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Mechanisms of HBx Mediated Liver Cancer: Multiple Pathways and Opportunities

Mark A. Feitelson et al.*
Department of Biology, Temple University, Philadelphia, PA USA

1. Introduction

Chronic hepatitis B virus (HBV) infection is associated with a high risk for the development of chronic liver diseases (CLDs) which include hepatitis, cirrhosis and hepatocellular carcinoma (HCC). HCC is among the top five most prevalent tumor types worldwide, has few effective treatment options, and is highly lethal. The pathogenesis of CLD and HCC is immune mediated, and the virus has developed a number of defense mechanisms that essentially prevent infected cells from being effectively eliminated by the immune system. This, in part, involves the sustained, high level expression of the virus encoded protein, hepatitis B x antigen (HBx). Recent work has shown that HBx blocks pathways of innate immunity (Kumar et al., 2011; Wei et al., 2010), thereby blunting the development of adaptive immunity that is central to virus elimination. In addition, HBx inhibits immune mediated apoptosis by multiple pathways, including those mediated by Fas and tumor necrosis factor alpha (TNFα). In this context, HBx has been shown to up-regulate TNFα expression (Lara-Pezzi et al., 1998), which is thought to kill uninfected hepatocytes more readily than infected cells, thereby promoting expansion of the virus within the liver, since virus infected hepatocytes would preferentially regenerate following a bout of chronic hepatitis. HBx also switches the growth signals mediated by elevated transforming growth factor beta 1 (TGFβ1) from that of negative growth regulation to that of positive growth regulation. TGFβ1 is a transcriptional target of HBx (Yoo et al., 1996), suggesting that HBx expression in the liver promotes fibrogenesis and the development of cirrhosis. Within the infected hepatocyte, HBx blocks the action of tumor suppressors, such as p53 and Rb (Feitelson et al., 2008), and up-regulates the expression of selected host genes that strongly promote hepatocarcinogenesis even in the absence of HBx (see below). Recent work has also

* Alla Arzumanyan[1], Tiffany Friedman[1], N. Lale Tufan[2], Zhaorui Lian[1], Marcia M. Clayton[1], Joyce Kang[3], Helena M. G. P. V. Reis[4], Jingbo Pan[5], Jie Liu[6], Patrick Arbuthnot[7] and Michael Kew[7]
[1]*Department of Biology, Temple University, Philadelphia, PA, USA*
[2]*Department of Medical Biology, Pamukkale University School of Medicine, Kinikli Denizli, Turkey*
[3]*Division of Medical Microbiology, Guiyang Medical College, Guizhou Province, People's Republic of China*
[4]*MIT Portugal Program, Av. Antonio Jose de Almeida, Lisboa, Portugal*
[5]*Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA*
[6]*Department of Digestive Diseases of Huashan Hospital, Fudan University, Shanghai, P. R. China*
[7]*Molecular Hepatology Research Unit, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa*

shown that HBx promotes phenotypic changes in hepatocytes characteristic of epithelial-to-mesenchymal transition (EMT). One of the molecular hallmarks of EMT, down-regulated expression of the cell adhesion molecule, E-cadherin, is blocked by sustained HBx expression via several mechanisms (Feitelson et al., 2009). HBx also overrides immune mediated apoptotic signals by constitutively activating key signaling pathways, such as nuclear factor kappa B (NF-κB), which is known to be hepatoprotective (Beg et al., 1995, 1996), and phosphatidylinositol 3-kinase (PI3K)/Akt, which is known to promote growth in many tumor types (Chung et al., 2004). The finding that HBx stabilizes β-catenin by a variety of mechanisms, and up-regulates ErbB2 (Liu et al., 2009), further underscores the importance of these actions in maintaining hepatocellular growth and survival required for virus propagation during the many years and decades that span chronic infection. Unfortunately, these same pathways are also those that contribute centrally to the development of HCC. This body of work provides many opportunities for the development of diagnostic markers that form a fingerprint of those chronically infected patients who are most likely to go on and develop HCC. These markers will serve as therapeutic targets for the repositioning of known drugs for this new indication, and/or the discovery of new drugs that will target rate limiting pathways during multi-step carcinogenesis. In doing so, this work proposes that the chemoprevention of cancer, instead of the treatment of tumor bearing patients, is worth pursuing, and could likely reduce or eliminate the morbidity and mortality associated with chronic HBV infection long before tumors appear. This represents an important challenge, since the knowledge gained will identify cause and effect relationships important for the identification of definitive biomarkers and pharmacological targets that participate decisively in tumorigenesis.

2. Relationship between HBx expression and the pathogenesis of CLD and (HCC): A model

HBx is one of four genes expressed by HBV during infection, and is known to have gene regulatory functions. Truncated envelope polypeptides that appear during chronic infection may also regulate gene expression and contribute to the pathogenesis of CLD and liver cancer (Chen et al., 2006; Lauer et al., 1992), but their contributions are less well characterized. HBx has been defined as a *trans*-activating protein that promotes virus gene expression and replication during infection (Belloni et al., 2009; Spandau & Lee, 1988; Tsuge et al., 2010). Experimental infection of newborn woodchucks with the related woodchuck hepatitis virus (WHV) results in the development of carriers in nearly 100% of cases, and most of these go on to develop severe chronic hepatitis and HCC (Tennant & Gerin, 2001). However, infection of neonatal woodchucks with an X protein negative clone of WHV failed to establish the chronic carrier state (Chen et al., 1993; Zoulim et al., 1994). This suggests that X protein promotes viremia. The impact of X protein on virus gene expression and replication is also supported by considerable *in vitro* data (Benhenda et al., 2009; Keasler et al., 2009; Tsuge et al., 2010). During the course of CLD, bouts of hepatitis are associated with hepatocellular destruction and regeneration. Among infected cells, the X open reading frame (ORF), which is at the end of the virus genome, becomes repeatedly integrated into host DNA at the replication forks that exist in host DNA during regeneration. This suggests that the intracellular levels of HBx increase with the severity and progression of CLD, and there is now considerable experimental evidence to support this hypothesis (Feitelson et al., 1993a; Jin et al, 2001; Wang et al., 1991a, 1991b). In fact, the highest levels of HBx expression

have been observed in cirrhotic nodules (Wang et al., 1991a, 1991b). As indicated above, HBx *trans*-activates HBV enhancers and promoters, thereby promoting long term virus replication. However, it is proposed that when the levels of intracellular HBx increase with time among patients with CLD, it *trans*-regulates the expression of many cellular genes as well (Balsano et al., 1994; Twu & Schloemer, 1987) by a variety of mechanisms. It is postulated that these changes in cellular gene expression help to make cells more permissive to continued virus replication, but also protect the cells from immune responses aimed at removal of infected hepatocytes. This is accomplished by triggering EMT (Du et al., 2010; Yang et al., 2009), by promoting up-regulated expression of selected oncogene associated pathways, and by turning off tumor suppressor, senescence and apoptotic pathways (Kew, 2011; Oishi et al., 2007; Park et al., 2011; Xu et al., 2010) that are often activated by immune responses against virus infected cells. The fact that HBx promotes cell cycle progression and cell growth (Feitelson et al., 2005), means that when this happens in normal hepatocytes, negative growth regulatory (senescence and tumor suppressor) pathways are triggered to reestablish homeostasis. The latter may underlie the putative "proapoptotic" properties of HBx observed in cell lines and *in vivo*, even though there is a considerable literature showing that HBx is also "anti-apoptotic" (Assrir et al., 2010). In this model, it is proposed that apoptosis is a cellular response to inappropriate growth stimulatory signals in the liver mediated by HBx during chronic infection and not due to an inherent property of HBx. Although there is considerable literature suggesting that HBx inhibits several DNA repair systems (e.g., Cheng et al., 2010; Martin-Lluesma et al., 2008; Mathonnet et al., 2004; Qadri et al, 2011), which would promote the development of mutations in the liver prior to the appearance of tumors, it appears that a major contribution of HBx to the pathogenesis of CLD is epigenetic. This is because many natural effectors of HBx correlate with HBx expression in chronically infected human livers and because mutations are not widespread in preneoplastic hepatocytes (Feitelson et al., 2002). The finding that HBx and its natural effectors (target genes) correlate in nontumor liver, but are mostly absent from adjacent tumor tissues, suggests that HBx and its target genes drive pathogenesis prior to the appearance of tumor, but are no longer rate limiting once tumors appear. In the latter case, it is proposed that epigenetic mechanisms mediated by HBx are replaced by genetic mechanisms that are independent of HBx. If so, then HBx may play a predominant role in the pathogenesis of CLD, but a more modest role in tumor progression.

3. Natural targets of HBx

Early work characterized HBx as a *trans*-regulatory protein that was initially shown to up-regulate the expression of almost every target gene that was evaluated using mostly reporter gene assays in transient transfected cell lines (Rossner, 1992). It seemed that in order to better understand what HBx was doing *in vivo*, the natural effectors and targets of HBx in the infected liver had to be identified and characterized. HBx targets that were up- or down-regulated were identified by microarray analysis, miRNA arrays, chromatin immunoprecipitation, and by other techniques (e.g., Hu et al., 2006; Sung et al., 2009; Wu et al., 2001, 2002). Some of the targets include telomerase (Liu et al, 2010), the ras pathway signaling molecule, RASSF1A (Yang, et al, 2010), the metastasis associated protein, MTA (Bui-Nguyen et al, 2010), β-catenin (Lian et al., 2006; Pan et al., 2007), E-cadherin (Liu et al., 2006), c-myc (Wu et al., 2001), and DNA methyltransferase 1 (Zheng et al., 2009). HBx is a protein binding protein that also regulates gene expression by activating a number of signal

transduction pathways in the cytoplasm (e.g., NF-κB, PI3K/Akt, JAK/STAT, PKC, AP-1, ras, src, Wnt and others) (Feitelson & Duan, 1997; Henkler & Koshy, 1996; Kew, 2011). Constitutive activation of these signaling pathways has been identified with up-regulated expression of specific target genes. For example, HBx mediated activation of the mitogen-activated protein kinase (MAPK) pathway has been shown to up-regulate the expression of hypoxia-inducible factor-1 alpha (HIF-1α) (Yoo et al., 2003), which promotes the survival of hepatocytes in cirrhotic nodules, where a hypoxic environment is known to exist during CLD. Further, HBx mediated constitutive activation of Wnt signaling is associated with up-regulated expression of c-myc and cyclin-D1, both of which promote hepatocellular growth. In the nucleus, HBx interacts with the basal transcription machinery (Haviv, et al., 1995, 1996), binds to the transcriptional scaffolds CBP/p300 (Cougot et al., 2007) and mSin3a (Arzumanyan et al., 2011), and alters the extent of DNA methylation and histone acetylation (Zheng et al., 2009). Further, there is increasing evidence that HBx alters the expression of host gene expression by up- or down-regulating selected miRNAs (Kong et al., 2011; Wu, et al., 2011). In many cases, the natural targets of these epigenetic changes have not been identified. The importance of doing so will provide both prognostic markers and therapeutic targets relevant to the pathogenesis of CLD and HCC, thus providing opportunities for earlier intervention.

3.1 HBx and fibrogenesis

3.1.1 Transforming growth factor beta 1 (TGFβ1)

The close association between intrahepatic expression of HBx and the severity of CLD suggests that HBx may take a part in driving pathogenesis. TGFβ1 is an important mediator of fibrosis and apoptosis in carriers with CLD (Castilla et al., 1991; Liu et al., 1999), as indicated by the direct correlation between serum TGFβ1 levels, elevated aminotransferases, and fibrosis scored in liver biopsy specimens (Flisiak et al., 2004). HBx has been shown to transcriptionally up-regulate the expression of TGFβ1 both in cell cultures and in HBx transgenic mice (Martin-Vilchez et al., 2008; Norton et al., 2004; Yoo et al., 1996). In liver tissue with HBx protein expression, phospho-Smad2 was detectable, suggesting a functional link between viral protein expression and TGF-β1 signaling. Phospho-Smad2 staining correlated significantly with fibrotic stage in patients with HBV infection and steatosis/steatohepatitis (Weng et al., 2009). HBx mediated up-regulation of TGFβ1 was further potentiated by suppressed expression of the natural inhibitor of TGFβ1, alpha-2-macroglobulin (α2M, Figure 1) (Pan et al., 2004). HBx may suppress α2M gene expression by either activation of NF-κB, which then blocks the activation of the α2M gene by STAT3, and/or by the HBx activation of PI3K, which then blocks α2M expression. Independent work showed that HBx also shifted TGFβ1 signaling from tumor suppression to tumor promotion in the livers of patients with chronic hepatitis B, and that this involved differential phosphorylation of smad3 *in vivo* (Murata et al., 2009). HBx was also shown to enhance TGFβ signaling by stabilizing a protein complex consisting of smad4 and components of the basic transcriptional machinery (Lee et al., 2001). The fact that HBx stimulates multiple signal transduction pathways (e.g., NF-κB, PI3K, MAPK, Wnt, ras, src, etc), combined with altered smad signaling, also appear to override the homeostatic and growth inhibitory properties of TGFβ1. This results in the development of a strong profibrogenic environment in the liver (Akhurst, 2002) which may underlie the close

relationship between HBx, inflammation, and fibrogenesis seen in earlier studies (Wang et al., 1991a, 1991b). In this context, hepatic inflammation, fibrosis and cell death were demonstrated in TGFβ1 transgenic mice (Sanderson et al., 1995), underscoring the contribution of elevated TGFβ1 expression to CLD. Interestingly, HBx also blocks TGFβ1 mediated growth inhibition and apoptosis, in part, through the up-regulation of PI3K (Shih et al., 2000), suggesting that HBx may confer resistance to TGFβ1 mediated growth inhibition, while uninfected cells remain sensitive, thereby favoring survival of virus infected hepatocytes. These observations are consistent with the strong correlation between HBx staining and the progression of CLD among HBV infected carriers (Jin et al, 2001; Wang et al., 1991a, 1991b).

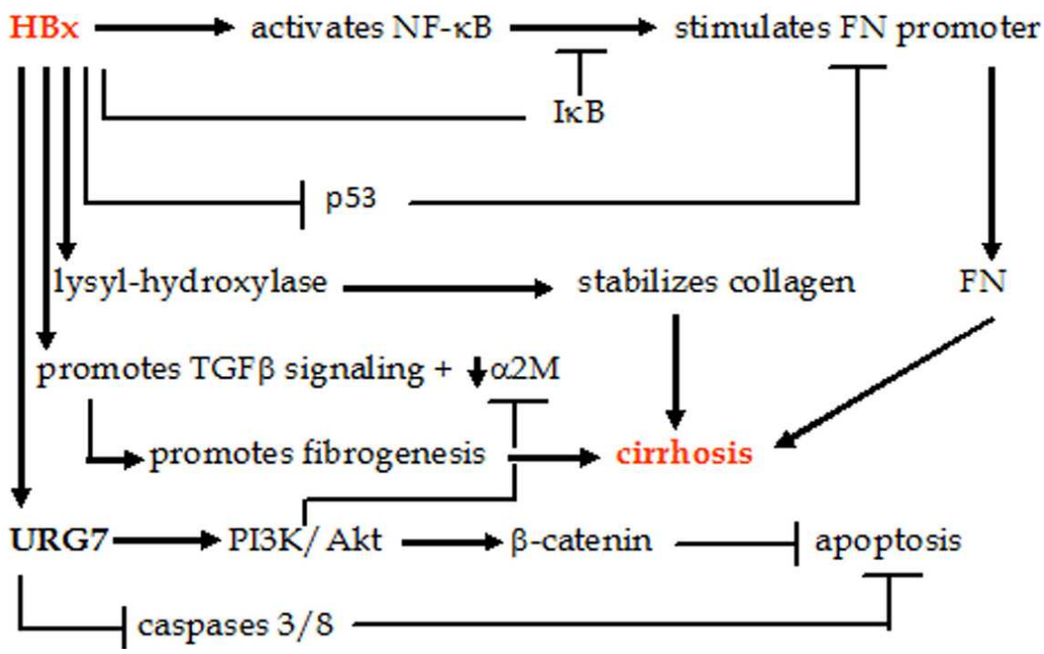

Fig. 1. Proposed model of how HBx may contribute to the development of cirrhosis. See the text for details.

3.1.2 Fibronectin (FN)

This close relationship is exemplified by the observations that HBx activation of NF-κB resulted in the stimulation of the fibronectin (FN) promoter (Figure 1), and that liver tissue samples from chronically infected patients showed a strong correlation between HBx and FN mRNA in hepatocytes from fibrotic and cirrhotic livers (Norton et al., 2004). In this context, the fact that HBx binds to and inactivates the tumor suppressor protein, p53, both *in vitro* and *in vivo* (Feitelson et al., 1993b; Ueda et al., 1995), and that p53 normally suppresses the FN promoter, suggest that inactivation of p53 also results in increased FN production. Interestingly, up-regulation of FN in HBx expressing cells also showed a modest (50%) decrease in adherence to FN (Lara-Pezzi et al., 2001a, 2001b) and depressed expression of the FN receptor, α5β1 integrin. There was also an observed decrease in the levels of collagen/laminin receptor α1 subunit in HBx positive compared to negative cells

(Lara-Pezzi et al., 2001a), suggesting that HBx promotes the detachment of infected cells from the extracellular matrix (ECM). This detachment was associated with increased cell migration, indicating that changes in the ECM-cell relationship probably also contributed to alterations in tissue morphology that accompany the development of cirrhosis. Since activated ras and src signaling depress α5β1 expression (Varner et al., 1995), that HBx stimulates ras and src signaling (Klein & Schneider, 1997), and that HBx disrupts adherens junctions in a src dependent manner (Lara-Pezzi et al., 2001b), it is likely that the activation of these signaling pathways by HBx contribute importantly to decreased integrin expression, decreased cell adhesion, and an increased propensity for cell migration and loss of tissue morphology in the infected liver, and to metastasis in already established tumors.

3.1.3 Lysyl hydroxylase (LH3)

As indicated above, the accumulation and remodeling of ECM is central to the development of fibrosis and cirrhosis. In this context, the finding that HBx up-regulates the expression of the enzyme, lysyl hydroxylase 3 (LH3) in liver cells, and that LH3 co-stains with HBx in livers of HBV infected patients (unpublished observations), suggests another mechanism whereby an HBx target gene may contribute to fibrosis (Figure 1). LH3 mediates the chemical cross-linking of several collagen and collagen-like molecules (Myullyla et al., 2007). This may promote stabilization of the ECM during chronic infection. Given that LH3 knockout mice with disrupted formation of basement membranes during embryogenesis resulted in embryonic lethality (Myullyla et al., 2007), the over-expression of LH3 during chronic HBV infection may promote the development and persistence of basement membranes that are characteristic of fibrosis. This would sever the intimate relationship between hepatocytes and the bloodstream observed in normal livers. Although LH3 is associated with the endoplasmic reticulum, it has also been found in the extracellular space and in serum (Salo et al., 2006), implying that LH3 serum levels may be elevated in the blood prior to the development of HCC.

3.1.4 Does HBx activate stellate cells?

It is also possible that HBx expression promotes stellate cell activation. Although there is little evidence that HBV infects stellate cells, when HBx was transfected into a human stellate cell line, it promoted proliferation and up-regulated expression of fibrosis related molecules (Guo et al., 2009). Independent work showed that HBx expressing hepatocytes induced paracrine activation of human and rat hepatic stellate cells. When these cells were exposed to conditioned medium from HBx-expressing hepatocytes, they showed increased expression of collagen I, connective tissue growth factor, alpha smooth muscle actin, matrix metalloproteinase-2, and TGFβ, together with an enhanced proliferation rate (Martin-Vilchez et al., 2008). More recently, hedgehog signaling and ligand production have been demonstrated to be activated in clinical samples from HBV (and hepatitis C virus) infected patients These ligands promoted the *in vitro* expansion of liver myofibroblasts, activated endothelial cells, and progenitors expressing markers of tumor stem/initiating cells (Pereira et al., 2010). Independent data has shown that hedgehog signaling is profibrogenic, in that it promotes activation and EMT in quiescent hepatic stellate cells (Choi et al., 2009), and in the context of cholestatic liver injury (Omenetti et al., 2011). Given that hedgehog signaling is

also known to promote tissue remodeling in the liver (Omenetti & Diehl, 2008), it is possible that this may contribute to the progression and formation of cirrhotic nodules in the liver of chronically infected patients. Preliminary data also suggests that HBx activates hedgehog signaling in liver cancer cells (Kim et al., 2011), although the role of this activation in hepatocarcinogenesis remains to be studied. Further, it is not clear whether the up-regulation of hedgehog ligands is activated by HBx, and whether this in some way contributes to fibrogenesis.

3.2 HBx up-regulated genes in chronically infected liver

3.2.1 Up-regulated gene, clone 7 (URG7)

Subtractive hybridization of mRNAs from HBx positive compared to negative human hepatoblastoma (HepG2) cells yielded a set of differentially expressed mRNAs that revealed additional mechanisms whereby HBx contributes to the pathogenesis of HCC. Several unique mRNAs were identified by subtractive hybridization, and among them were a number of previously uncharacterized transcripts. One of them, URG7, encoded a 99 amino acid polypeptide with no distinguishing functional motifs (Lian et al., 2001), was found to down-regulate the expression of the TGFβ1 inhibitor, α2M (Figure 1), suggesting that it contributes to the development and progression of fibrosis. It appears to do so by activation of PI3K, by stabilization of β-catenin, and by blocking the activities of caspase 8 and 3 (Pan et al., 2007) (Figures 1 and 2). Among its many activities, HBx also activates PI3K (Lee et al., 2001), stabilizes β-catenin (Lian et al., 2006), and blocks caspase 3 (Gottlob et al., 1998), suggesting that these functions may be carried out by URG7. Further data showed that both HBx and URG7 activated fragments of the β-catenin promoter, and also promoted expression of β-catenin target genes. These include c-myc (Terradillos et al., 1997), multi-drug resistance gene 1 (MDR1) (Doong et al., 1998) and cyclin D1 (Park et al., 2006). While the activation of β-catenin target genes by URG7 suggests that the latter promotes tumor formation, URG7 did not promote growth of HepG2 cells in soft agar, nor did it accelerate the outgrowth of HepG2 based tumors in SCID mice (Lian et al., 2001). Its role in blocking apoptosis, however, is shared with that of β-catenin (Chen et al., 2001). Importantly, one of the major characteristics of tumor cells is resistance to immune mediated apoptosis. The finding that URG7 is over-expressed in infected liver, but not in HCC cells from clinical specimens, suggests that resistance to apoptosis precedes the development of tumor, and that it probably protects HBV infected cells from immune damage and elimination. On the molecular level, caspase 8, which is just up-stream of caspase 3, transmits death signals from Fas (T cell) and from TNFα signaling (Figure 2). In this context, it had previously been shown that HBx blocks Fas mediated killing in primary human hepatocytes (Diao et al., 2001), which may actually be mediated by URG7. Further, the finding that HBx activates NF-κB (Su & Schneider, 1996), that activated NF-κB protects hepatocytes from cell death (Beg et al., 1995, 1996), and that NF-κB transcriptionally activates URG7 (Pan et al., 2001), suggest a pathway that promotes persistence of the carrier state (and sustained HBV replication) even in the presence of recurring immune responses spanning many years. The findings of elevated TNFα production in human hepatocytes infected with HBV, and that HBx targets this up-regulation (Lara-Pezzi et al, 1998), not only suggests that TNFα is a target for HBx, but is also consistent with the strong correlation between HBx expression and inflammatory liver disease (Jin et al., 2001; Wang et al., 1991a, 1991b).

Additionally, the observation that HBx activates the expression of Fas ligand in HCC cell lines (Shin et al., 1999), may provide a way for virus infected cells to escape direct T cell killing by inducing apoptosis in such T cells. This would not only promote chronicity, but in tumor cells, an escape from immune elimination.

3.2.2 Up-regulated gene 11 (URG11) and hepatocarcinogenesis

Another transcript identified by subtractive hybridization in HBx positive compared to negative HepG2 cells encoded a novel protein provisionally designated as URG11 (Lian et al., 2003). The protein product was about 70kDa (673 amino acids) in size and contained five von Willebrand factor type-C repeats and one C-type lectin domain. Functional characterization showed that over-expression of URG11 significantly stimulated cell growth in culture, anchorage-independent growth in soft agar, accelerated tumor formation, and yielded larger tumors in SCID mice injected subcutaneously with HepG2 cells. Further work showed that HBx *trans*-activated URG11, and that URG11 *trans*-activated the β-catenin promoter. URG11 specific siRNA inhibited the growth of HBx expressing liver cells in serum free medium. The latter was associated with depressed levels of β-catenin. As

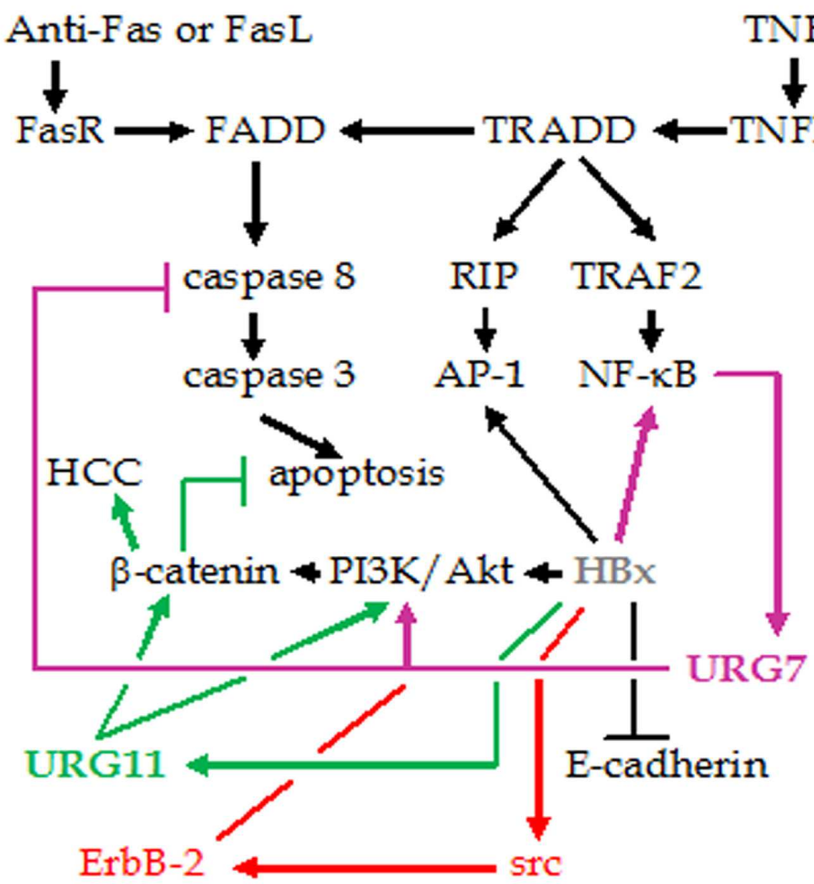

Fig. 2. Model showing selected steps of how HBx inhibits apoptosis and promotes tumorigenesis. HBx alters pathways involving URG7 (in purple), URG11 (in green), and ErbB-2 (in red). See the text for additional details.

with URG7, there was extensive co-staining between HBx and URG11 in chronically infected liver (Lian et al., 2006) but not in tumor. This suggests that URG11 promotes hepatocellular growth prior to the appearance of HCC. The ability of URG11 specific siRNA to block the growth of liver tumor cells both *in vitro* and *in vivo*, not only underscores the importance of elevated URG11 to cell growth, but also suggests that it may be a novel target for the development of specific therapeutics against HCC (Fan et al., 2011). Independent work has recently shown that URG11 was induced under hypoxic conditions in human kidney tubule cells (Du et al., 2010). The latter was associated with increased levels of HIF-1α, which is also known to be a target of HBx (Holotnakova et al., 2010). Importantly, HIF-1α is known to *trans*-activate VEGF *in vivo* (Yoo et al., 2003), suggesting that neovascularization may occur in cirrhotic nodules prior to the appearance of HCC. If this occurs during the pathogenesis of chronic hepatitis B, it would most likely be observed in cirrhotic nodules, since this represents a hypoxic environment characterized by high levels of HBx expression (Wang et al., 1991a, 1991b). Interestingly, elevated expression of URG11 in kidney tubule cells was also associated with suppression of E-cadherin, and up-regulation of the mesenchymal markers vimentin and alpha-SMA, suggesting that URG11 is associated with EMT. In chronic HBV infection, the development of cirrhosis is accompanied by considerable alterations in the tissue architecture within the liver, implying that URG11 may also play a significant role in tissue remodeling during the pathogenesis of chronic infection.

3.2.3 Elevated vascular endothelial growth factor receptor 3 (VEGFR-3)

Vascular endothelial growth factor receptor 3 (VEGFR-3), which is associated with angiogenesis, is a receptor tyrosine kinase that is expressed in lymphatic endothelial cells (Iljin et al., 2001). Binding of VEGFR-3 to the ligands VEGF-C or VEGF-D stimulate lymphangiogenesis (Alitalo & Carmeliet, 2002), while in carcinogenesis, the production of VEGFs by tumors promote metastases and result in decreased survival (Su et al., 2006). Elevated VEGF has been found in patients with HCC (Dahr et al., 2002, Poon et al., 2003). VEGFR-3 is also expressed in tumor cells from several tumor types (Bando et al., 2004, Su et al., 2006), including HCC (Dahr et al., 2002), implying the existence of an autocrine/paracrine loop that promotes tumor development independent of lymphangiogenesis (Su et al., 2006). In HCC, elevated VEGFR-3 is associated with portal vein invasion of tumors, increased hepatic tumor recurrence, and shorter survival (Dhar et al., 2002), suggesting that VEGFR-3 is important in the pathogenesis of HCC. In this context, differential display of HBx positive compared to negative cells showed that HBx up-regulated the expression of an mRNA which encoded a splice variant of VEGFR-3 (Lian et al., 1997). This was verified at the mRNA and protein levels in HBx positive compared to negative HepG2 cells. In infected liver, expression of VEGFR-3 was prominent in nodules of HCC and correlated with HBx expression. VEGFR-3 stimulated cell cycle in culture, anchorage independent growth in soft agar, and accelerated tumor formation and larger tumor size in SCID mice injected with HepG2 cells over-expressing VEGFR-3. Further work showed that over-expression of VEGFR-3 in the absence of HBx resulted in activation of PI3K/Akt, which then activated β-catenin gene expression (Figure 2), and with inactivation of the tumor suppressor, PTEN. Interestingly, HBx also mediates these changes, suggesting that they may be actually carried out by up-regulation of VEGFR-3. These findings also suggest that in addition to lymphangiogenesis, VEGFR-3 may promote tumorigenesis in HBx associated HCC.

3.2.4 Elevation of β-catenin and suppression of E-cadherin

Constitutive activation of β-catenin is characteristic of many tumor types (Fukuchi et al., 1998; Morin et al., 1997). This results in constitutive Wnt signaling, where β-catenin translocates to the nucleus and stimulates the expression of genes that promote tumorigenesis (Clevers & van de Wetering, 1997; Peifer & Polakis, 2000; Terradillos et al., 1997). Importantly, β-catenin mutations are found in small HCCs and in preneoplastic liver (Calvisi et al., 2001; Terris et al., 1999,), suggesting they occur early in tumor development. The finding of frequent β-catenin mutations in a subset of human HCC (de La Costa et al., 1998, Miyoshi et al., 1998), especially in HBV-negative tumors (Hsu et al., 2000), implies that the majority of β-catenin activation must occur by mechanisms other than mutation. In the chronically infected liver, HBx has been shown to be associated with the constitutive activation of wild type β-catenin. The finding that the activation of wild type β-catenin was associated with URG11 (Lian et al., 2006) and URG7 (Pan et al., 2007), underscores the importance of this activation in hepatocarcinogenesis. Moreover, β-catenin appears to be stabilized by a number of mechanisms, including *trans*-activation of the β-catenin promoter (Lian et al., 2006; Pan et al., 2007), inhibition of proteasomal degradation (Cui et al., 2006; Zhang et al., 2000), and suppression of E-cadherin expression (Arzumanyan et al., 2011; Lee et al., 2005; Liu et al., 2006) (Figure 2). The latter is of particular importance because suppression of the cell adhesion protein, E-cadherin, is a hallmark of EMT, which is important to the pathogenesis of CLD and HCC. The importance of suppressed E-cadherin expression is further underscored by the findings that this occurs by DNA methylation of the E-cadherin promoter (Lee et al., 2005; Liu et al., 2006), by the inhibition miR-373 expression by HBx, and by HBx mediated stimulation of histone deacetylase (HDAC) at the E-cadherin promoter (Arzumanyan et al., 2011). Independent of the mechanism involved, suppression of E-cadherin has important ramifications upon β-catenin. Normally, β-catenin participates in cell adhesion by serving as a link between E-cadherin and the cytoskeleton. When E-cadherin expression is suppressed, β-catenin is released from this role and translocates to the nucleus where it activates genes that promote cell growth. Thus, in the presence of HBx, there is an inverse correlation with E-cadherin expression, and a direct correlation with the accumulation of cytoplasmic and nuclear β-catenin at the expense of membranous β-catenin, both in cultured cells and in clinical specimens (Arzumanyan et al., 2011; Lian et al., 2006; Liu et al., 2006). This suggests a tight coupling between EMT and the promotion of hepatocellular growth prior to the development of HCC (Du et al., 2010).

3.2.5 Elevated expression of ErbB-2

Another natural effector of HBx is ErbB-2 (Liu et al., 2009). ErbB-2 (HER2 or neu) is a member of the epidermal growth factor receptor tyrosine kinases that is involved in the transmission of differentiation and proliferation signals (Olayioye et al., 2000, Yarden & Sliwkowski, 2001). High levels of ErbB-2 have been shown in various types of cancers (Sauter et al., 1993; Slamon et al., 1987; Tanner et al., 1996), and in some tumors, over-expression is associated with poor prognosis. In breast cancer, up-regulated ErbB-2 appears to be an early event, since it appears in tumor and nontumor tissue (Menard et al., 2002). In HCC, elevated ErbB-2 has been reported in hyperplastic nodules (Niu & Wang, 2005) and in 30-40% of HCCs (Chen et al., 2002; Neo et al., 2004). However, ErbB-2 was not found in HCC

tissues from other studies (Alitalo & Carmeliet, 2002; Hsu et al., 2002; Vlasoff et al., 2002). The finding that HBx up-regulates and stabilizes β-catenin (Lian et al., 2006), which in some tumors is activated by elevated levels of ErbB-2, suggested that constitutive expression of β-catenin may be associated with elevated ErbB2. Accordingly, when HBx positive and negative cells were subjected to proteomics analysis, ErbB-2 was up-regulated in HBx expressing but not control cells. ErbB-2 was also strongly up-regulated in HBV infected liver, where it correlated with HBx expression, and weakly in some HCC nodules (Liu et al., 2009). Among tumor bearing patients, strong ErbB-2 staining in the liver was associated with dysplasia, and a shorter survival after tumor diagnosis. This implies that elevated ErbB-2 is an early marker of HCC. Treatment of HBx expressing cells with ErbB-2 specific siRNA not only reduced ErbB2 expression, but also reduced the expression of β-catenin, suggesting that ErbB-2 contributed to the stabilization of β-catenin. ErbB-2 specific siRNA also partially blocked the ability of HBx to promote DNA synthesis and growth of cells *in vitro* (Liu et al., 2009). These results suggested that ErbB-2/β-catenin up-regulation contributed to HBx mediated hepatocellular growth. The additional finding that HBx stimulates expression of the epidermal growth factor receptor (EGFR or ErbB1) (Menzo et al.,1993), and that EGFR signaling stabilizes β-catenin (Takahashi et al., 1997), suggested that EGF signaling may be strongly activated in patients at high risk for HCC or with already established tumors. This suggests that elevated ErbB-2 may be rate limiting in tumor formation, and if so, may be a therapeutic target (Altimari et al., 2003). Further, the accumulation of wild type β-catenin in the presence of elevated ErbB-2 correlated with the activation of PI3K/Akt signaling, which is known to be activated by HBx and ErbB-2 (Lian et al., 2006; Shih et al., 2000; Yarden & Sliwkowski, 2001) (Figure 2). PI3K/Akt activity may also be stimulated by src, the latter of which is activated by HBx, early in tumor development (Lara-Pezzi et al., 2001b; Shih et al., 2003). Further, the peptidyl prolyl isomerase, Pin1, is up-regulated in HCC, and is known to stabilize both HBx (Pang et al., 2007) and ErbB-2 (Lam et al., 2008), suggesting a variety of possible mechanisms underlying the close HBx/ErbB-2 relationship.

3.2.6 Other natural target genes of HBx

In addition to transcriptional regulation of gene expression, HBx up-regulates expression of the ribosomal protein, S15a (Lian et al., 2004) and down-regulates expression of the translation initiation factor, Sui1 (Lian et al., 1999). S15a is a highly conserved protein (Chan et al., 1994; Reed, 1980; Schaap et al., 1995) that promotes mRNA/ribosome interactions early in translation (Lavoie et al., 1994). S15a also stimulates growth in yeast (Pringle et al., 1981; Reed, 1980,), in plants (Bonhan-Smith & Moloney, 1994; Bonham-Smith et al., 1992) and in human lung carcinoma cells (Akiyama et al., 2000). The observation that S15a stimulates hepatocellular growth and survival *in vitro*, and tumor formation *in vivo*, suggests that it also plays a role in hepatocarcinogeneis, and that HBx contributes to transformation, in part, at the level of protein translation by up-regulated expression of S15a (Lian et al., 2004). As stated above,, HBx was also shown to depress the expression of the translation initiation factor, sui1. Sui1, whose function is to work with eIF-2 to enable the initiator $tRNA^{MET}$ to establish ribosomal recognition of an AUG codon (Yoon and Donahue, 1992), suggests that the expression of hu-sui1 contributes to the regulation of protein translation. *In*

vivo work showed that sui1 was expressed in nontumor liver but not in tumor cells from patients with HCC. Sui1 inhibited cell growth in culture, in soft agar, and partially inhibited tumor formation in nude mice, suggesting that suppression of sui1 may result in the abrogation of negative growth regulation that contributes to the development of HCC (Lian et al., 1999). Given that S15a and sui1 are both involved in regulating translation, it is likely that HBx also contributes to HCC by altering gene expression at multiple steps within translation, although the mRNAs that are differentially translated remain to be identified.

HBx also stimulates the expression of the novel protein, URG4 (Tufan et al., 2002). URG4, encodes a protein of about 104 kDa that was strongly expressed in HBV- infected liver and in HCC cells, where it co-stained with HBx, and was weakly expressed in uninfected liver, suggesting URG4 was an effector of HBx *in vivo*. Over-expression of URG4 without HBx in human hepatoblastoma cells promoted hepatocellular growth and survival in tissue culture and in soft agar, and accelerated tumor development in nude mice (Tufan et al., 2002). URG4 over-expression was associated with elevated cyclin D1 expression, and treatment of such cells with URG4 specific siRNA reduced both cyclin D1 expression and inhibited cell cycle progression (Tufan et al., 2010). These observations suggest that URG4 may be an oncogene that contributes to HBV associated HCC. Independent work showed that over-expression of URG4 in osteosarcoma tissues directly correlated with tumor recurrence and metastasis, as well as with the proliferative activity of osteosarcoma cells. Patients with high expression of URG4 had shorter survival time, suggesting that URG4 might be rate limiting in carcinogenesis and a valuable prognostic marker in osteosarcoma patients (Huang et al., 2009). Thus, URG4 may contribute to carcinogenesis outside of the liver.

HBx also appears to up-regulate the expression of insulin - like growth factor 2 (IGF-2) and the IGF-1 receptor in HCC (Kim et al., 1996; Su et al., 1994). The finding that insulin-like growth factor-2 expression, which is normally observed only in fetal liver (Soares et al., 1985), is elevated in HCCs (D'Arville et al., 1991, Cariani et al., 1991), and in premalignant proliferative nodules in the liver (Cariani et al., 1988; D'Arville et al., 1991), suggest that its reactivation may be an early step in the development of this tumor type. The elevation of IGF-2 expression in HCCs from HBV infected but not uninfected patients, combined with the finding of a strong correlation between IGF-2 and HBx in the liver by immunohistochemical staining (Su et al., 1994), suggest that IGF-2 may be a natural target of HBx during chronic infection. In human hepatoma cell lines, IGF-2 was expressed strongly in growing cells, but was undetectable in confluent cultures (Su et al., 1994), suggesting that it was associated with cell proliferation. At the molecular level, the tumor suppressor, PTEN normally suppresses IGF-2 expression (Kang-Park et al., 2003), but in the presence of HBx, PTEN expression is blocked, resulting in activation of IGF-2 (Chung et al., 2003). Normally, PTEN is up-regulated by another tumor suppressor, p53, but since HBx binds to and inactivates p53 (Feitelson et al., 1993b; Wang et al., 1994), PTEN expression also drops (Chung et al., 2003). In addition, HBx activation of Sp1 via protein kinase C (PKC) and p44/p42MAPK signaling pathways are also operative in promoting IGF-2 gene expression (Kang-Park et al., 2001). These multiple pathways underscore the importance of IGF-2 up-regulation in hepatocarcinogenesis. Finally, the finding that HBx stimulates the expression of the IGF-1 receptor in human HCC cell lines (Kim et al., 1996), which binds both IGF-1 and IGF-2, suggests that HBx may set up an autocrine loop that enhances cell growth. Thus, the

up-regulated expression of IGF-2, which appears to be a target of HBx *in vivo*, may promote hepatocarcinogenesis.

The finding that HBx interacts with and inhibits the function of the proteasome (Huang et al., 1996) suggests another mechanism whereby HBx could alter gene expression at a post-translational level. This inhibition appears to be important in supporting HBx *trans*-activation activity (Hu et al., 1999). Given that HBx *trans*-activates virus gene expression and replication, when mutants of the X protein that bound to and inhibit the proteasome were introduced into WHV, and the resulting virus used for experimental infection, no or transient viremia was observed. In contrast to wild type WHV, which resulted in a high carrier rate among experimentally infected woodchucks, none of the animals infected with the X mutant developed the carrier state (Zhang et al., 2001). Further work *in vitro* showed that in the presence of proteasome inhibitors, replication of the wild-type virus was not affected, while the replication of the X-negative HBV or WHV was enhanced and restored to the wild-type levels. Similar results were obtained in mouse models replicating wild type and X mutant HBV (Zhang et al., 2010). Thus, HBx appears to affect hepadnavirus replication through a proteasome-dependent pathway (Zhang et al., 2004). Moreover, in the livers of transgenic mice where the levels of HBx expression increased with age, there was a parallel age related decreases in the peptidase activities of the proteasome in the liver (Hu et al., 2006). Microarray analysis showed that many of the genes affected involved transcription and cell growth. For example, insulin-like growth factor-binding protein 1 was down-regulated in the HBx mouse liver (Hu et al., 2006), while *in vitro*, HBx stabilized c-myc (Kalra & Kumar, 2006) and the protooncoprotein, pituitary tumor-transforming gene 1 (PTTG1) (Molina-Jimenez et al., 2010), by blocking ubiquitination and proteasomal degradation. HBx also differentially regulated the level of β-catenin through two ubiquitin-dependent proteasome pathways depending upon the status of p53 (Jung et al., 2007). Given that HBx expression is dominant in liver compared to HCC tissue (Wang et al., 1991a, 1991b), it was not surprising to find an elevated proteasomal activity in HCC compared to surrounding nontumor liver, both in HBx transgenic mice that developed tumors, and in clinical samples from patients with HCC (Cui et al., 2006). These observations suggest that changes in proteasome function accompany the pathogenesis of CLD and HCC, and that these changes appear to be related to the levels of HBx .

4. Conclusions

Tumorigenesis is a multi-step process, and as outlined above, HBx impacts upon this process by targeting selected pathways and genes in natural infection. For most of the target genes presented here, up-regulated or down-regulated expression was established by comparison of gene expression profiles in HBx positive compared to negative cells, suggesting that they were due to the properties of HBx. Clinical validation was carried out on liver and tumor tissues obtained from HBV infected patients. For up-regulated genes, there was strong co-staining between HBx and the putative target, while for down-regulated genes, there was an inverse relationship by immunohistochemistry, and in many cases, northern blotting or RT/PCR analyses as well. Moreover, many of the natural targets of HBx discussed herein were characterized to gain at least a preliminary outline as to their contribution to the pathogenesis of HCC. The overall results show that HBx contributes to

multiple steps in hepatocarcinogenesis, and that the pleiotrophic nature of HBx, known for many years, is now being better understood by the functions of the proteins encoded by these target genes. These data provide crucial information as to the steps in the pathogenesis of HCC that are likely to be rate limiting, which is very important for the application of therapeutic approaches to known targets and for the development of therapeutics to novel targets. The hope embodied in these studies is that they will lead to the development of diagnostic/prognostic biomarkers and/or therapies that will specifically target gene products whose functions appear to be rate limiting in tumorigenesis. The fact that most of the up-regulated genes are over-expressed in liver, and much less often in tumor, means that specific therapies could be devised to ultimately reduce the risk for development of HCC, and if this is achieved, this would open up the probability that chemoprevention could become a realistic approach to treating patients at high risk for the appearance of cancer. This approach will not only be useful, for approaching cancer prevention in the liver, but if one or more of the URGs described above are also elevated in precancerous lesions from other tissue types, the approach would become more widespread in preventing the development of other tumors. In doing so, this has the possibility of establishing a different paradigm for therapeutic approaches against cancer.

5. Acknowledgements

This work was supported by grants CA111427, CA104025, and AI076535 awarded to Dr. Mark Feitelson

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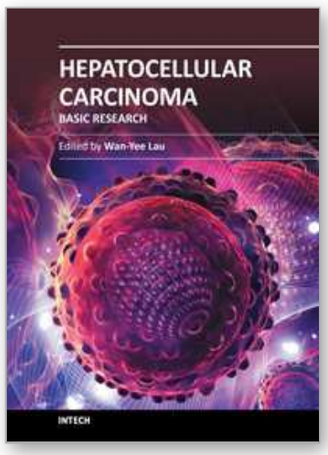

Hepatocellular Carcinoma - Basic Research
Edited by Dr. Joseph W.Y. Lau

ISBN 978-953-51-0023-2
Hard cover, 402 pages
Publisher InTech
Published online 10, February, 2012
Published in print edition February, 2012

Hepatocellular Carcinoma represents a leading cause of cancer death and a major health problem in developing countries where hepatitis B infection is prevalent. It has also become increasingly important with the increase in hepatitis C infection in developed countries. Knowledge of hepatocellular carcinoma has progressed rapidly. This book is a compendium of papers written by experts to present the most up-to-date knowledge on hepatocellular carcinoma. This book deals mainly with the basic research aspect of hepatocellular carcinoma. The book is divided into three sections: (I) Biomarkers / Therapeutic Target; (II) Carcinogenesis / Invasion / Metastasis; and (III) Detection / Prevention / Prevalence. There are 18 chapters in this book. This book is an important contribution to the basic research of hepatocellular carcinoma. The intended readers of this book are scientists and clinicians who are interested in research on hepatocellular carcinoma. Epidemiologists, pathologists, hospital administrators and drug manufacturers will also find this book useful.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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