Modeling Hepatitis B Virus X–Induced Hepatocellular Carcinoma in Mice With the Sleeping Beauty Transposon System

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The mechanisms associated with hepatitis B virus (HBV)–induced hepatocellular carcinoma (HCC) remain elusive, and there are currently no well-established animal models for studying this disease. Using the Sleeping Beauty transposon as a delivery system, we introduced an oncogenic component of HBV, the hepatitis B virus X (HBx) gene, into the livers of fumarylacetoacetate hydrolase (Fah) mutant mice via hydrodynamic tail vein injections. Coexpression of Fah complementary DNA from the transposon vector allowed for the selective repopulation of genetically corrected hepatocytes in Fah mutant mice. The process of hydrodynamic delivery induced liver inflammation, and the subsequent selective repopulation of hepatocytes carrying the transgene(s) could provide useful genetic information about the mechanisms of HBV-induced hyperplasia. Short hairpin RNA directed against transformation-related protein 53 (shp53) or other tumor suppressor genes and oncogenes [e.g., constitutively active neuroblastoma RAS viral (v-ras) oncogene homolog with Gly12Val substitution (NRASG12V)] could also be codelivered with HBx by this system so that we could determine whether oncogenic cooperation existed. We found that the expression of HBx induced the activation of β-catenin expression in hydrodynamically injected livers, and this indicated its association with the Wnt signaling pathway in HBV-induced hyperplasia. HBx coinjected with shp53 accelerated the formation of liver hyperplasia in these mice. As expected, constitutively active NRASG12V alone was sufficient to induce liver hyperplasia, and its tumorigenicity was augmented when it was coinjected with shp53. Interestingly, HBx did not seem to cooperate with constitutively active NRASG12V in driving liver tumorigenesis.

Conclusion: This system can be used as a model for studying the various genetic contributions of HBV to liver hyperplasia and finally HCC in an in vivo system. (HEPATOLOGY 2010;000:000-000.)

The activation of proto-oncogenes and the loss of tumor suppressor genes generated by epigenetic and genetic mechanisms have been implicated in the tumorigenesis of hepatocellular carcinoma (HCC). Presently, there is no consensus on the number of different HCC molecular subtypes, although a recent metaanalysis based on gene expression and genetic changes has suggested three main subtypes. Hepatitis B virus (HBV) infection appears to play multiple roles in hepatocellular carcinogenesis. The study of HBV pathogenesis has been difficult because there currently is no good animal model that combines hepatocyte necrosis and repopulation along with facile viral gene delivery (GD).

The unique regulatory component gene X of HBV encodes a 17-kDa protein called hepatitis B virus X (HBx; 154 amino acid residues). The HBx gene has been shown to induce cell proliferation and proapoptotic and stress responses, activate certain signal transduction pathways, and promote angiogenesis.

Abbreviations: Ab, antibody; ACTB, β-actin; AFP, alpha-fetoprotein; AKT, v-akt murine thymoma viral oncogene homolog 1; ALT, alanine aminotransferase; CTNNB1, β-catenin; FAH, fumarylacetoacetate hydrolase; FVB, inbred mouse strain FVB/N; GD, gene delivery; GFP, green fluorescent protein; HBV, hepatitis B virus; HBx, hepatitis B virus X; HCC, hepatocellular carcinoma; HE, hematoxylin-eosin; IHC, immunohistochemistry; NRASG12V, neuroblastoma RAS viral (v-ras) oncogene homolog with Gly12Val substitution; pAKT, phosphorylated v-akt murine thymoma viral oncogene homolog 1; PHK, post-hydrodynamic injection; PI3K, phosphoinositide 3-kinase; RT-PCR, reverse-transcription polymerase chain reaction; SB, Sleeping Beauty; shp53, short hairpin RNA directed against transformation-related protein 53; STAT3, signal transducer and activator of transcription 3; TP53, tumor protein p53.

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pathways and DNA repair mechanisms, and induce transformation.\textsuperscript{5} HBx as a transgene in mice has produced variable effects.\textsuperscript{6,7} It remains unclear whether and how HBx can induce HCC in transgenic mice.

The oncogenic mechanisms of HBx are also controversial. HBx has been variably reported to activate signal transducer and activator of transcription 3 (STAT3) and WNT/\beta-catenin (CTNNB1) or bind to and inactivate tumor protein p53 (TP53).\textsuperscript{7-11} The critical activators of HBx in HCC induction have been difficult to identify because no efficient and rapid system for \textit{in vivo} GD and oncogenesis has been available. In order to elucidate the effect of HBx \textit{in vivo}, we used the Sleeping Beauty (SB) transposon system to deliver this transgene stably via hydrodynamic tail vein injections into the livers of fumarylacetoacetate hydrolase (Fah)–deficient mice.\textsuperscript{12} Because mutations in TP53 are common in patients with HBV-induced HCC, we cointroduced a transposon containing short hairpin RNA directed against transformation-related protein 53 (shp53) to study this relationship. Also, to elucidate whether any relationship exists between HBx infection and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) mutations, a transposon containing a constitutively active neuroblastoma RAS viral (v-ras) oncogene homolog with Gly12Val substitution (NRAS\textsuperscript{G12V}) was also cointroduced with HBx. Using this model, we were able to mimic HBx expression after HBV infection and then the subsequent repopulation of HBV-infected hepatocytes in the liver.

### Materials and Methods

**Generation of Fah-Deficient/Rosa26-SB11 Mice.** All animal work was conducted according to an institutionally approved animal welfare protocol. The generation, maintenance, and genotyping of doubly transgenic mice (Fah\textsuperscript{--/--}/Rosa26-SB11)\textsuperscript{13,14} are described in the Supporting Methods.

**Generation of Transgenes Used for Hydrodynamic Tail Vein Injections.** We generated pKT2/GD plasmids carrying HBx, NRAS\textsuperscript{G12V}, green fluorescent protein (Gfp), an empty vector, or a transposon vector containing shp53 (pKT2/GD-HBx, pKT2/GD-NRAS, pKT2/GD-Gfp, pKT2/GD-empty, and pT2/shp53, F1 respectively; Supporting Information Fig. 1A)\textsuperscript{15} with standard molecular cloning techniques. The steps are described in detail in the Supporting Methods.

**Hydrodynamic Tail Vein Injections.** Twenty micrograms of each construct was hydrodynamically injected into 4- to 6-week-old, doubly transgenic male mice as described previously.\textsuperscript{16} These mice were normally maintained on 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione drinking water, but this was replaced with normal drinking water immediately after the hydrodynamic injection of transposon vector(s).

**Liver Analysis.** Whole livers were removed and weighed, and the number of visible macroscopic hyperplastic nodules was counted. Reasonably sized nodules were carefully removed for DNA and RNA extraction. Histological sections were also taken from larger nodules for hematoxylin-eosin (HE) or immunohistochemistry (IHC) analyses as described in the Supporting Methods.

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Fig. 1. Proliferative and oncogenic potential of HBx in a selectively repopulating liver. (A) RT-PCR analyses of liver nodules and adjacent normal livers at 139 days PHI expressing HBx and Fah transgenes. (B) An RT-PCR semiquantitative analysis demonstrated significantly higher Afp expression in the aforementioned hyperplastic nodules versus the adjacent normal livers ($P = 0.0076$, unpaired $t$ test) with respect to Actb levels.
Blood Serum Analyses. Alanine aminotransferase (ALT) levels in blood serum samples were analyzed by Marshfield Laboratories (Marshfield, WI).

Reverse-Transcription Polymerase Chain Reaction (RT-PCR). The protocol is described in detail in the Supporting Methods.

Results

Effect of the HBx Transgene During Selective Liver Repopulation. Mice injected with HBx alone (Supporting Information Fig. 1A) were observed for up to 139 days post–hydrodynamic injection (PHI; n = 16). The detection of luciferase activity at 48 days PHI indicated selective repopulation of the liver as a result of stable transgene integration into the mouse genome mediated by SB transposition (Supporting Information Fig. 1B, top). The majority of HBx animal livers displayed no evidence of hyperplasia (88%). However, two HBx animals sacrificed at 74 and 139 days PHI displayed livers with hyperplastic nodules (Supporting Information Fig. 1C). Hyperplastic nodules isolated at 139 days PHI were positive for HBx transcripts by RT-PCR (Fig. 1A). These hyperplastic nodules expressed high levels of alpha-fetoprotein (Afp), a known diagnostic marker for HCC, in comparison with the adjacent normal liver (Fig. 1A). According to semiquantitative RT-PCR analyses, the arbitrary expression levels of Afp with respect to β-actin (Actb) were 0.31 ± 0.13 and 0.96 ± 0.042 (means and standard deviations) in normal livers and hyperplastic nodules, respectively (P = 0.0076; Fig. 1B). In order to visualize the selective hepatocyte repopulation process, control mice injected with Gfp alone (Supporting Information Fig. 1A) were observed for up to 113 days PHI (n = 4). The detection of luciferase activity at 48 days PHI also indicated selective repopulation of the liver (Supporting Information Fig. 1B, bottom). These Gfp mice were sacrificed at 82 and 113 days PHI (n = 4). Although no hyperplastic nodules were initially detected at 82 days PHI (n = 2), a single Gfp-negative nodule was detected at 113 days PHI (n = 2). Viewed with fluorescent imaging, the Gfp expression pattern confirmed that the liver
repopulation process occurred uniformly (Supporting Information Fig. 1D). Importantly, control mice coinjected with an empty vector and shp53 (empty/shp53; Supporting Information Fig. 1A) were negative for hyperplasia up to 139 days (n = 9). Interestingly, Ki67 staining did not show a significant increase in the mitotic index for Gfp animals (data not shown). However, there were higher levels of Ki67 staining in HBx animals (Fig. F2 2B). The liver weight percentage of HBx mice was significantly higher than that of Gfp mice (P < 0.01) and empty/shp53 controls (P < 0.001; Fig. 3B), and this indicates that HBx may have a proliferative effect on hepatocytes. Mice injected with HBx alone had high levels of Ctnnb1 expression by IHC, and this was mainly localized in the cellular membrane of repopulated hepatocytes (Fig. 4). Livers of HBx mice had hardly detectable levels of phosphorylated v-akt murine thymoma viral oncogene F5 homolog 1 (pAkt; Fig. 5) and displayed more CD45 staining cells by IHC in comparison with control Gfp animals (Supporting Information Fig. 4). Interestingly, ALT levels among HBx, empty/shp53, and Gfp representative animals were not significantly different (Table T1).

Synergistic Effects of HBx and shp53 Transgenes for Tumorigenesis During Selective Liver Repopulation. Mice injected with HBx and shp53 (HBx/shp53; Supporting Information Fig. 1A) were observed for up to 139 days PHI (n = 16). HBx/shp53 animals were sacrificed at various time points between 63 and 139 days PHI. Although no hyperplastic nodules were initially detected at 63 days PHI, the HBx/shp53 mouse liver had a rough surface texture (Supporting Information Fig. 2A, middle), and this indicated possible hyperproliferation and/or hyperplasia. The roughly textured liver was also Gfp-positive (detection of the Gfp reporter gene within shp53) and nodular in appearance when it was viewed under a fluorescent microscope (Supporting Information Fig. 2B, middle). Empty/shp53 mice were sacrificed at various time points.
 between 63 and 139 days PHI (n = 9). No hyperplasia was seen in the liver of the empty/shp53 mouse at 63 days PHI (Supporting Information Fig. 2A, left), and uniform Gfp expression was detected throughout the liver (Supporting Information Fig. 2B, left). In contrast, 86% of HBx/shp53 mice (n = 7) sacrificed at
approximately 70 days PHI had multiple hyperplastic nodules (Supporting Information Fig. 2A, right) that were Gfp-positive (Supporting Information Fig. 2B, right). Livers of empty/shp53 mice observed at various time points were normal, and the Gfp expression throughout the livers was relatively uniform (data not shown). The majority of hyperplastic nodules isolated from HBx/shp53 animals at 72 days PHI were Gfp-positive, and the presence of HBx and/or shp53 was confirmed by both RT-PCR (Supporting Information Fig. 2C) and IHC (Fig. 2A). Semiquantitative RT-PCR analysis demonstrated no statistical differences in Afp expression levels between hyperplastic nodules and adjacent normal livers isolated from 72-day PHI HBx/shp53 animals (Supporting Information Fig. 2D). However, significant differences in Afp expression levels

Fig. 5. The oncogenic potential of NRAS is associated with the P3k/Akt signaling pathway. IHC staining is shown for serial liver sections taken from experimental animals injected with the indicated transgene(s) with an antibody against pAkt (see Fig. 4 for descriptions of the HBx, HBx/shp53, NRAS, NRAS/shp53, and HBx/NRAS/shp53 mice). The left column shows sections incubated with anti-pAkt; the right column shows sections with no primary antibody. Magnified inserts in the upper left corners are indicated by dashes. Arrows and arrowheads indicate pAkt cytoplasmic and nuclear staining, respectively, detected in indicated cells; P indicates parenchymal; and T indicates hyperplastic nodule (scale bar = 100 μm).
Table 1. ALT Serum Levels in Representative Experimental Animals Injected With Various Transgenes

<table>
<thead>
<tr>
<th>Injected Transgene(s)*</th>
<th>Number of Tested Animals</th>
<th>PHI (Days)</th>
<th>ALT in Blood Serum (U/L)†</th>
<th>Nodules/Appearance‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gfp</td>
<td>4</td>
<td>82 and 113</td>
<td>61.3 ± 22.3</td>
<td>Normal to 1 nodule</td>
</tr>
<tr>
<td>Empty/shp53</td>
<td>5</td>
<td>70 to 131</td>
<td>87.0 ± 28.3</td>
<td>Normal</td>
</tr>
<tr>
<td>HBx</td>
<td>9</td>
<td>71 to 78</td>
<td>79.7 ± 29.5</td>
<td>Rough to 3 nodules</td>
</tr>
<tr>
<td>HBx/shp53</td>
<td>9</td>
<td>71 to 74</td>
<td>146.8 ± 83.2</td>
<td>Rough to 3 nodules</td>
</tr>
<tr>
<td>HBx/NRAS</td>
<td>5</td>
<td>71 to 72</td>
<td>43.2 ± 25.5</td>
<td>Slightly rough to 1 nodule</td>
</tr>
<tr>
<td>HBx/NRAS/shp53</td>
<td>6</td>
<td>61 to 72</td>
<td>157 ± 110.9</td>
<td>Normal to 35 nodules</td>
</tr>
<tr>
<td>NRAS</td>
<td>5</td>
<td>82</td>
<td>57.6 ± 42.2</td>
<td>Normal to 8 nodules</td>
</tr>
<tr>
<td>NRAS/shp53</td>
<td>6</td>
<td>61 and 71</td>
<td>89.7 ± 19.0</td>
<td>1 to 40 nodules</td>
</tr>
</tbody>
</table>


†Data presented as mean and standard deviations.

‡Normal indicates no abnormalities; slightly rough indicates that the liver texture was slightly rough in appearance; rough indicates that the liver texture was rough in appearance; and nodule indicates hyperplastic liver nodule detection.

were seen between (1) empty/shp53 and HBx/shp53 normal livers (P = 0.0035) and (2) normal empty/shp53 livers and HBx/shp53 nodules (P < 0.0001; Supporting Information Fig. 2D). HBx was detected in HBx/shp53 livers (Fig. 2A), and these animals generally had higher levels of Ki67 by IHC in comparison with animals injected with HBx alone (Fig. 2B). Although HBx alone was capable of inducing hyperplasia at low penetration (74 days PHI) or after prolonged latency (139 days PHI), its oncogenic potential was augmented, as shown by reduced latency to 71 days PHI and greater tumor multiplicity, with the coinjection of the shp53 transgene. HBx/shp53 mice had levels of Ctnnb1 by IHC comparable to those of mice injected with HBx alone (Fig. 4). Expression of Ctnnb1 was mainly localized to the cellular membrane of HBx repopulated hepatocytes (Fig. 4). In addition to membranous Ctnnb1 staining, cytoplasmic staining was also detected in some hepatocytes of HBx/shp53 animals (Fig. 4). Hyperplastic nodules taken from an HBx/shp53 animal were weakly positive for pAkt (Fig. 5) and displayed more CD45 staining cells by IHC in comparison with Gfp animals (Supporting Information Fig. 4). ALT levels in representative HBx/shp53 experimental animals were marginally significantly higher (P < 0.05) than those in Gfp or HBx animals, and they displayed a trend (not statistically significant) toward higher ALT levels in comparison with empty/shp53 animals (Table 1).

**HBx Does Not Cooperate With NRAS<sup>G12V</sup> in Accelerating Liver Tumorigenesis During Selective Liver Repopulation.** Hyperplasia was detected in 60% of mice injected with NRAS<sup>G12V</sup> (NRAS; Supporting Information Fig. 1A) at 82 days PHI (n = 5; Supporting Information Fig. 3A, left). Histological analyses of F6b these hyperplastic nodules by HE staining (Fig. 6A, left) and IHC confirmed the detection of NRAS in these nodules (Fig. 6B). This tumorigenic potential was augmented when mice were coinjected with shp53 (NRAS/shp53), as shown by the accelerated detection of hyperplasia by 61 days PHI (n = 2). By 71 days PHI, hyperplasia was detected in all remaining NRAS/shp53 mice (n = 4; Supporting Information Fig. 3A, middle). NRAS/shp53 livers were Gfp-positive macroscopically (Supporting Information Fig. 3C, left) and were shown to express Gfp and NRAS by RT-PCR (Supporting Information Fig. 3D). Histological analyses of these hyperplastic nodules by HE staining (Fig. 6A, right) and IHC confirmed that NRAS and shp53 contributed to the tumorigenesis (Fig. 6C). NRAS/shp53 animals displayed a trend (not statistically significant) toward higher ALT values in comparison with NRAS or Gfp animals (Table 1). NRAS/shp53 livers displayed more CD45-positive staining cells than Gfp or NRAS (Supporting Information Fig. 4). In contrast, in mice coinjected with HBx and NRAS transgenes (HBx/NRAS; n = 5), only one hyperplastic nodule was isolated from a single experimental mouse at 70 days PHI (Supporting Information Fig. 3A, right). Besides this, the livers isolated from remaining HBx/NRAS mice were macroscopically normal in appearance (data not shown). Interestingly, HBx/NRAS livers displayed more CD45-positive staining cells than Gfp or NRAS livers (Supporting Information Fig. 4). ALT levels in HBx/shp53 animals were significantly higher than those in HBx/NRAS animals (P < 0.01), and marginally significantly higher levels (P < 0.05) were seen in HBx and NRAS/shp53 animals versus HBx/NRAS animals (Table 1). When all three transgenes were coinjected (HBx/NRAS/shp53), 67% of the mice (n = 6) sacrificed at 61 and 71 days PHI displayed multiple hyperplastic nodules (Supporting Information Fig. 3B). The majority of nodules were Gfp-positive (Supporting Information Fig. 3C, right) and were
shown to express Gfp by RT-PCR (Supporting Information Fig. 3D). Expression of the injected transgenes was detected in the majority of normal livers and hyperplastic nodules isolated from both groups (HBx/shp53 and NRAS/shp53; Supporting Information Fig. 3D). ALT levels for HBx/shp53 and HBx/NRAS/shp53 animals were similar (Table 1). Semi-quantitative RT-PCR analyses also demonstrated a significant difference in the expression levels of Afp in hyperplastic nodules versus adjacent normal livers (P...
have higher liver to whole mass percentages, and this indicates that HBx may have a hyperproliferative effect during HBV-induced liver tumorigenesis (Fig. 3B). Tumor latency was reduced and the oncogenic effect was augmented when HBx was coinjected with shp53. This is especially important because approximately 50% of human patients with HCC have mutations in the TP53 gene. HBx has been shown to bind TP53 and inactivate its activity, but our data indicate that this mechanism must not impair TP53 function sufficiently for tumor formation. Therefore, TP53 mutant hepatocytes in a patient who acquires an HBV infection would likely lead to an enhanced risk of transformation to HCC.

Our results indicate that HBx does not seem to cooperate with constitutively active NRAS to induce liver tumorigenesis in HBx/NRAS animals. This was evident from the relatively low ALT levels in serum (Table 1), the low tumor multiplicity, and the liver weight to whole mass percentage (Fig. 3A,B) in HBx/NRAS mice. HBx has been suggested to up-regulate the Ras signaling pathway. Perhaps HBx expression and activated Ras are redundant in this transformation assay. Even when all the transgenes were coinjected (HBx/NRAS/shp53), there was only a marginally significant increase (P < 0.05) in tumorigenicity in comparison with HBx alone. Moreover, no significant increase in tumorigenicity was seen in HBx/NRAS animals versus animals with NRAS alone (Fig. 3A). Our results indicate that HBx up-regulates the Wnt signaling pathway, and this may play a role in liver tumorigenesis (Fig. 4), whereas constitutively active NRAS seems to induce hyperplasia, probably via the RAF/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase and/or phosphoinositide 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT) associated pathways (Fig. 5). In addition, we detected high levels of CD45-positive staining cells in livers of animals injected with HBx alone or in combination with other transgenes (Supporting Information Fig. 4). These cells could represent infiltrating lymphocytes, which are often associated with HBV infection. Indeed, the HBx protein would be predicted to act as a foreign antigen in our system, unlike previously reported HBx transgenic mouse models. HBx-induced inflammation may play some role in HCC progression; this hypothesis could be tested with our model. Elevated pAkt levels were detected by IHC in experimental animals injected with NRAS alone or in combination with shp53, and this indicates that NRAS is likely signaling via the PI3K/Akt pathway (Fig. 5). HBx has been previously shown to activate the WNT1/
CTNNB1 signaling pathway in human hepatoma cell lines. HBx antigen has also been associated with the accumulation of CTNNB1 in the cytoplasm and/or nucleus and the up-regulation of the HBx antigen effector up-regulated gene 11, which results in increased activation of CTNNB1. The CTNNB1 staining pattern can be correlated with the histopathological types of liver tumors. The absence of nuclear staining and strong membranous staining with rare, weak cytoplasmic expression of Ctnnb1 suggested that the hyperplastic nodules induced by HBx or HBx/shp53 were adenocarcinomas or poorly differentiated HCC (Fig. 4). We did not detect any activation of Stat3 in liver tumors expressing HBx by IHC in our experimental cohorts with a phospho-Stat3 (Tyr705)–specific antibody (data not shown) despite the previous suggestion that HBx activates Stat3. Finally, it should be stated that there will be a subpopulation of Fah-null cells that can escape the selection process by activating the survival Akt pathway caused by hepatic stress. These cells can evolve by acquiring additional mutations and result in hyperplastic nodules not associated with the injected transgenes. Examples of such background Fah-negative nodules were seen in HBx/shp53 and HBx/NRAS/shp53 mice (Supporting Information Figs. 2C and 3D, respectively). These nodules were negative for the injected transgenes by RT-PCR. Such background tumors occur only at a low rate and can be segregated from transgene-induced tumors by molecular and biochemical tests.

Nevertheless, our experience shows that the Fah-deficient mouse model, in combination with the SB transposon system, is useful for in vivo functional validation of HBV genes in liver hyperplastic induction. Therefore, our present study reinforces the previous observations associated with HBV infection and validates the use of our mouse model in studying HBV-induced liver hyperplasia and its progression to HCC.

References