Abstract

Chronic hepatitis B virus (HBV) infection is a major risk factor for hepatocellular carcinoma (HCC). Hepatitis B virus X protein (HBx) is a HBV protein that has multiple cellular functions, but its role in HCC pathogenesis has been controversial. The farnesoid X receptor (FXR) is a nuclear receptor known to have activities in anti-inflammation and inhibition of hepatocarcinogenesis. However, whether or how FXR can impact HBV/HBx-induced hepatocarcinogenesis remains unclear. In this study, we showed that HBx can interact with FXR and function as a co-activator of FXR. Expression of HBx in vivo enhanced FXR responsive gene regulation. HBx also increased the transcriptional activity of FXR in luciferase reporter gene assay. The HBx-FXR interaction was confirmed by co-immunoprecipitation and GST-pull down assays, and the FXR AF-1 domain was mapped to bind to the third α helix in the C-terminal of HBx. We also found that the C-terminal truncated variants of HBx, which were found in clinical HCC, were not effective in transactivating FXR. Interestingly, recruitment of the full-length HBx, but not the C-terminal truncated HBx, enhanced the binding of FXR to its response element. In vivo, FXR ablation markedly sensitized mice to HBx-induced hepatocarcinogenesis.

Conclusions—We propose that transactivation of FXR by the full-length HBx may represent a protective mechanism to inhibit HCC, and this inhibition may have been compromised upon the
appearance of the C-terminal truncated HBx, or when the expression and/or activity of FXR is decreased.

**Keywords**
nuclear receptor; co-activator; gene regulation; HCC; transgenic mice

**Introduction**
Hepatocellular carcinoma (HCC) is a deadly malignancy (1, 2). Accumulating evidence suggests that hepatitis B virus (HBV) X protein (HBx), a 154-amino acids viral protein encoded by the HBV genome, plays an important role in the pathogenesis of HBV-associated HCC. Early work showed that the expression of HBx under the control of its own regulatory elements led to the development of HCC in transgenic mice (3). Anti-HBx antibody can be detected in the serum or tissue samples of HCC patients (4, 5). However, the oncogenic phenotype of HBx is not without controversies. A strain of independently generated ATX transgenic mice, in which the expression of HBx was under the control of the human α1-antitrypsin gene promoter, failed to develop spontaneous HCC (6), although they were more sensitive to diethylnitrosamine-induced carcinogenesis (7, 8).

HBx can be detected in both the cytoplasm and nucleus, and both populations of HBx contribute to the transcriptional regulation. The cytosolic HBx affects transcription by modulating cell signaling in the cytoplasm, whereas the nuclear HBx can directly impact on the chromatin and transcription (2). As a transcriptional regulator, an intriguing feature of HBx is that it does not bind to DNA directly. Instead, HBx exerts its regulatory function mainly via the protein-protein interactions (2).

The mechanism by which HBx may promote HCC remains poorly defined. Among the proposed mechanisms, HBx has been shown to induce oxidative stress in the liver cells (9). In addition to its carcinogenic activity, HBx can also trigger inflammation (10), an important pathogenic event associated with HCC. Interestingly, in addition to the full-length HBx protein, the C-terminal truncation mutants of HBx have been reported in HCC patients (11, 12), although the conclusions are not without controversies (13). However, the significance of the truncated HBx proteins in the pathogenesis of HCC remains to be defined.

The farnesoid X receptor (FXR) is a nuclear receptor highly expressed in the liver and intestine. FXR exhibits diverse functions, ranging from maintaining the homeostasis of bile acids (14) to regulating the metabolism of glucose and lipids (15), inhibiting liver fibrosis (16), promoting liver regeneration (17), reducing liver inflammation (18), and inhibiting hepatic (19, 20) and intestinal (21, 22) carcinogenesis. The FXR−/− mice in the C57BL/6 background developed spontaneous liver tumors (19). FXR inhibited NF-κB-mediated inflammation (18), which may have contributed to the anti-hepatocarcinogenic activity of FXR. In addition to FXR, the FXR target gene small heterodimer partner (SHP) has been proposed as a tumor suppressor gene that inhibits cellular proliferation, activates apoptosis and suppresses cell migration (23-26).
Knowing that HBx and FXR have the opposite role on the pathogenesis of HCC and their effect on inflammation might be a shared mechanism by which these two proteins impact hepatocarcinogenesis, we propose that there might be a functional crosstalk between HBx and FXR. In this study, we showed that HBx enhanced the transcriptional activity of FXR by interacting with FXR and functioning as a FXR co-activator. We also found that the C-terminal truncated HBx variants were not effective in co-activating FXR. Moreover, FXR ablation markedly sensitized mice to HBx-induced hepatocarcinogenesis in vivo.

Experimental Procedures

Animals

The ATX HBx transgenic (ATX) mice have been previously described (6). The FXR−/− mice were purchased from the Jackson Laboratory. The ATX mice in the ICR background were bred with the FXR−/− mice in the C57BL/6 background to generate the ATX-FXR−/− mice. All strains of mice used for the tumorigenic studies are in the mixed background of ICR and C57BL/6. The use of mice in this study has complied with all relevant federal guidelines and institutional policies.

Hydrodynamic liver transfection, plasmids, cell culture, transient transfection and luciferase reporter gene assay, real-time PCR, immunoprecipitation, Western blotting, immunofluorescence and immunohistochemistry, glutathione S-transferase pull-down assay, electrophoretic mobility shift assay, and chromatin immunoprecipitation assay

See Supplemental Materials and Methods for details.

Statistical analysis

Experimental values are expressed as the mean ± standard deviation. Statics were analyzed by Student t tests or Fisher’s exact test. P values of less than 0.05 were considered to be statistically significant.

Results

Expression of HBx in the mouse liver induces the expression of FXR target genes

To examine the functional crosstalk between HBx and FXR, we evaluated the expression of FXR target genes in ATX mice that expresses HBx in the liver (6). The expression of the ATX transgene was confirmed at the mRNA and protein levels (Fig. 1A). The amount of liver lysate required for the detection of HBx in the ATX mice was similar to the amount of liver lysate needed for the detection of the woodchuck WHx protein (27), indicating the level of HBx in ATX mice is comparable to that observed in the woodchuck infection model. SHP and Cyp7a1 are two genes known to be positively and negatively regulated by FXR, respectively. As shown in Fig. 1B, compared to the WT mice, the hepatic expression of SHP was increased and the expression of Cyp7a1 was decreased in ATX mice. The transgene had little effect on the expression of FXR, CAR and the CAR target gene Cyp2b10 (Fig. 1C).

The HBx-responsive regulation of FXR target genes was independently observed in WT mice whose livers were transiently transfected with the expression vector for HBx. In this
experiment, WT mice were hydrodynamically transfected with HBx or the control vector before being treated with the vehicle or the FXR agonist GW4064. The expression of HBx in the transfected livers was confirmed by RT-PCR (Fig. 1D). Expression of HBx increased and decreased the basal expression of SHP and Cyp7a1, respectively (Fig. 1E). Upon the GW4064 treatment, the respective expression of SHP and Cyp7a1 was induced and decreased in both the vector and HBx transfected groups. In GW4064-treated mice, the respective expression of SHP and Cyp7a1 in HBx-transfected mice was significantly higher and lower than their vector-transfected counterparts (Fig. 1E). Transfection of HBx had little effect on the expression of Cyp2b10 (Fig. 1F).

**HBx augments FXR-mediated transcriptional activity in reporter gene assay**

To determine whether HBx can increase the transcriptional activity of FXR, HepG2 cells were co-transfected with the FXR expression vector and FXR responsive reporter gene tk-EcRE-Luc, in the absence or presence of HBx. Transfection of HBx alone had little effect on the reporter activity, consistent with the broadly-activating albeit weak transcriptional transactivation ability of HBx (28). However, HBx dramatically increased the GW4064 responsive reporter activation (Fig. 2A). The potentiation of the FXR-mediated transactivation by HBx was also observed when the transfected cells were treated with the FXR activator CDCA (Fig. 2B). HBx potentiated GW4064-responsive reporter activity in a dose dependent manner (Fig. 2C). The HBx effect appeared to be FXR specific, because HBx had little effect on the activity of CAR in cells co-transfected with the CAR responsive reporter gene tk-PBRE-Luc (29) (Fig. 2D).

**HBx is recruited to the FXR target gene promoter and enhances the binding of FXR to its response element**

The potentiation of FXR transcriptional activity by HBx suggested that HBx might be recruited to the FXR target gene promoter, which was tested by chromatin immunoprecipitation (ChIP) assay. Transfection of HBx into HepG2 cells increased the basal and GW4064-responsive expression of SHP gene (Fig. 3A). To determine whether HBx can be recruited to the FXR target gene promoters, FXR was transfected into HepG2 cells with or without the co-transfection of Flag-HBx. Transfected cells were treated with vehicle or GW4064 before ChIP analysis using an anti-Flag antibody. As shown in Fig. 3B, Flag-HBx was recruited onto the FXR response element (FXRE) on the BSEP (top panel) and SHP (bottom panel) gene promoters, and this recruitment was enhanced in cells treated with GW4064. Electrophoretic mobility shift assay (EMSA) showed that HBx alone was not able to bind to the prototypical IR1 type of FXRE, but the addition of HBx dose dependently increased the FXR-RXR heterodimer binding to FXRE (Fig. 3C). The enhancement of binding was FXR specific, because the addition of HBx cannot increase the binding of CAR-RXR heterodimer to the CAR-responsive phenobarbital response element (PBRE) (29) (Fig. 3D). The enhancement of FXR's binding to its FXRE by HBx was also confirmed by ChIP assay (Fig. 3E).

**HBx interacts with FXR and both proteins are co-localized in the nucleus**

To understand the co-activation of FXR by HBx, we first used GST-pull down assay to determine whether HBx can interact with FXR. As shown in Fig. 4A, GST-HBx specifically
interacted with $^{35}$S-FXR, and this interaction was disrupted when unlabeled FXR protein was added to the reaction. The HBx-FXR interaction was further confirmed by co-immunoprecipitation assay. In this experiment, plasmids encoding the full-length HA-tagged FXR and Flag-HBx were transiently transfected into cells individually or in combination. After 24-hr treatment with vehicle or GW4064, cells were lysed and immunoprecipitated with an anti-Flag antibody, and the presence of FXR in the immunoprecipitate was detected by Western blotting using an anti-HA antibody. The HBx-FXR interaction was detected when both proteins were transfected, but the interaction was not enhanced by the GW4064 treatment (Fig. 4B). Immunofluorescence analysis showed that HA-FXR and Flag-HBx were co-localized in the nucleus when both were transfected into HepG2 cells (Fig. 4C). The co-localization of endogenous FXR and HBx was shown by immunohistochemistry on human HCC sections (Fig. 4D).

Mapping the interface of FXR-HBx interaction

We used GST pull-down assay to map the FXR-HBx interaction domains. The full-length HBx protein contains three α-helixes as outlined in Fig. 5A. To determine which α-helixes were important for the interaction, we constructed GST fusion proteins containing individual α-helixes or the full-length HBx (Fig. 5B) and tested their abilities to interact with $^{35}$S-labeled full-length FXR. We showed that the second and third α-helixes, but not the first α-helix, were capable of binding to FXR (Fig. 5C, right panel). The use of second and third α-helixes to interact with FXR was in contrast to the case of RXR, in which the first α-helix was used for the interaction with RXR (Fig. 5C, left panel), consistent with a previous report (30). To examine the functional relevance of the FXR-interacting α-helixes, we constructed two deletion mutants of HBx that lacked the second α-helix (deletion of amino acids 73-100, or Δ73-100) or the third α-helix (Δ100-120). Compared to the full-length HBx, Δ100-120 was largely ineffective in co-activating FXR, whereas Δ73-100 remained partially effective to co-activate FXR (Fig. 5D). These results suggested that the third α-helix of HBx was most functionally relevant in transactivating FXR.

To map the domain of FXR that interacts with HBx, we constructed FXR fragments that contained either AF-1+DBD or LBD as outlined in Fig. 6A, and tested for their ability to interact with the full-length HBx using GST pull-down assay. To our surprise, it was the AF-1+DBD fragment (Fig. 6B, left panel), instead of the LBD (Fig. 6B, middle panel) of FXR that interacted with HBx. Further GST pull-down analysis showed that it was the AF-1, but not the DBD, that was responsible for the interaction with HBx (Fig. 6C). We then used co-IP assay to confirm the mapping. As shown in Fig. 6D, the AF+DBD fragment co-IP with HBx. Moreover, it was the AF1, but not the DBD of FXR that co-IP with HBx (Fig. 6E). The LBD of FXR failed to interact with HBx in the co-IP assay (Fig. 6E).

The C-terminal truncated HBx proteins are not effective in transactivating FXR, and preferential loss of full-length HBx and increased expression of C-terminal truncated HBx in HCC

Previous studies suggested that the C-terminal truncated HBx variants may be present in HCC patients, and these variants may have played distinct roles in hepatocarcinogenesis (12, 31). We constructed the C-terminal truncated HBx-C40 (deletion of the C-terminal 40 aa)
(Fig. 7A), a truncation similar to those reported in HCC patients (11). Compared to the full-length HBx, HBx-C40 was not effective in potentiating the transcriptional activity of FXR in reporter assay (Fig. 7B), and failed to increase the binding and recruitment of FXR to FXRE as shown by EMSA (Fig. 7C) and ChIP (Fig. 7D), respectively.

To support the notion that truncated HBx may contribute to hepatocarcinogenesis, we profiled the expression of full-length and C-terminal truncated HBx by RT-PCR in 20 cases of HBV-positive HCC tumor tissues and compared the expression to the paired adjacent non-tumor tissues. We observed a preferential loss of full-length HBx and increased expression of C-terminal truncated HBx in tumor tissues as shown by RT-PCR (Supplemental Fig. 1). We also performed IHC on HBV-positive HCC and adjacent non-tumor tissues using two antibodies, Anti-HBx (aa 50-88) and Anti-HBx (aa 139-154). The full-length HBx can be recognized by both antibodies, whereas the C-terminal truncated HBx can only be detected by Anti-HBx (aa 50-88). As shown in Fig. 7E, both antibodies were efficient in detecting HBx in non-tumor tissues. In the tumor tissues, however, HBx was detected only by Anti-HBx (aa 50-88) but not by Anti-HBx (aa 139-154), suggesting the loss of full-length HBx in the tumor tissues. The IHC results were consistent with a previous report (31). When the expression of FXR in HCC was analyzed, we found that in patients expressing exclusively the c-terminal truncated HBx (c-HBx) or both the full-length and c-HBx, although the expression of FXR was markedly lower in tumor tissues compared to adjacent non-tumor tissues as previously reported (32), the expression of FXR within tumor or non-tumor tissues was not different between the full-length HBx cohort and the c-HBx cohort (Fig. 7F).

**FXR ablation sensitizes the carcinogenic activity of HBx in vivo**

To determine whether co-activation of FXR by HBx played a hepatoprotective role in the carcinogenic effect of HBx, we bred the ATX mice with the FXR−/− mice to generate the ATX mice deficient of FXR (ATX-FXR−/−). WT, FXR−/−, ATX and ATX-FXR−/− mice were sacrificed at 15 months of age and examined for spontaneous liver tumors. As shown in Fig. 8A and summarized in Fig. 8B, approximately 50% of the ATX-FXR−/− mice developed grossly identifiable liver tumors at 15 months of age, whereas no tumors were found in WT, ATX and FXR−/− mice. The tumorigenesis in the ATX-FXR−/− mice was confirmed by H&E staining and immunostaining for the cell proliferation marker Ki-67 (Fig. 8C). The livers of many of the ATX-FXR−/− mice without grossly identifiable tumors exhibited pre-neoplastic features, such as steatosis, necrosis and infiltration of inflammatory cells (Fig. 8D). It is interesting to note that the incidence of tumor and pre-neoplastic lesions in the female ATX-FXR−/− mice was significantly higher than in their male counterparts (Fig. 8E). Consistent with the tumor incidence and the gender effect, we observed a significantly increased expression of pro-inflammatory genes *TNFα* and *iNOS* in the ATX-FXR−/− mice when male and female mice were analyzed in combination (Fig. 8F). Within the genotype of ATX-FXR−/−, the expression of *TNFα* and *iNOS* was substantially higher in female mice than in the males (Fig. 8F).

Considering that SHP has been proposed to be a tumor suppressor gene (23), we also profiled the expression of *SHP* along with *Cyp7a1*, *CAR* and *Cyp2b10*. The expression of
SHP was not increased in ATX-FXR\textsuperscript{−/−} mice (Supplementary Fig. 2A), suggesting that the induction of SHP in ATX mice was FXR dependent. The expression of Cyp7a1 was elevated in ATX-FXR\textsuperscript{−/−} mice (Supplementary Fig. 2B), likely due to the dominating effect of FXR ablation on the expression of this gene. Although the expression of CAR was not changed (Supplementary Fig. 2C), the expression of Cyp2b10 was increased both in FXR\textsuperscript{−/−} and ATX-FXR\textsuperscript{−/−} mice (Supplementary Fig. 2D), which might have been caused by the accumulation of hepatic bile acids due to the loss of FXR (33).

To gain more insight into the heightened carcinogenesis in ATX-FXR\textsuperscript{−/−} mice, we profiled the expression of epithelial-mesenchymal transition (EMT) genes E-cadherin and N-cadherin and extracellular matrix (ECM) degradation genes MMP13, TIMP-1 and vimentin that are known to be involved in hepatocarcinogenesis. The expression of E-cadherin was decreased in ATX mice regardless of the FXR genotype (Supplementary Fig. 3A), consistent with a previous report (34). However, the expression of N-cadherin was not affected (Supplementary Fig. 3B). The expression of MMP13 and TIMP-1 was increased in both FXR\textsuperscript{−/−} and ATX-FXR\textsuperscript{−/−} mice (Supplemental Fig. 3C), but the expression of vimentin was unchanged (Supplemental Fig. 3D).

We also measured the expression of genes that are involved in TGF\(\beta\) signaling, cell cycle and apoptosis. The expression of TGF\(\beta\)1 and TGF\(\beta\)2 and their receptors was not affected (Supplemental Fig. 4A). The expression of Cyclin D1 and Cyclin E1 was not affected either (Supplemental Fig. 4B). The expression of Bcl-2 was increased in both FXR\textsuperscript{−/−} and ATX-FXR\textsuperscript{−/−} mice (Supplemental Fig. 4C).

**Discussion**

HBx is considered to be an important co-factor in HBV-associated HCC (1, 3, 28). The HBx proteins are often stably expressed in the hepatocytes and serum of HCC patients throughout the entire hepatocarcinogenesis from chronic HBV infection to the development of HCC.

Since HBx has been implicated in the pathogenesis of HCC, most of the previous work on HBx has been focused on its detrimental effects on liver cells, such as the mitochondria damage, oxidative injury, and inflammation. In the current study, we showed that HBx functions as a potent co-activator of FXR, a nuclear receptor known for its anti-inflammation and anti-hepatocarcinogenic activities. HBx interacts with FXR and increases the transcriptional activity of FXR. Moreover, FXR ablation sensitizes the carcinogenic activity of HBx \textit{in vivo}. While no spontaneous tumors were found in the ATX mice or the FXR\textsuperscript{−/−} mice, nearly half of the ATX-FXR\textsuperscript{−/−} mice developed liver tumors at 15 months of age. The lack of spontaneous liver tumor in the ATX mice was consistent with a previous report (6). Although FXR\textsuperscript{−/−} mice in the C57BL/6 background developed spontaneous liver tumors by the age of 15 months (19), we did not observe liver tumors in our cohort of FXR\textsuperscript{−/−} mice that were maintained in the mixed background of ICR and C57BL/6, likely due to the differences in the genetic background. Based on our observations, we propose that transactivation of FXR by HBx may represent a protective mechanism to inhibit inflammation and the subsequent carcinogenesis. The protective role of FXR in the context of HBV infection and HBx expression is consistent with the notion that viral infection per se is rarely oncogenic in
hepatocarcinogenesis and “second hit” may be necessary to manifest the oncogenic activity of HBV/HBx. The “second hit” may include a decreased expression and/or activity of FXR, as well as the appearance of C-terminal truncated HBx that fails to transactivate FXR. Indeed, the expression of FXR is markedly decreased in human HCC (32). The anti-HCC effect of FXR may have also been contributed to by its target gene SHP, which itself was proposed to be a tumor suppressor gene (23-26). The SHP induction observed in ATX mice was absent in ATX-FXR<sup>−/−</sup> mice, suggesting the SHP induction in ATX mice was FXR dependent and the loss of SHP induction may have also contributed to the increased hepatocarcinogenesis in ATX-FXR<sup>−/−</sup> mice.

The notion that activation of FXR by HBx may function as a protective response was also supported by our observation that the C-terminal truncation mutants of HBx lost their ability to co-activate FXR. The C-terminal truncated HBx proteins have been suggested to be present in HCC patients, which may have played distinct roles in hepatocarcinogenesis (11, 12, 31), although these conclusions are not without controversies (13). We showed the expression level of FXR was indistinguishable between HCC that express the full-length HBx and HCC that express the truncated HBx. It is tempting for us to speculate that the C-terminal truncated HBx may facilitate hepatic carcinogenesis by failing to mount a FXR-mediated protective response. In addition to its effect of anti-inflammation and anti-carcinogenesis, activation of FXR by HBx may also be beneficial by improving hepatic metabolism and preventing the deleterious effect of toxic metabolites, such as toxic bile acids that are known to increase HBV viral gene expression (35).

In addition to transactivating FXR, HBx has also been shown to affect the transcriptional activity of nuclear receptors (NRs) LXR, PPARγ and RXR. There were notable differences between FXR and other NRs in their mode of interactions as well as in the functional outcomes of their interactions with HBx. For example, we showed that HBx enhances the transcriptional activity of FXR through interacting with the AF-1 domain of FXR, and the second and third α-helixes of HBx are important for the interaction. In contrast, HBx also co-activates LXR and PPARγ, but the HBx-LXR and HBx-PPARγ interactions were mediated by the LBD of LXR (36, 37) and DBD of PPARγ (38), respectively. At the functional outcome level, HBx co-activates FXR, whereas the consequence of the PPARγ-HBx interaction is the inhibition of the transcriptional activity of PPARγ (38). HBx can also bind to the LBD of RXR, but the interaction interface was localized to the N-terminal first α-helix of HBx (30). RXR is the obligatory heterodimerization partner of FXR. Since HBx uses distinct interface to interact with FXR and RXR, it is interesting to know whether HBx can simultaneously bind to both FXR and RXR.

Epidemiological and animal studies have shown males are more susceptible to HCC, the mechanism of which was suggested to be due to the stimulatory effect of androgens and the protective effect of estrogens. We were surprised to find that the incidence of liver tumors in the ATX-FXR<sup>−/−</sup> mice was higher in female than in male mice. The expression of pro-inflammatory genes was also higher in female ATX-FXR<sup>−/−</sup> mice than in their male counterparts. A potential explanation is that FXR is more protective in female mice, so FXR ablation in females showed a more dramatic sensitizing effect. The expression of ER and AR was not affected in the ATX-FXR<sup>−/−</sup> mice (data not shown). The detailed mechanism for the
gender specific effect of FXR ablation in HBx-induced hepatocarcinogenesis remains to be understood. It will be interesting to know whether the association between HCC and decreased expression of FXR is more prominent in female patients.

In summary, we have uncovered a novel function of HBx in co-activating FXR. Our results suggested that co-activation of FXR by HBx may represent a protective mechanism to inhibit hepatocarcinogenesis. This protective mechanism may be compromised in patients that carry the C-terminal truncated HBx proteins, or whose expression or activity of FXR is compromised.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


Abbreviations

AF-1  activation function 1

c-HBx  c-terminal truncated HBx

DBD  DNA-binding domain

FXR  farnesoid X receptor

FXRE  FXR response element

HBV  hepatitis B virus

HBx  hepatitis B virus X protein

HCC  hepatocellular carcinoma

LBD  ligand-binding domain

SHP  small heterodimer partner
Figure 1. Expression of HBx in mouse liver induces the expression of FXR target genes

(A) Hepatic expression of HBx in the ATX mice was shown by RT-PCR (left panel) and immunoprecipitation followed by Western blotting (right panel). Lanes represent individual mice. (B and C) The mRNA expression of Cyp7a1 and SHP (B), and FXR, CAR and Cyp2b10 (C) were measured by real-time PCR. (D) The expression of HBx in mouse livers hydrodynamically transfected with vector or HBx encoding plasmid was measured by RT-PCR. Lanes represent individual mice. (E and F) The mRNA expression of Cyp7a1 and SHP (E), and Cyp2b10 (F). *, P<0.05; **, P<0.01.
Figure 2. HBx augments the transcriptional activity of FXR in reporter gene assay

(A and B) HepG2 cells were transfected with tk-EcRE-Luc, the expression vectors of FXR and/or HBx, and the transfection efficiency control pCMX-β-gal. Transfected cells were treated with vehicle (DMSO), GW4064 (2 μM) (A), or CDCA (B) for 24 hrs before being harvested for luciferase and β-gal assays. The transfection efficiency was normalized against the β-gal activity. (C) HepG2 cells were transfected with plasmids encoding tk-EcRE-Luc, FXR, and increasing amounts of HBx. Transfected cells were treated with vehicle or GW4064 for 24 hrs before cell harvesting. (D) HepG2 cells were transfected with plasmids encoding tk-PBRE-Luc, and CAR and/or HBx. Transfected cells were treated with vehicle (DMSO) or TCPOBOP (2 μM) for 24 hrs before cell harvesting.
Figure 3. HBx is recruited to the FXR target gene promoter and enhances the binding of FXR to its response element

(A) HepG2 cells were transfected with a plasmid encoding FXR in the absence or presence of the co-transfection of the HBx plasmid. Transfected cells were treated with vehicle (DMSO) or GW4064 (2 μM) for 24 hrs before cell harvesting and real-time PCR to measure the expression of SHP. (B) ChIP assay to demonstrate the recruitment of Flag-HBx onto the BSEP and SHP gene promoters. Cells are the same as described in (A). ChIP assay was performed using an antibody against Flag or control IgG. The final DNA extracts were analyzed by real-time PCR using the primer pairs encompassing the BSEP/FXRE (top panel) and the SHP/FXRE (bottom panel). (C) HBx enhanced the binding of the FXR-RXR heterodimers to FXRE in a dose-dependent manner as shown by electrophoretic mobility shift assay (EMSA). (D) HBx had little effect on the binding of the CAR-RXR heterodimers to the CAR-responsive phenobarbital response element (PBRE) as shown by EMSA. (E) Increased binding of FXR to BSEP/FXRE by HBx as shown by ChIP assay.
Figure 4. HBx interacts with FXR and both proteins are co-localized in the nucleus
(A) The HBx-FXR interaction was demonstrated by GST pull-down assay using purified GST-HBx and $^{35}$S-labeled FXR. The interaction was efficiently competed by the addition of unlabeled FXR protein. (B) The HBx-FXR interaction was confirmed by co-immunoprecipitation assay. HepG2 cells were transfected with HA-FXR and Flag-HBx individually or in combination. Transfected cells were treated with vehicle or GW4064 for 24 hrs before cell harvesting. The cell extracts were immunoprecipitated with an anti-Flag antibody and blotted with an anti-HA antibody. (C) Co-localization of transfected Flag-HBx and HA-FXR in the nucleus as shown by immunofluorescence. (D) Co-localization of the endogenous FXR and HBx as shown by immunohistochemistry using serial paraffin sections. The arrows indicate positive staining.
Figure 5. Mapping of the HBx domain that interacts with FXR

(A) Schematic representation of the full-length HBx and deletion variants. (B) Coomassie blue staining showing the successful expression and purification of the GST fusion proteins. (C) The interaction between $^{35}$S-FXR and the second and third alpha helixes was demonstrated by GST pull-down assay (right half). The interaction between $^{35}$S-RXR and the first alpha helix was included as a positive control (left half). (D) HBx deletion mutants that lack the second or third alpha helix were not effective in co-activating FXR. HepG2 cells were transfected with tk-EcRE-Luc, FXR, and the full-length or deletion mutants of HBx. Transfected cells were treated with vehicle or GW4064 for 24 hrs before cell harvesting.
Figure 6. Mapping of FXR domain that interacts with HBx

(A) Schematic representation of the full-length FXR and deletion variants. (B) Binding of FXR AF1+DBD, but not LBD, to HBx as shown by GST pull-down assays. (C) Binding of FXR AF1, but not DBD, to HBx as shown by GST pull-down assays. (D) Binding of FXR AF1+DBD to HBx as shown by co-immunoprecipitation assay. (E) Binding of FXR AF1, but not DBD or LBD, to HBx as shown by co-immunoprecipitation assay.
Figure 7. The C-terminal truncated HBx proteins are not effective in transactivating FXR, and preferential loss of full-length HBx and increased expression of C-terminal truncated HBx in HCC

(A) Schematic representation of the full-length HBx and C-terminal truncated HBx C40. (B) The full-length HBx, but not C40, was efficient in co-activating FXR in reporter assays. HepG2 cells were transfected with plasmids encoding tk-EcRE-Luc, FXR, and the full-length HBx or C40. Transfected cells were treated with DMSO or GW4064 for 24 hrs before cell harvesting. (C) C40 failed to enhance the binding of FXR-RXR to FXRE as shown by EMSA. (D) C40 failed to enhance the recruitment of FXR to BSEP/FXRE as shown by ChIP. (E) Immunohistochemical detection of the full-length and C-terminal truncated HBx. (F) The expression of FXR in tumor tissues and the adjacent non-tumor tissues as shown by real-time PCR.
Figure 8. FXR ablation sensitizes the carcinogenic activity of HBx in vivo

(A) Gross appearance of the livers from 15-month-old mice. (B) Prevalence of spontaneous HCC (>0.5mm) in WT (n=7), ATX (n=7), FXR−/− (n=14) and ATX-FXR−/− (n=16) mice at the age of 15 months. (C) The liver histology was assessed by H&E staining and Ki-67 immunostaining. Arrowheads indicate positive Ki-67 staining. (D) Representative pre-neoplastic lesions in the liver of ATX-FXR−/− mice that include steatosis, hepatocyte necrosis and inflammatory infiltration. (E) Prevalence of spontaneous HCC and pre-neoplastic lesions in male and female ATX-FXR−/− mice. (F) The expression of pro-inflammatory genes was measured by real-time PCR. Results in the left and middle panels include both male and female mice, whereas results in the right panel compare the gender effect. *, P<0.05; **, P<0.01.