesion at the plasma membrane; in a cytoplasmic complex with axin, APC, and GSK3β, where it is serine and threonine phosphorylated and targeted for degradation; or as a nuclear cofactor in complex with TCF/LEF transcription factors (Hülsken et al., 1994; von Kries et al., 2000; Behrens et al., 1998).

β-catenin is found at the plasma membrane when bound tightly to epithelial cadherin (E-cadherin) (Fig. 4). There it is a central component that links E-cadherin to the actin cytoskeleton in cell-cell adhesion (McCrea et al., 1991; Rimm et al., 1995). Together with α-catenin and p120-catenin (p120\text{ctn}) this functional complex is necessary for adhesion and the maintenance of epithelial cell layers. α-Catenin is an actin binding protein that links the cadherin complex to the actin cytoskeleton (Rimm et al., 1995) while p120\text{ctn} regulates cadherin surface levels by antagonizing endocytosis (Davis et al., 2003) and promoting cadherin clustering (Yap et al., 1998).

The structural and functional integrity of the cadherin-catenin complex is regulated by phosphorylation. Phosphorylation of E-cadherin at serine and threonine residues results in an increased stabilization of the cadherin-catenin complex (Lickert et al., 2000). Conversely, tyrosine phosphorylation of β-catenin by Src kinases, disrupts binding to the cadherin-catenin complex. Specifically, phosphorylation of β-catenin at tyrosine 142 disrupts binding with α-catenin (Piedra et al., 2003; Ozawa and Kenler, 1998), while phosphorylation at tyrosines
or 654 reduces β-catenin’s affinity for E-cadherin (Roura et al., 1999; Rhee et al., 2002). These phosphorylation events result in dissociation of β-catenin from the complex. In order to maintain the cadherin-catenin complex, the cytoplasmic domain of cadherin utilizes a tyrosine phosphatase, PTP1B, to maintain β-catenin in a de-phosphorylated state (Lilien et al., 2005). In general, activation of tyrosine kinases results in a loss of cadherin mediated cell-cell adhesion and an increase in the level of cytoplasmic β-catenin, in a free, un-complexed and tyrosine-phosphorylated form (Müller et al., 1999). In this way, E-cadherin acts as a negative regulator of β-catenin signaling as it binds β-catenin at the cell surface thereby sequestering it from the nucleus (Fagotto et al., 1996). Therefore, phosphorylation dependent release of β-catenin from the cadherin complex not only regulates the integrity and function of the adhesion complex, but also provides an alternative mechanism for activating β-catenin signaling (Nelson et al., 2004).

In addition to its obligatory role in cadherin-mediated adhesion, β-catenin is also implicated as an integral component in the Wnt signaling pathway (Hinck et al., 1994; Papkoff et al., 1996). Cytoplasmic β-catenin that is tyrosine phosphorylated and thus, free from the cadherin-catenin complex functions in transducing Wnt signals from the cell surface to the nucleus (Fig. 4). Reception of the Wnt signal at the cell surface by Frizzled family members results in inactivation of the negative regulator of β-catenin, GSK3β, allowing for stabilization and accumulation of cytoplasmic β-catenin (Cook et al., 1996). Once accumulation occurs, β-catenin translocates to the nucleus where it
interacts with the N-terminus region of members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of DNA binding proteins (Behrens et al., 1996; Huber et al., 1996; Aoki et al., 1999) and acts as a cofactor to activate transcription of Wnt responsive genes and other genes involved in cell proliferation (such as c-Myc, cyclin D1 and EpCAM) (He et al., 1998; Shtutman et al., 1999; Tetsu et al., 1999; Yamashita et al., 2007).

In the absence of the Wnt signal, cytoplasmic β-catenin rapidly degrades and only tyrosine phosphorylated β-catenin and β-catenin complexed with cadherins are protected from degradation (Figure 4). This is because Wnt signaling inactivates cytoplasmic glycogen synthase kinase 3 beta (GSK3β) (Cook et al., 1996). Otherwise, GSK3β phosphorylates β-catenin at specific serine and threonine residues targeting it for ubiquitination and degradation, thus maintaining cytoplasmic β-catenin at a low level (Aberle et al., 1997) (Fig. 4). Phosphorylation of β-catenin by GSK3β occurs within a multi-protein complex containing the adenomatous polyposis coli (APC) tumor suppressor protein and axin (Behrens et al., 1998; Ikeda et al., 1998). Typically, phosphorylation of N-terminus residues marks β-catenin for degradation. These include serines 33, 37 and 45 as well as threonine 41 and provide a docking site for the E3 ubiquitin ligase, β-TRCP1 (Kikuchi et al., 2006). Conversely, phosphorylation of C-terminus residues by other kinases favors the Wnt signaling pathway. These include serines 657 and 552, as well as tyrosines 654, 489 and 142.

The evolutionarily conserved Wnt signaling pathway has pivotal roles during the development of many organ systems, and its deregulation is a key
factor for the initiation of various tumors. For example, aberrant activation of β-catenin plays a major role in promotion of neoplastic growth (Aberle et al., 1996). Accordingly, the subsequent relocation of β-catenin into the nucleus is capable of inducing a gene expression pattern favoring tumor invasion and tumor cell proliferation (Inagawa et al., 2002). In fact, nuclear accumulation of β-catenin has been found in breast (Uchino et al., 2010), colorectal (Miyamoto et al., 2004), ovarian (Ling Poon et al., 2011), liver (Park et al., 2001) and other cancers. Furthermore, the development of malignant tumors is in part characterized by the ability of a tumor cell to overcome cell–cell adhesion and to invade surrounding tissue (Perl et al., 1998). Pathways that stimulate the tyrosine phosphorylation and subsequent release of β-catenin from the cadherin-catenin complex may be implicated in achieving this particular phenotype.

There is growing evidence that the Wnt signaling is also important in stem cell self-renewal and in carcinogenesis (Reya and Clevers, 2005; Kleber and Sommer, 2004; James et al., 2005). Wnt signaling results in the accumulation of β-catenin, which promotes transcription of target genes in the nucleus in association with TCF/Lef1 and DNA-binding transcriptional regulators of the canonical Wnt signaling pathway (Katoh, 2007; Dreesen and Brivanlou, 2007). 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 a mechanism whereby β-catenin regulates the expression of “stemness” genes (Lowry et al., 2005). Importantly, in the absence of β-catenin, TCF proteins function as transcriptional repressors instead (Marson et al., 2008;
Pereira et al., 2006). Thus, presence of β-catenin is rate-limiting in the activation of these “stemness” genes. Supporting this idea, studies with hepatocytes have revealed that diminished nuclear β-catenin causes differentiation into mature hepatocytes while nuclear accumulation induces dedifferentiation into immature hepatocyte progenitors (Marson et al., 2008; Zulehner et al., 2010).

Importantly, increased β-catenin activation is sufficient to cause hepatocyte proliferation and expansion of the liver. The activated Wnt/β-catenin pathway is now considered to be one of the main driving forces of hepatocarcinogenesis (Taniguchi et al., 2008; van Zijl et al., 2009). In fact, about 50%–70% of HCCs demonstrate an abnormal β-catenin protein accumulation in the cytoplasm and nucleus (de La Coste et al., 1998; Suzuki et al., 2002; Wong et al., 2001). β-Catenin activation is also implicated in HBV-mediated HCC.

Increasing evidence suggests that HBx promotes tumorigenesis, in part, by constitutively activating wild type β-catenin (Lian et al., 2006; Fodde & Brabletz, 2007). A possible mechanism whereby HBx activates β-catenin involves the inactivation of its regulator, GSK3β. HBx is capable of activating both the Erk (Ding et al., 2005) and PI3K (Lee et al., 2001) signaling pathways. These activated kinases can phosphorylate and inactivate GSK3β resulting in subsequent activation of β-catenin (Ding et al., 2005; Lee et al., 2001; Cha et al., 2004; Fig. 4). HBx has also been shown to induce adherens junction disruption in a Src kinase-dependent manner, resulting in accumulation of cytoplasmic β-catenin (Lara-Pezzi et al., 2001; Fig. 4). The fact that β-catenin is capable of activating “stemness” pathways and that HBx not only activates the activity of β-
catenin but also transcriptionally activates β-catenin in up to 80% of HCCs (Ding et al 2004; Lian et al., 2006; Fodde and Brabletz, 2007) underscores the potentially close relationship between HBx, activated β-catenin, and stem cell renewal.

**Figure 4. Role of β-catenin in Wnt signaling and the cadherin complex.** General summary of the canonical Wnt signaling pathway (right) and β-catenin's role as a cytoskeletal adhesion intermediate (left). Three modes of HBx-mediated activation of β-catenin are also shown (Figure adapted from Howard et al., 2003).

1.7 **E-cadherin**

E-cadherin is a single-span calcium-dependent transmembrane glycoprotein that provides a physical link among adjacent cells and is crucial for the establishment and maintenance of cell polarity and the structural integrity of
As the core molecule of adherens junctions, E-cadherin connects neighboring epithelial cells by calcium-dependent homotypic interactions of its extracellular tail (Nollet et al., 2000). Intracellular domains bind tightly to a multi-protein complex comprising α-, β- and p120 catenins which in turn anchors to the actin cytoskeleton thereby mediating mechanical stability (Guarino et al., 2007; Wheelock and Johnson, 2003; Xu et al., 2009). By linking together the cells, E-cadherin maintains epithelial cells in a stationary, non-motile state and, therefore, any event that perturbs the cadherin/catenin/cytoskeleton complex leads to reorganization of the actin cytoskeleton and destabilization of cell-cell adhesion (Guarino et al., 2007; Hajra and Fearon, 2002). In this regard, abnormalities in expression and cellular distribution of E-cadherin are frequently associated with de-differentiation and invasiveness in a variety of human malignancies including primary HCC (Endo et al., 2000). E-cadherin is also a critical factor in the process of intra-hepatic metastasis of HCC (Osada et al., 1996). It has been reported that E-cadherin expression may be regulated at genetic (e.g. E-cadherin coding CDH1 gene mutation, LOH) and/or epigenetic (e.g. CDH1 promoter methylation) levels (Jiao et al., 2002).

The developmental process whereby epithelial intercellular adhesion is lost and fibroblastoid properties are gained, is known as the epithelial-mesenchymal transition (EMT). The invasiveness of human cancers relies on this transition and targets E-cadherin expression to achieve it (Vleminckx et al., 1991; Iwatsuki, 2009). In fact, 50-80% of highly metastatic carcinomas
downregulate expression or function of E-cadherin or α-catenin, (Endo et al., 2000). Re-establishing the function of E-cadherin reverts tumor cell lines to a benign epithelial phenotype (Vleminckx et al., 1991) and arrests tumor development (Perl et al., 1998). This is in part due to the reversal of EMT to mesenchymal-epithelial transition (MET) that occurs when E-cadherin is activated. Another characteristic change during EMT is the translocation of β-catenin into the nucleus after dissociation of the membrane complex. Indeed, both the down-regulation of E-cadherin and the increased expression of non-membranous β-catenin are frequently found in HCC cases (Osada et al., 1996; Endo et al., 2000).

Accumulating evidence suggests that E-cadherin silencing in cancer progression involves epigenetic mechanisms that include hypermethylation at CpG islands and/or histone deacetylation of the CDH1 gene promoter region. In fact, hypermethylation of the CDH1 promoter has been identified in several human tumors, including prostate, breast, and liver (Conacci-Sorrell, 2002). DNA methylation often causes downregulation of tumor suppressor genes in cancer cells by making the DNA inaccessible for basal transcription machinery like RNA polymerase II. Specifically, a large CpG island in the 5’ proximal promoter region of the E-cadherin gene, which is located on 16q22.1, is known to have aberrant DNA methylation in cancer progression (Osada et al., 1996; Van Roy et al., 2008). This mechanism is described to increase with the progression of HCC, which implicates its significance in hepatocarcinogenesis (Kanai, 1997).
Furthermore, HBV-related tumors also show the association between CpG methylation and loss of E-cadherin. Specifically, E-cadherin was shown to be suppressed at both the mRNA and protein levels in HBx positive cells (Liu et al., 2006). This can be explained by the ability of HBx to increase the activity of DNA-methyltransferases (DNMTs) by inducing DNMT1 and DNMT3A. DNMT1 is responsible for maintaining proper methylation states after replication while DNMT3A is responsible for de novo methylation and the establishment of new methylation patterns (Lee et al., 2005). The HBx-mediated increase in activity of these methyltransferases provides the strong foundation to promote tumorigenesis through aberrant hypermethylation (Tischoff et al., 2008).

Recent evidence indicates another important epigenetic mode of E-cadherin repression. The zinc finger transcription factor Snail is a well-known inducer of EMT due to its strong repression of E-cadherin transcription (Batlle, 2000). Snail is composed of a highly conserved C-terminus region, containing four to six zinc fingers and a basic helix-loop-helix (bHLH) domain that functions in binding DNA at E-box motifs. The E-boxes are made up of the following sequence: CANNTG, and are regulatory elements upstream of the CDH1 gene and other Snail-related genes (Nieto, 2002). Upon binding to the three E-boxes of the CDH1 promoter (Giroldi et al, 1997; Batlle, 2000) it is capable of recruiting the mSin3A/HDAC complex (Peinado, 2004). The interaction between Snail and the mSin3A/HDAC complex creates a multi-molecular complex that is dependent on the SNAG domain of Snail (Peinado et al., 2004).
It was demonstrated that 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 specifically over-expressed in the cells located at the invasive front of tumors (Guarino et al., 2007), promoting the acquisition of invasive properties by tumors. Snail genes are activated by most pathways triggering epithelial/mesenchymal transition (Xu et al., 2009). Such pathological activation of Snail and repression of E-cadherin are observed during fibrosis and tumor progression (Moustakas and Heldin, 2007). These pathways occur in HBV-mediated HCC as well, but whether or not HBx upregulates Snail to utilize this mechanism of E-cadherin repression is unknown.

1.8 mSin3A/HDAC

It is now widely accepted that cancer is in part an epigenetic disease (Feinberg et al., 2006). Epigenetic modifications require the dynamic activity of DNMTs, histone modifying enzymes, and their corresponding interacting cellular factors (Li et al., 2005; Russo et al., 1996). The major enzymatic histone-modifiers include histone deacetylases (HDACs), histone methyltransferases (HMTs), and their antagonists, histone acetyl-transferases (HATs) and histone demethylases (Strahl and Allis, 2000; Sterner and Berger, 2000). The pattern of covalent modifications of histone tails and the apparent association of these modifications with DNA methylation machinery is often referred to as the histone
code (Jenuwein and Allis, 2001). Given the complexity of these epigenetic pathways, the susceptibility for dysregulation defines its role is carcinogenesis.

Overexpression and aberrant activity of HDACs have been shown in a variety of cancers, including breast, prostate, colorectal and HCC (Glozak and Seto, 2007). Recent evidence revealed that high HDAC1 expression in HCC has a direct correlation with its aggressiveness (Rikimaru, 2007). Furthermore inhibition of estrogen receptor alpha expression by HDAC1 activity implicates its role in breast cancer progression (Kawai et al., 2003). Of the four classes of histone deacetylases, class I HDACs are strictly nuclear. These include HDAC1, HDAC2 and HDAC3, however HDAC1/2 are ubiquitously expressed and seem to be involved in more-general cellular processes, making them greater targets for carcinogenic manipulation. These enzymes are only functional when bound to a scaffolding complex such as the mSin3A, NuRD or CoREST complexes (Ahringer, 2000; Zhang et al., 1999; You et al., 2001). HDAC1/2 have no intrinsic DNA binding domain and must be recruited to DNA through interactions with transcription factors or other co-factors.

HDACs function by removing acetyl groups from lysine residues on histones tails or non-histone proteins. This removal changes amides on the lysine amino acids into amines, which carry a positive charge. For histones, a positive charge attracts negatively charged phosphate groups on the DNA backbone, resulting in the condensed heterochromatic DNA that is associated with repressed gene transcription (Marks et al., 2003). Therefore, deacetylation of histone tails by HDAC1/2 negatively regulates gene expression. Deacetylation of lysine residues
on non-histone proteins has several effects. In some cases the protein becomes inactivated, as seen when deacetylation of the C-terminal region of p53 causes a conformational change so that the C-terminus sterically hinders the central DNA binding domain, rendering it inactive (Gu and Roeder, 1997). In other cases deacetylation may target a protein for degradation since unacetylated lysine residues can be targeted for ubiquitination (Ito et al., 2002).

As previously stated, the enzymatic function of HDAC1/2 requires the association with a scaffolding complex. The mSin3A/HDAC co-repressor complex, is known for various binding capabilities. The large, 150 kD mSin3A protein acts as a scaffold, assembling a variety of transcription factors and cofactors together with the enzymatic activity of HDACs to repress specific target genes and/or gene products (Ayer et al., 1995) (Figure 5). The protein-protein interactions associated with mSin3A are mediated by its four imperfect repeats of paired amphipathic helices (PAHs 1-4) (Wang et al., 1990; Sahu et al., 2008). The core components assemble closer to the C-terminal end, in between PAH3 and 4 at the HDAC interaction domain (HID), while most other binding partners associate near PAH 1 and 2. The core components include mSin3A, HDAC1, HDAC2, SDS3, RBBP4, RBBP7, SAP30, and SAP18, each of which contribute to the structural integrity of the complex but also have individual roles (Ayer et al., 1995; Zhang et al., 1997; Hassig et al., 1997; Laherty et al., 1997). For example SDS3 is crucial for the stability of the whole complex and is a necessity for the sustained association between mSin3A and HDAC1/2 (Lechner et al., 2000). The retinoblastoma binding proteins 4 and 7 (RBBP4/7) maintain association
between the complex and the nucleosome after the complex has been recruited there (Zhang et al., 1997; Zhang et al., 1999). SAP30 and SAP18 serve as bridging molecules, both among the core components, and between the complex and other co-repressors such as RBP1 (Zhang et al., 1997; Zhang et al., 1998; Lai et al., 2001; Espinas et al., 2000). These and other protein-protein interactions are integral to the function of the complex since it has no intrinsic DNA binding domain.

Figure 5. The mSin3A/HDAC complex. The core complex is comprised of members with specialized roles in stabilizing interactions within the complex as well as with other factors (Grzenda et al., 2009; Wang et al., 1990; Ayer et al., 1995; Hassig et al. 1997; Laherty et al. 1997; Zhang et al. 1997; Zhang et al. 1998; Espinas et al., 2000; Lechner et al., 2000; Lai et al. 2001; Silverstein et al. 2005; Sahu et al., 2008)
1.9 Overview and Specific Aims

The mechanism of HBV mediated HCC is not clear. However, sustained production of HBx is associated with hepatocellular transformation, and represents a major contribution of HBV to HCC (Henkler and Koshy, 1996). Independent observations suggest that HCC arises from dysfunctional liver stem cells (Sun et al., 2008), which raises the question as to whether HBx contributes to liver stem cell dysfunction.

CSCs comprise a minor population of cells that re-establish the phenotypic heterogeneity in the primary tumor and exhibit self-renewing capability (Marquardt and Thorgeirsson, 2010). Oct-4, Klf-4, Nanog and their binding partners act as key regulators of pluripotency in early mammalian development (Niwa, 2007). These “stemness” genes are re-expressed in cancer cells (Monk and Holding, 2001), and their reactivation contributes to tumorigenesis in somatic tissues (Hochedlinger et al., 2005).

EpCAM is a marker of hepatic stem cells and CSCs (Schmelzer et al., 2006) and is present in cases of HCC (Yamashita et al 2009; Breuhahn et al., 2006). β-catenin, like EpCAM, is important in stem cell self-renewal and in carcinogenesis (Takigawa and Brown, 2008), in part, by binding the promoters of Oct-4 and Nanog (Pereira et al., 2006). β-catenin acts as a transcriptional activator in the nucleus, but promotes cell adhesion when bound to E-cadherin (Guarino et al., 2007). In this way, E-cadherin acts as a negative regulator of β-
catenin, sequestering it from the nucleus. Importantly, HBx transcriptionally activates β-catenin in up to 80% of HCCs (Ding et al., 2005), while also down-regulating E-cadherin underscoring the potentially close relationship between HBx, activated β-catenin, suppressed E-cadherin, and stem cell renewal (Figure 6).

![Figure 6](image_url)

**Figure 6. Hypothesized convergence of relevant signaling pathways in the presence of HBx.** HBx represses E-cadherin, activates β-catenin, and potentially upregulates EpCAM allowing for translocation into the nucleus and the formation of the transcription initiating complex (Modified from Munz et al., 2009).

Altered expression and cellular distribution of E-cadherin is frequently associated with invasiveness in human cancers including HCC (Osada et al., 1996). In HBV mediated HCC, HBx is known to suppress E-cadherin expression by promoting methylation of CDH1 (Park et al., 2007; Lee et al., 2005). Another
epigenetic mechanism that represses transcription of the E-cadherin gene involves the recruitment of mSin3A/HDAC to the E-boxes of the CDH1 promoter by transcription factor Snail (Peinado et al., 2004; Giroldi et al., 1997).

Importantly, HCC is characterized by high levels of HDAC1 expression (Rikimaru et al., 2007). In this context, HBx was shown to recruit HDAC1 to repress, for example, insulin-like growth factor binding protein 3 transcription (Shon et al., 2009). Since HBx-HDAC interaction (Shon et al., 2009; Zheng et al., 2009) as well as physical interactions between Snail-HDAC-mSin3A (Peinado et al., 2004) have already been proven, it is possible that HBx can also interact with mSin3A, and recruit this complex to the E-cadherin promoter. This may provide a previously unexplored, alternative epigenetic mechanism of E-cadherin repression by which HBx contributes to the pathogenesis of HBV-associated hepatitis and to HCC.

It is hypothesized that HBx may be central in the down-regulation of E-cadherin through histone deacetylation and subsequent activation of β-catenin and EpCAM, as well as up-regulation of “stemness” markers contributing to the pathogenesis of hepatocellular carcinoma (Figure 7).
Figure 7. Model outlining the putative relationship between HBx and “stemness” in the pathogenesis of HBV associated HCC.

Thus, the specific aims of the study are

a. To analyze the expression of EpCAM and β-catenin in the presence of HBx in vitro and in clinical samples.

b. To evaluate the mechanism of HBx-mediated mSin3A/HDAC repression of E-cadherin expression in vitro and in clinical samples.

c. To explore the ability of HBx to promote properties that are characteristic of cancer stem cells in vitro and in clinical samples.
CHAPTER 2
MATERIALS AND METHODS

2.1  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). Cells were cultured in MEM (Invitrogen, Carlsbad, CA) supplemented with 100 mM nonessential amino acids, 100 mM sodium pyruvate, and 10% fetal bovine serum (Invitrogen), at 37°C in a humidified 5% CO₂ incubator.

2.2  Patient Samples

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 hepatitis B surface antigen (HBsAg) positive in blood; 41 were males, the age range was from 35-69 (average: 48), and all were of Chinese ethnicity. All samples were used for diagnostic purposes, and then used for this study. Ten uninfected human liver tissues (Abcam, Cambridge, MA) were used as controls. The use of samples was approved by the Institutional Review Boards at all participating universities.
2.3 **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 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, Oct-4, E-cadherin, β-catenin, Snail-1, Lamin A and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), Nanog (Cell Signaling, Danvers, MA), Klf-4, EpCAM, and mSin3A (Abcam Cambridge, MA), Ac-Lys H3 (Active Motif). The blots were developed using the ECL plus detection kit (Amersham, Piscataway, NJ) and exposed to Kodak imaging films (Kodak BioMax, Rochester, NY). Signal intensities were quantified using ImageJ software (NIH).

2.4 **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), anti-EpCAM (Millipore, Billerica MA) 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.

2.5 Statistics

Statistical values for cell migration were defined using an unpaired Student’s t-test, in which \( P < 0.05 \) was considered significant. The relationship between HBx and EpCAM, β-catenin, E-cadherin, mSin3A and Snail-1 obtained by immunohistochemistry was determined using 2 x 2 comparisons in the Chi square (\( \chi^2 \)) test. Statistical significance was considered when \( P < 0.05 \).

2.6 Immunoprecipitation (IP) Assay

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.

2.7 Chromatin Immunoprecipitation (ChIP) Assay

HepG2X and HepG2CAT cells were cross-linked with 1% formaldehyde for 20 minutes 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 (Invitrogen, Carlsbad, CA), dounce homogenized in hypotonic buffer (Active Motif) and centrifuged 10 minutes 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 Sonifier® 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 NaHCO₃), 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’- GGGCTGGAGTCTGAAGTGA - 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.
2.8 Treatment of Cells with Trichostatin A (TSA)

Suspensions of $3.5 \times 10^5$ 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.

2.9 Immunofluorescence Microscopy

Cells were plated on collagen type I coated slides, fixed and permeabilized with 95% ethanol–5% acetic acid and incubated at 4°C overnight with antibodies against HBx and mSin3A, (Santa Cruz Biotechnology Inc). Cells were subsequently incubated with 1:250 dilution of FITC- and Rhodamine-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) one hour at room temperature. Sections were washed and mounted in Vectashield aqueous mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Fluorescent specimens were analyzed using an ECLIPSE Ti inverted microscope (Nikon, Melville, NY), lenses with hardened filters (Nikon Inc.), and a Nikon DS-Fi1 camera, which was operated by NIS Elements computer software (Nikon Inc.). Pictures were taken at resolutions 1280 x 960 pixels and 625 x 469 pixels and 400x magnification. Pictures with zoom were enlarged at 200%. The merge panel is overlay of the two images. (All antibody catalog numbers listed previously).
2.10 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, Corning, NY) 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, Melville, NY).

2.11 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, Franklin Lakes, NJ) according to enclosed instructions. Cell migration was observed after 24h by hematoxylin and eosin (H & E) staining.
CHAPTER 3

RESULTS

3.1 Analysis of EpCAM and β-catenin expression in the presence of HBx

3.1.1 HBx up-regulates EpCAM and β-catenin in vitro

Selected markers were characterized by western blotting (Fig. 8). In whole cell extracts, HBx was associated with up-regulation of β-catenin (2.5 ± 0.7-fold) and EpCAM (3 ± 1.2-fold). In HepG2 cells, β-catenin is present as wild type (upper band) and truncated mutant (lower band) due to partial exon 3 deletion (Carruba et al., 1999). HBx up-regulates wild type β-catenin without modifying the levels of truncated β-catenin, rendering the presence of the truncated form irrelevant. Moreover, β-catenin in HepG2 cells was studied in a number of articles elucidating the mechanism whereby HBx up-regulates β-catenin (Ding et al., 2005). Previous work confirmed that nuclear β-catenin in HBx positive HCC is wild type by DNA sequence analysis of β-catenin exons obtained from micro-dissected human liver tumor tissue from 50-µm sections (Lian et al., 2006). This is the first work to show up-regulation of EpCAM in HBx expressing cells.
Figure 8. Upregulation of “stemness”-associated markers in the presence of HBx. Representative western blots with HepG2X and HepG2CAT cells using total (50 µg) extracts. β-actin is the loading control.
3.1.2 Relationships between HBx, EpCAM and β-catenin in clinical samples

Experiments were conducted to determine whether the results from western blotting could be validated in vivo. Thus, clinical samples containing HCC and nontumor liver were stained for HBx, β-catenin, and EpCAM (Table 1). 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%) (Fig 9). In 82% of cases with tumor and nontumor tissues, HBx staining was stronger and more widespread in liver compared to tumor, as previously reported (Wang et al., 1991). Ten commercially available liver sections from uninfected individuals were uniformly negative for HBx. HBx staining was cytoplasmic in all cases, and one case showed nuclear staining in nontumor liver. EpCAM was present in 26 cases in the tumor compartment (66%) (Fig. 10A). Membranous EpCAM staining (Fig. 10B) was observed in the nontumor liver among 21 patients (60%), even though EpCAM is not expressed in uninfected, healthy liver (Schmelzer et al., 2006). In tumors from 3 patients, nuclear EpCAM was also detected (Fig. 10C). With regard to β-catenin, staining was observed in the tumor compartment from 28 patients (74%), and in the nontumor compartment from 19 patients (61%). Although membranous β-catenin is characteristic of normal, uninfected liver, all cases of tumor and nontumor tissues showed both membranous and
cytoplasmic staining for β-catenin, consistent with β-catenin activation, as previously shown (Lian et al., 2006). β-catenin was also observed in the nuclei of two tumors (data not shown).

<table>
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<th>Positive staining in nontumor (# of cases)</th>
<th>Positive staining in HCC (# of cases)</th>
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<tbody>
<tr>
<td>HBx</td>
<td>34 (100%)</td>
<td>26 of 40 tumors (65%)</td>
</tr>
<tr>
<td>EpCAM</td>
<td>21 (60%)</td>
<td>26 (66%)</td>
</tr>
<tr>
<td>Correlation with HBx</td>
<td>$\chi^2 = 11.8; P &lt; 0.001$</td>
<td>$\chi^2 = 2.03; P &gt; 0.1$</td>
</tr>
<tr>
<td>β-catenin</td>
<td>19 (61%)</td>
<td>28 (74%)</td>
</tr>
<tr>
<td>Correlation with HBx</td>
<td>$\chi^2 = 5.88; P &lt; 0.02$</td>
<td>$\chi^2 = 0.23; P &gt; 0.5$</td>
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Table 1. Correlation between HBx and “stemness”-associated markers in HBV-mediated HCC. Summary of HBx, EpCAM and β-catenin staining and correlation of these markers with HBx expression in clinical samples.
Figure 9. Immunohistochemical detection of HBx in HBV-mediated HCC clinical samples. (A) Negative staining for HBx in HCC and (B) positive staining for HBx in nontumor liver on the same tissue from a representative patient (x200). (C) Nuclear staining of HBx in HCC (tumor) (x400).
Figure 10. Immunohistochemical detection of EpCAM in HBV-mediated HCC clinical samples. (A) Membranous staining for EpCAM in tumor (x400),
and (B) nontumor (x200). (C) Mixed membranous and nuclear staining for EpCAM in tumor (x200).

Additional analyses were conducted to determine the relationship between HBx and these markers. In the tumor compartment, co-staining between HBx and EpCAM was seen in only 16 cases (Fig. 11A-D) ($\chi^2 = 2.03; P > 0.1$), and between HBx and β-catenin in 19 cases ($\chi^2 = 0.23; P > 0.5$). Hence, there appears to be no correlation between HBx and these markers in tumors. In contrast, in the nontumor compartment, where HBx is strongly expressed, co-staining between HBx and EpCAM was seen in 21 cases ($\chi^2 = 11.8; P < 0.001$), and between HBx and β-catenin in 19 cases ($\chi^2 = 5.88; P < 0.02$), suggesting a strong correlation between HBx and these markers (Table 1).

Analysis of relationship between β-catenin and EpCAM staining and HCC grade showed an inverse correlation between EpCAM staining and HCC in grade 1 ($\chi^2 = 10.2257; P < 0.005$; number of tumors (n) = 8) and grade 2 ($\chi^2 = 5.3831; P < 0.025$; n = 17), but not in grades 3 ($\chi^2 = 1.3; P > 0.05$; n = 5) and 4 ($\chi^2 = 1.06; P > 0.3$; n = 11). There was no correlation between β-catenin staining and tumor grade. Experiments with a larger number of samples should be conducted to address these relationships more thoroughly. These data suggest that HBx is associated with the re-activation of “stemness”-associated EpCAM and β-catenin.
Figure 11. Immunohistochemical detection and correlation of HBx and EpCAM in consecutive HBV-mediated HCC clinical samples. Staining for (A,C) HBx and (B,D) EpCAM in consecutive HCC sections from two representative patients (x400).
3.2 Evaluation of the mechanism of HBx-mediated mSin3A/HDAC repression of E-cadherin expression

3.2.1 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 prepared and 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. 12A). In nuclear extracts, HBx was associated with up-regulation of mSin3A (2.5 ± 1.1-fold) and Snail-1 (2.0 ± 0.7-fold) (Fig. 12B). These data show that HBx suppresses E-cadherin and stimulates expression of the scaffold, mSin3A, and its associated transcription factor, Snail-1.

![Figure 12. Expression of HBx, E-cadherin, mSin3A and Snail-1 in the presence of HBx. Representative western blots with 50 µg of (A) total or (B) nuclear extracts of HepG2X and HepG2CAT cells. β-actin and lamin A are the loading controls.](image-url)
3.2.2 Interaction between HBx and mSin3A in vitro

The interaction between HBx and HDAC (Shon et al., 2009; Zheng et al., 2009), as well as physical interactions between Snail-HDAC1-mSin3A (Peinado et al., 2004) have been previously demonstrated. Therefore it is possible that HBx can also interact with mSin3A, and recruit this complex to the E-cadherin promoter.

To determine whether HBx physically interacts with mSin3A, reciprocal immunoprecipitation experiments were performed with whole cell lysates isolated from HepG2X and HepG2CAT cells. Prior work showed that HBx is present in both 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 demonstrated by western blotting with anti-HBx (Fig. 13A, lane 1). Immunoprecipitation experiments with anti-mSin3A showed HBx in cell lysates from HepG2X but not HepG2CAT cells (Fig. 13A, lanes 2 and 3, respectively).

Reciprocal immunoprecipitation experiments with anti-HBx, followed by western blotting with anti-mSin3A showed strong reactivity with mSin3A in HepG2X but not HepG2CAT cells (Fig. 13B, 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. 13C), anti-mSin3A (green staining in Fig. 13D), and DAPI (blue staining in Fig. 13E). When the images were merged, HBx-mSin3A co-
localization was observed in the nuclei of HepG2X cells as small orange regions (Fig. 13F). These results support the hypothesis of HBx-mSin3A nuclear co-localization.
Figure 13. HBx and mSin3a interact. 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 (C) anti-HBx, (D) mSin3A, and (E) DAPI. (F) Merged image of HBx and mSin3A staining showing co-localization of mSin3A and HBx in the nucleus (arrow).
3.2.3 HBx promotes recruitment of mSin3A to the E-cadherin promoter

HBx was previously shown to recruit DNA-methyltransferases to suppress E-cadherin expression (Park et al., 2007; Zheng et al., 2009). HBx was also shown to recruit HDAC1 to repress, for example, insulin-like growth factor binding protein 3 transcription (Shon et al., 2009). Thus, ChIP assays were performed to determine if HBx also promotes recruitment of the mSin3A/HDAC complex, which is known to suppress the expression of E-cadherin (Peinado et al., 2004). In this context, the human CDH1 contains three regulatory elements consisting of a CANNTG motif (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/HDAC complex to the E-boxes (Batlle et al., 2000), experiments were designed to target this fragment (which included all three E-boxes) for PCR amplification following ChIP (Fig. 14A).

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. 14B). Since HBx can interact with HDAC1 (Shon et al., 2009; Zheng et al., 2009) and with mSin3A (Fig. 13), these results confirm that HBx mediates CDH1 deacetylation by recruiting the mSin3A/HDAC complex.
Figure 14. mSin3A/HDAC occupies the E-cadherin promoter in the presence of HBx. (A) Presented sequence for the human \textit{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. The target fragment included three E-boxes (1, 2, 3) that are Snail binding sites (Batlle et al., 2000). (B) ChIP assays of 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.
3.2.4 HDAC inhibition reveals relationship between all markers

HepG2X and HepG2CAT cells were treated with the 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. 15). This was accompanied by increased levels of E-cadherin in HepG2X cells (7-fold), decreased levels of wild type β-catenin (5-fold) and EpCAM (9.5-fold) with little change in HepG2CAT cells. Taking into consideration that HBx can directly interact with HDAC (Shon et al., 2009; Zheng et al., 2009) and with mSin3A (Fig. 15), the finding that inhibition of HDAC1 reverses the effects of HBx on E-cadherin, confirms that HBx mediates CDH1 deacetylation by recruiting the mSin3A/HDAC complex. These results also show that once HBx repression of E-cadherin is reversed by TSA, alterations in β-catenin and EpCAM are also observed, suggesting these events are linked.
Figure 15. HDAC inhibition reveals relationship between all markers. Western blots showing levels of acetyl-H3, E-cadherin, β-catenin and EpCAM after treatment of HepG2X and HepG2CAT cells with TSA. The blots are representative of three independent experiments.
3.2.5 Relationships between HBx, E-cadherin, mSin3A and Snail-1 in clinical samples

Clinical samples containing HCC and nontumor liver were stained for HBx, E-cadherin, mSin3A and Snail-1 (Table 2). Among 42 patients, 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%). HBx staining was stronger and more widespread in liver compared to tumor, as previously reported (Wang et al., 1991), hence the positive HBx staining in all 33 nontumor livers (100%). For E-cadherin, exclusively membranous staining was detected in 10 of 39 tumors (26%), and in 21 of 33 cases where nontumor liver was available (64%), and in non-infected control samples (Fig. 16). These results are similar to those previously published (Liu et al., 2006). Nuclear mSin3A staining was observed in 20 of 39 tumors (51%) and in the nontumor liver from 19 of 33 patients (58%) (Fig. 17A). mSin3A and Snail-1 staining in uninfected, healthy liver was undetectable (Fig. 17B and C). With regard to Snail-1, staining was observed in the tumor compartment from 22 of 39 patients (56%), and in the nontumor compartment from 17 of 33 patients (52%) where these tissue samples were available to evaluate (Fig. 17A). Hence, mSin3A and Snail up-regulation was observed in HBV associated HCC.
Table 2. Correlation between HBx and E-cadherin, mSin3A and Snail-1 in HBV-mediated HCC. Summary of HBx, E-cadherin, mSin3A and Snail-1 staining and correlation of these markers with HBx expression in clinical samples.

<table>
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<th>Positive staining in nontumor (# of cases)</th>
<th>Positive staining in HCC (# of cases)</th>
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<tbody>
<tr>
<td><strong>HBx</strong></td>
<td>33 (100%)</td>
<td>27 of 39 tumors (69%)</td>
</tr>
<tr>
<td><strong>E-cadherin</strong></td>
<td>21 (64%)</td>
<td>10 (26%)</td>
</tr>
<tr>
<td>Correlation with HBx</td>
<td>$\chi^2 = 5.04; P &lt; 0.025$</td>
<td>$\chi^2 = 0.0037; P &gt; 0.95$</td>
</tr>
<tr>
<td><strong>mSin3A</strong></td>
<td>19 (58%)</td>
<td>20 (51%)</td>
</tr>
<tr>
<td>Correlation with HBx</td>
<td>$\chi^2 = 10.8; P &lt; 0.005$</td>
<td>$\chi^2 = 0.64; P &gt; 0.75$</td>
</tr>
<tr>
<td><strong>Snail-1</strong></td>
<td>17 (52%)</td>
<td>22 (56%)</td>
</tr>
<tr>
<td>Correlation with HBx</td>
<td>$\chi^2 = 8.41; P &lt; 0.005$</td>
<td>$\chi^2 = 0.113; P &gt; 0.7$</td>
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Figure 16. Immunohistochemical detection of E-cadherin in uninfected hepatocytes. E-cadherin staining in uninfected tissue (commercial slide). Sample is shown x400.
Figure 17. Immunohistochemical detection of HBx, Snail-1, mSin3a, and E-cadherin in clinical samples. Representative staining for (A) HBx, Snail-1, mSin3A, and E-cadherin in consecutive tissue sections from a patient with HBV infected liver. Samples are shown x400. Representative staining for (B) mSin3A and (C) Snail-1 from uninfected human liver (commercial slide) and from tissue of a patient with HCC. Samples are shown x400.
Additional analyses were conducted to determine the relationship between HBx and these markers. In the nontumor compartment, there was an inverse correlation between HBx and E-cadherin in 21 cases ($\chi^2 = 5.04; P < 0.025$). As expected, there was no correlation between HBx and E-cadherin in the tumor compartment, with co-staining in only 7 cases ($\chi^2 = 0.004; P > 0.95$). HBx and mSin3A were significantly associated in the nontumor liver ($\chi^2 = 10.8; P < 0.005$) but not in the tumor compartment ($\chi^2 = 0.64; P > 0.75$). Likewise, HBx and Snail-1 were significantly associated in nontumor liver ($\chi^2 = 8.41; P < 0.005$) but not in the tumor ($\chi^2 = 0.113; P > 0.7$). 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., 1991). 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 ($\chi^2 = 24.5; P < 0.001$) and in tumor ($\chi^2 = 16.8; P < 0.001$). 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 inverse relationships between the expression of E-cadherin with mSin3A or Snail-1 in both tumor and nontumor compartments, is consistent with their expected roles in the suppression of E-cadherin in vivo.
3.3 Exploration of the ability of HBx to promote properties that are characteristic of cancer stem cells

3.3.1 HBx up-regulates “stemness” markers \textit{in vitro}

In nuclear extracts, up-regulation of Oct-4 (1.6 ± 0.9-fold), Nanog (3.4 ± 1.3-fold), and Klf-4 (3.5 ± 0.8-fold) (Fig. 18) was observed in HepG2X compared to control cells. These data suggest that HBx is associated with the re-activation of “stemness” transcription factors Oct-4, Nanog, and Klf-4.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure18}
\caption{Upregulation of “stemness” markers in the presence of HBx. Representative western blots using nuclear extracts (100 \, \mu g) from HepG2X and HepG2CAT cells.}
\end{figure}
3.3.2 Relationship between HBx and “stemness” markers in clinical samples

Clinical samples containing HCC and nontumor liver were stained for Oct-4 and Nanog. Two and six tumor samples were positive in scattered nuclei, respectively (Fig. 19A and B). Unfortunately, the available antibodies for Klf-4 showed strong non-specific cytoplasmic staining, and, therefore, were not used. Although “stemness” markers were observed in just a few HBV associated HCC(s), this is consistent with published data showing rare CSCs in clinical samples (Tang et al., 2008).

Figure 19. Immunohistochemical detection of “stemness” markers in HBV-mediated HCC clinical samples. Representative staining of (A) Oct-4 and (B) Nanog in human HCC tissue sections from HBV carriers (x200).
3.3.3 HBx-expressing cells exhibit clonogenic potential

HepG2X and HepG2CAT cells were tested for their ability to form spheres in 3D culture systems (clonogenic potential). It has been shown that stem/progenitor cells and CSCs can form spheroids in vitro suggesting the presence of self-renewing cells (Fang et al., 2005; Barclay et al., 2008). In this work, HBx-expressing cells formed spheroids by day 3 after inoculation. About 4-fold more spheroids were derived from HepG2X compared to HepG2CAT cells after 16 days in culture. Some of these spheroids exceeded 100 µm in diameter (Fig. 20A,C). In contrast, HepG2CAT cells formed mostly disorganized clusters that became adherent, with less floating spheroids (Fig. 20B). Primary spheroids enzymatically dissociated to single cells gave rise to secondary spheroids.
Figure 20. HBx-expressing cells exhibit clonogenic potential. Representative images of spheroids derived from (A) HepG2X cells, (B) HepG2CAT cells, and (C) relative numbers of spheroids for each culture. Data are shown as mean ± SD.
3.3.4 HBx-expressing cells exhibit invasive potential

Cell migration was then examined by Matrigel invasion assay and hematoxylin and eosin (H&E) staining after 24 hrs. About 3.5-fold more HepG2X than HepG2CAT cells migrated through Matrigel basement membrane matrix (*P* < 0.001) (Fig. 21). Thus, HepG2X cells showed more pronounced phenotypes consistent with stem cell behavior compared to HepG2CAT control cells. Hence, the phenotypic characteristics presented above were consistent with the induction of "stemness" markers by HBx.

![HepG2X and HepG2CAT cells](image)

**Figure 21. HBx-expressing cells exhibit invasive potential.** Migration of HepG2X and HepG2CAT cells through Matrigel basement membrane.
CHAPTER 4

DISCUSSION

In this study, the pluripotent stem cell transcription factors Oct-4, Nanog, and Klf-4, and the "stemness"-associated markers EpCAM and β-catenin, were upregulated in HBx-expressing cells (Figs. 18 and 8). These observations were validated in tumor and adjacent nontumor liver from HBV-infected patients (Figs. 19 and 10). HBx-expressing cells also showed enhanced migration capabilities and sphere-forming activity in vitro (Figs. 21 and 20). The tight epidemiologic association between chronic HBV infection and the development of HCC (Feitelson, 1999), combined with evidence that HBx and CSCs contribute importantly to tumor development, suggest that HBV may promote hepatocarcinogenesis, in part, by triggering "stemness." This may be mediated by HBx-associated transcriptional trans-regulation and altered epigenetic regulation of host gene expression (Zheng et al., 2009; Shon et al., 2009) and activation of cellular kinases that alter signal transduction (Henkler and Koshy, 1996; Feitelson, 1999). 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 (Feitelson and Duan, 1997; Diamantis et al., 1992). As
chronic infection proceeds, virus replication decreases and HBx expression from integrated templates increases (Feitelson and Duan, 1997). During this process, HBx impacts on the host pathways and gene targets mentioned earlier. Additionally, 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 (Feitelson and Duan, 1997; Diamantis et al., 1992). 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. 18) and Oct-4 and Nanog were detectable in several HCC nodules (Fig. 19). Independent evidence showed strongly positive clusters of Oct-4-positive cells in HCC (Tang et al., 2008). Importantly, CSCs comprise a minor population of cells that re-establish the phenotypic heterogeneity in the primary tumor and exhibit self-renewing capability on serial passaging (Marquardt and Thorgeirsson, 2010). Oct-4, Sox-2, Nanog, and their binding partners act as key regulators of pluripotency in early mammalian development (Niwa, 2007). These "stemness" genes are re-expressed in cancer
cells, and the reactivation of these factors contributes to tumorigenesis in somatic tissues (Monk and Holding, 2001). 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 (Uche et al., 2005). It is proposed that some of these same pathways are turned on by HBx in hepatocarcinogenesis.

HBx contributes to the pathogenesis of HCC through several pathways, resulting in the sustained activation of EpCAM. Prior studies have shown that EpCAM is present on subsets of normal epithelia, numerous tissue stem and progenitor cells, and most carcinomas including HCCs (Yamashita et al., 2009; Breuhahn et al., 2006). Although normal adult hepatocytes do not express EpCAM (Schmelzer et al., 2006; Breuhahn et al., 2006), it is striking that HBV-infected hepatocytes do (Fig. 10B), implying that HBx confers "stemness" properties on at least some infected cells. The significant co-staining 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. 10C). 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 (Lu et al., 2010). 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 β-catenin
EpCAM has been shown to be a direct transcriptional target of the Wnt/β-catenin signaling with two TCF binding elements identified in the EpCAM promoter (Yamashita et al., 2007). Nuclear accumulation of β-catenin induced, whereas the degradation of β-catenin or inhibition of TCF/β-catenin complex formation reduced EpCAM gene expression in cultured human hepatocytes and HCC cell lines (Yamashita et al., 2007). When xenografted in NOD-SCID mice, only EpCAM-positive cells could efficiently initiate the development of invasive tumors, even after serial transplantation, demonstrating that EpCAM-positive cells display CSC-like characteristics (Yamashita et al., 2009). Thus, the HBx upregulation of β-catenin (Fig. 8) and β-catenin–mediated induction of EpCAM may be critical in initiating and maintaining CSCs growth.

β-Catenin also functions in regulation of cell adhesion when in complex with transmembrane glycoprotein, E-cadherin. E-cadherin is important for the maintenance of cell polarity and the structural integrity in tissue, and it happens to be downregulated by HBx (Liu et al., 2006). HBx-mediated repression of E-cadherin releases β-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 β-catenin (Muller et al., 1999; Lara-Pezzi et al., 2001). In the nucleus, β-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 β-catenin regulates the expression of "stemness" genes (Takigawa and Brown, 2008; Pereira et al., 2006). Hence, the finding that HBx constitutively activated wild-type β-catenin in up to 80% of HCCs (Ding et al., 2005) underscores the potentially close relationship between HBx, activated β-catenin, suppressed E-cadherin and stem cell renewal.

The main adhesion molecule of epithelia, E-cadherin, maintains cells in a stationary, non-motile state (van Roy and Berx, 2008). Abnormalities in the expression and cellular distribution of E-cadherin are frequently associated with dedifferentiation, invasiveness and poor prognosis in a variety of human malignancies, including HCC (Endo et al., 2000; Osada et al., 1996). Re-establishing the functional E-cadherin complex in tumor cell lines results in a reversion from an invasive to a benign epithelial phenotype (Perl et al., 1998). Downregulation of E-cadherin induces epithelial-to-mesenchymal transition 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 downregulated expression of E-cadherin (Liu et al., 2006), underscores the importance of suppressed CDH1 to HBV-associated HCC.

Epigenetic modulation of transcriptional activity of the target genes could be an important mechanism for HBx mediated transformation, as HBx does not bind to 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 et 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 50-Aza-20dC (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. 12, 13 and 14) indicating that additional mechanisms target suppression of E-cadherin in chronic hepatitis B.

Histone deacetylation triggers chromatin remodeling which yields a more compact chromatin structure, and represses gene transcription by limiting the accessibility of transcription factors to target genes (Ropero and Esteller, 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 \textit{in vitro} (Figure 13), and correlates with both mSin3A and Snail-1 \textit{in vivo} (Figure 17), supports this hypothesis. An important characteristic of the interaction between DNA methylation and HDAC activity, particularly in maintaining the aberrant silencing of hypermethylated genes in cancer, is that the methylation seems to function as the dominant event that seals transcriptional repression and histone deacetylation is secondary to DNA methylation (Cameron et al., 1999). Thus, methylation and deacetylation could function together to potentiate the repressed state of E-cadherin. In fact, it was shown that methyl-CpG-binding protein 2 recruits mSin3A/HDAC to the methylated \textit{CDH1} promoter, leading to histone-3 deacetylation (Takeno et al., 2004). In this context, it is documented that HBx promotes both hypermethylation of \textit{CDH1} (Liu et al., 2006) and histone deacetylation through recruitment of the mSin3A/HDAC complex to \textit{CDH1} (Figure 14). Furthermore, it is expected that these mechanisms are also operative with regard to other HBx target genes in hepatocarcinogenesis.

Interestingly, treatment of HepG2X and HepG2CAT cells with the HDAC inhibitor TSA, resulted in increased expression of E-cadherin and decreased expression of “stemness”-associated markers, EpCAM and β-catenin (Figure 15). Thus, HDAC inhibition not only reversed the effects of HBx, but also revealed an interdependent relationship between “stemness”-associated markers and E-cadherin.

An important connection between HBx and Snail may exist within the TGF-β pathway. TGF-β participates in tumor progression through its EMT-
induction activity. HBx is known to indirectly induce TGF-β expression through the activation of Smad4 (Yoo et al., 1996; Lee et al., 2001) and the repression of TGF-β inhibitor, alpha2-macroglobulin (α2-M) (Pan et al., 2004). Interestingly, TGF-β transcriptionally induces Snail gene expression via Smad3 or via activation of the Erk and PI3K pathways (Peinado et al., 2003). Importantly, this process has been shown to occur in hepatocytes (Spagnoli et al., 2000). Thus, HBx may indirectly upregulate Snail by activating the TGF-β pathway.

Collectively, this study supports the hypothesis that HBx promotes stemness in the pathogenesis of hepatocellular carcinoma. The mechanisms by which HBV contributes to the pathogenesis of HCC are still not well understood. However, this study suggests one possible mechanism and contributes to the elucidation of the role of HBx in triggering transformation of hepatocytes. Furthermore, the significant correlation between epigenetic E-cadherin repressors, “stemness” markers and HBx suggest possible detectable markers for earlier detection of HCC in chronically infected HBV patients.
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