Hepatitis B virus X protein induces epithelial–mesenchymal transition by repressing E-cadherin expression via upregulation of E12/E47

Soo Shin Kim,† Sujeong Yeom,† Juri Kwak, Hyung-Jun Ahn and Kyung Lib Jang

Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea

Previous reports have demonstrated that hepatitis B virus (HBV) X protein (HBx) represses E-cadherin expression to induce epithelial–mesenchymal transition (EMT), an essential component of cancer progression to more aggressive phenotypes characterized by tumour invasion, migration and metastasis; however, the underlying mechanism for this phenomenon is still unclear. In this study, we found that ectopic expression of HBx in human hepatocytes using overexpression and 1.2-mer WT HBV replicon systems upregulated levels of the transcriptional repressors E12 and E47, resulting in inactivation of the E-cadherin promoter, containing three E-box motifs, and subsequent repression of its expression. E12/E47 knockdown using a specific small interfering RNA almost completely abolished the potential of HBx to repress E-cadherin expression. HBx inhibited the ubiquitin-dependent proteasomal degradation of E12/E47 without affecting their expression at the transcriptional level. Upregulation of E12/E47 by HBx ultimately led to EMT in human hepatocytes, as demonstrated by morphological changes, altered protein levels of EMT markers, including E-cadherin, plakoglobin, fibronectin, vimentin and N-cadherin, and increased capacity for cell detachment and migration.

INTRODUCTION

Hepatitis B virus (HBV) is strongly associated with the development of hepatocellular carcinoma (HCC) (Neuveut et al., 2010; Xu et al., 2014). One of the ORFs encoded by the HBV genome is an oncogenic X protein (HBx), which is the most frequently integrated viral sequence found in HCC (Paterlini et al., 1995). HBx is likely to be implicated at several different steps of HCC development (Neuveut et al., 2010; Xu et al., 2014). Although most efforts in the study of the role of HBx have focused on its involvement in the genesis of HCC, it is also involved in tumour progression, invasion and metastasis. For example, HBx induces extensive morphological changes and cytoskeleton rearrangements in liver cells (Lara-Pezzi et al., 2001c). In addition, it induces adherence junction disruption and modulates integrin-mediated adhesion to the extracellular matrix (Lara-Pezzi et al., 2001a, b). Furthermore, HBx can promote invasion and metastasis of HCC by changing the proteolytic enzymes secreted by liver cancer cells (Chan et al., 2004). Despite our increasing knowledge about HBx in cellular transformation and immortalization, its role during tumour progression and the underlying mechanism are relatively unknown.

The cell–cell adhesion molecule E-cadherin plays essential roles in normal physiological processes such as development, cell polarity and tissue morphology (van Roy & Berx, 2008). Several studies have provided consistent evidence of its role as a tumour suppressor (Hirohashi & Kanai, 2003). Loss or alteration of E-cadherin expression has been observed in a variety of different tumour types, including HCC (Hirohashi & Kanai, 2003; Kanai et al., 1997). In addition, transfection of E-cadherin cDNA into invasive carcinoma cells leads to a significant reduction in their invasive capacity in vitro (Kanai et al., 1997), and activation of E-cadherin results in growth retardation of tumour cell lines (Watabe et al., 1994). Aberrant cellular distribution of E-cadherin or repression of its expression is also observed during epithelial–mesenchymal transition (EMT), an essential component of cancer progression to more aggressive phenotypes characterized by tumour dedifferentiation, infiltration and metastasis (Kalluri & Weinberg, 2009; Le Bras et al., 2012; Wells et al., 2008).

Chronic HBV infection is closely associated with downregulation of E-cadherin (Wei et al., 2002). In addition, several reports have provided direct evidence for a role of HBx in the downregulation of E-cadherin (Arzumanyan et al., 2012; Lee et al., 2005; Liu et al., 2006). Two different mechanisms can be considered for the repression of E-cadherin expression by HBx in HCC.

†These authors contributed equally to this work.
First, the E-cadherin gene is frequently inactivated via promoter hypermethylation in HBV-associated HCC (Kanai et al., 1997; Lee et al., 2003; Wei et al., 2002; Yang et al., 2003). Indeed, HBx represses E-cadherin expression via promoter hypermethylation both in vitro and in vivo (Arzumanyan et al., 2012; Lee et al., 2005; Tian et al., 2013). This mechanism may explain altered E-cadherin expression in pre-neoplastic and early tumours (Lee et al., 2003; Yang et al., 2003). Secondly, E-cadherin expression is usually regulated at the transcriptional level by EMT regulators such as Snail, Slug, Zeb1, Zeb2, Twist and E47 via E-box motifs (Tania et al., 2014; Teng & Li, 2014). It is thus possible to speculate that HBx represses E-cadherin expression by upregulating the levels and/or activities of these transcriptional repressors. The relative importance of the two mechanisms and their possible cross-talk in the regulation of E-cadherin expression by HBx are unknown. Therefore, we first investigated in the present study whether the two mechanisms are actually involved in the repression of E-cadherin expression by HBx. Next, we attempted to identify a transcriptional repressor(s) responsible for the repression of E-cadherin expression by HBx. We also investigated the mechanism by which HBx upregulates the levels of these repressors. Lastly, we attempted to provide evidence that HBx induces EMT by downregulating E-cadherin expression.

RESULTS

HBx represses E-cadherin expression via promoter hypermethylation and upregulation of transcription repressors acting on E-box motifs

According to previous reports, HBx represses E-cadherin expression via DNA methylation (Arzumanyan et al., 2012; Lee et al., 2005; Tian et al., 2013). Consistently, we found that HBx induced DNA methylation of E-cadherin (Fig. 1a) and downregulated its promoter activity (Fig. 1c) and protein level (Fig. 1d) in a human hepatoma cell line, HepG2. In addition, the potential of HBx to induce these effects was severely impaired by treatment with a universal DNA methyltransferase inhibitor, 5-aza-
2′-deoxycytidine (5-Aza-2′dC), confirming that DNA methylation is a mechanism by which HBx represses E-cadherin expression. However, the potential of HBx to inhibit the promoter activity of E-cadherin (Fig. 1c) and downregulate its protein level (Fig. 1d) were still active under the condition where 5-Aza-2′dC almost completely abolished the HBx-mediated DNA methylation of E-cadherin (Fig. 1a, b), suggesting that another mechanism(s) in addition to DNA methylation is involved in the repression of E-cadherin expression by HBx. Further evidence supporting this hypothesis was obtained with E-cadmE123-luc in which three E-box motifs on the E-cadherin promoter were inactivated through point mutations. The basal reporter activity of E-cadmE123-luc was much higher compared with that of E-cad-luc, probably due to removal of the E-box motifs (Fig. 1c), which has been demonstrated previously (Batlle et al., 2014; Teng & Li, 2014). HBx could significantly downregulate the reporter activity from E-cadmE123-luc while this effect almost completely disappeared in the presence of 5-Aza-2′dC (Fig. 1c), suggesting that HBx downregulates E-cadherin promoter activity via a transcriptional repressor(s) that acts on E-box motifs. Taken together, we concluded that HBx represses E-cadherin expression by inactivating its promoter via DNA methylation and transcription repressors acting on E-box motifs.

**HBx represses E-cadherin expression by upregulating E12/E47 levels**

As the DNA methylation-mediated repression of E-cadherin expression by HBx is relatively well established (Kanai et al., 1997; Lee et al., 2003; Wei et al., 2002; Yang et al., 2003), we next investigated the mechanism by which HBx downregulates its promoter activity via E-box motifs. For this purpose, we first examined the effects of HBx on transcriptional factors that are known to repress E-cadherin expression by acting on E-box motifs (Tania et al., 2014; Teng & Li, 2014). As a result, we found that HBx upregulated levels of Snail, Slug, E12/E47 and Zeb1, while it had little effect on the levels of Zeb2 and Twist.
We focused on E12/E47 because their levels were dramatically upregulated in the HBx-expressing cells. Transient expression of HBx dose-dependently upregulated the levels of E12/E47 in both HepG2 and Huh-7 cells, resulting in downregulation of E-cadherin (Fig. 2b). Levels of E12/E47 and E-cadherin in these cells were similarly affected by transient transfection with 1.2-mer WT HBV replicon (1.2-mer WT) but not its HBx-null counterpart (1.2-mer HBx-null) (Fig. 2c). In addition, HBx upregulated the levels of E12/E47 but downregulated that of E-cadherin in HBV-positive human liver samples (Park et al., 2005) (Fig. 2d). These results indicated that E12/E47 upregulation and subsequent E-cadherin downregulation is a general effect of HBx in human hepatocytes.

Next, we attempted to prove that upregulation of E12/E47 is responsible for the repression of E-cadherin expression by HBx. Ectopic expression of E47 in the absence of HBx dose-dependently decreased the luciferase activity from E-cad-luc but not that of E-cadmE123-luc (Fig. 3a) and thereby downregulated the levels of E-cadherin in HepG2 cells (Fig. 3b), indicating that E47 is an effective transcription repressor for E-cadherin expression in HepG2 cells. In addition, E12/E47 knockdown using a specific small interfering RNA abolished the potential of HBx derived

![Fig. 2a](image)

![Fig. 2b](image)

![Fig. 2c](image)

![Fig. 2d](image)

![Fig. 2e](image)

![Fig. 2f](image)
HBx upregulates E12/E47 levels by inhibiting their ubiquitin-dependent proteasomal degradation

We next investigated the mechanism by which HBx upregulates E12/E47 levels. According to data from the luciferase assay shown in Fig. 4(a), the E2A promoter activity was weakly affected by ectopic expression of HBx. Accordingly, E12/E47 mRNA levels were unaffected by HBx expression (Fig. 4b). These results suggested that HBx does not activate transcription of E12/E47 to upregulate their protein levels. Therefore, we explored whether HBx affects the protein stability of E12/E47. Treatment with a proteasomal inhibitor, MG132, almost completely abolished the effect of HBx on E12/E47 levels in both HBx overexpression (Fig. 4c) and HBV replicon systems (Fig. 4d), indicating that HBx upregulates E12/E47 levels by increasing their stability without affecting their transcription.

To confirm that HBx actually inhibits ubiquitin (Ub)-dependent proteasomal degradation of E12/E47, we from either HBx expression vector or 1.2-mer WT to downregulate both the promoter activity and protein level of E-cadherin (Fig. 3d, e). Moreover, according to the data from a chromatin immunoprecipitation (ChIP) assay, the E-cadherin promoter recruited more E12/E47 in the HBx-expressing cells (Fig. 3f), presumably due to their upregulation in these cells. Taken together, these results indicated that HBx represses E-cadherin expression by upregulating E12/E47 levels in human hepatocytes.

**Fig. 4.** HBx inhibits ubiquitin-dependent proteasomal degradation of E12/E47. (a) E2A-luc was co-transfected with an empty vector, pHA-HBX3 (HBx), 1.2-mer WT or 1.2-mer HBx-null into HepG2 cells, followed by a luciferase assay (n=4). (b) RNA levels of E12/E47, HBx and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in HepG2 cells transiently transfected with an empty vector, pHA-HBX3 (HBx), 1.2-mer WT or 1.2-mer HBx-null were determined by reverse transcription PCR. (c) HepG2 cells with or without HBx expression were either mock treated or treated with MG132 for 4 h before harvesting, followed by Western blotting. (d) HepG2 cells with or without an HBV replicon were either mock treated or treated with MG132 for 4 h, followed by Western blotting. (e) HepG2 cells with an empty vector, pHA-HBX3 (HBx), 1.2-mer WT or 1.2-mer HBx-null were transiently co-transfected with Ub- and E47-expression plasmids for 48 h. Total E12/E47 proteins including Ub-conjugated ones were immunoprecipitated with an anti-E12/E47 antibody and subjected to Western blotting to measure the levels of the Ub-complexed E12/E47 using an anti-Ub antibody (upper panel). Levels of E12/E47 and γ-tubulin in the total lysates were measured by Western blotting (lower panels).
introduced a haemagglutinin (HA)-tagged Ub along with E47 into HepG2 cells in the presence or absence of HBx and immunoprecipitated the Ub-complexed products. As a result, ubiquitination of E12/E47 was severely impaired in HepG2 cells stably transfected with either HBx expression vector or 1.2-mer WT HBV replicon but not with an HBx-null replicon, resulting in upregulation of E12/E47 in HBx-expressing cells (Fig. 4e). Taken together, our results indicated that HBx upregulates E12/E47 levels by inhibiting their degradation through the Ub–proteasome system.

HBx induces EMT by downregulating E-cadherin levels

E-cadherin is a cell–cell adhesion molecule, and loss of its expression is a hallmark of EMT (Hirohashi & Kanai, 2003; Le Bras et al., 2012; Wells et al., 2008). Therefore, we investigated whether HBx induces EMT in HepG2 cells as a consequence of E-cadherin downregulation. Both the parental HepG2 and HepG2 cells with the HBx-null HBV replicon were tightly connected to each other (Fig. 5a). In contrast, the HBx-overexpressing HepG2 cells and cells transfected with 1.2-mer WT did not form such tight intercellular connections, and instead were loosely connected to each other (Fig. 5a). In addition, mesenchymal markers such as fibronectin, vimentin and N-cadherin were upregulated, while epithelial markers including E-cadherin and plakoglobin were downregulated in the HBx-expressing cells and cells with the integrated 1.2-mer WT (Fig. 5b). In addition, compared with the parental cells and cells with 1.2-mer HBx-null, the HBx-expressing cells and cells with 1.2-mer WT exhibited decreased cell–cell interactions, as demonstrated by their
lower cell aggregation ability (Fig. 5c). Moreover, according to the results of the wound-healing assay, the HBx-expressing cells and cells with 1.2-mer WT showed a relatively fast migratory behaviour, colonizing 75.0 and 76.9% of the wounded surface, respectively, in 24 h, whereas in the parental cells and cells with 1.2-mer HBx-null at this time point, 69.3 and 61.5% of the wounded surface remained uncovered, respectively (Fig. 5d). Taken together, these results indicated that HBx induces EMT in HepG2 cells via downregulation of E-cadherin.

**DISCUSSION**

EMT is a key event in the tumour invasion process whereby epithelial cells lose polarity and cell–cell contacts, undergo dramatic remodelling of the cytoskeleton and acquire migration capacity (Kalluri & Weinberg, 2009; Le Bras et al., 2012). One of the hallmarks of EMT is the functional loss of E-cadherin, which is currently thought to be a suppressor of invasion during carcinoma progression (Birchmeier & Behrens, 1994; Le Bras et al., 2012; Wells et al., 2008). Immunohistochemical studies of E-cadherin expression in HBV-related HCC have demonstrated significant downregulation of E-cadherin in the tumour tissue compared with adjacent non-tumour tissues (Yoshiura et al., 1995). Also, both in vitro and in vivo studies have suggested that HBx is implicated in the downregulation of E-cadherin (Arzumanyan et al., 2012; Lee et al., 2005; Liu et al., 2006). Consistently, the present study showed that HBx could induce EMT by downregulating levels of E-cadherin in hepatoma cells. Therefore, knowledge of the molecular mechanism that HBx represses E-cadherin expression or interferes with its function is of prime importance in understanding the late stages of HCC progression.

According to the previous reports, HBx represses E-cadherin expression via promoter hypermethylation (Arzumanyan et al., 2012; Lee et al., 2005; Tian et al., 2013). Consistently, the present study showed that HBx induces promoter hypermethylation and subsequently downregulated protein levels of E-cadherin. Previous studies also have demonstrated that HBx represses E-cadherin expression via upregulation of Snail (Liu et al., 2012). The present study showed that several other repressors including Slug, E12/E47 and Zeb1, in addition to Snail, were upregulated in the HBx-expressing cells. In addition, we provided several lines of evidence that E12/E47 plays a key role in the repression of E-cadherin expression by HBx. First, E12/E47 expression was dramatically upregulated in the HBx-expressing cells. Secondly, ectopic expression of E47 in HepG2 cells decreased the E-cadherin promoter activity in an E-box-dependent manner, resulting in downregulation of its protein levels. Thirdly, E12/E47 silencing almost completely abolished the potential of HBx to downregulate both the promoter activity and protein levels of E-cadherin. Therefore, it is possible to speculate that HBx represses E-cadherin expression via at least two different mechanisms.

Epigenetic changes are usually associated with altered E-cadherin expression in pre-neoplastic and early tumours (Lee et al., 2003; Yang et al., 2003). Upregulation of E12/E47 thus might be associated with the non-epigenetic regulation of E-cadherin expression at the later stages of HCC development. Otherwise, HBx may simply adopt the two mechanisms together to effectively regulate E-cadherin expression at two different levels, i.e. transcriptional regulation and chromosome remodelling. The latter explanation appears to be more plausible if considering the presence of cross-talk between site-specific transcription factors and DNA methylation states (Blattler & Farnham, 2013). Interestingly, the CpG sites whose methylation was induced by HBx were clustered at or near the Sp1-binding sites on the E-cadherin promoter, while the E-box motifs were not subjected to the HBx-induced DNA methylation (Fig. 1b). Therefore, it is possible to speculate that HBx effectively represses E-cadherin expression not only by interrupting the action of positive regulators such as Sp1 via DNA methylation of their binding sites but also by upregulating the levels of negative regulators whose activities are little affected by DNA methylation. However, their relative contributions to E-cadherin downregulation could be different depending on the stages of HCC development.

The E2A gene encodes the two class I basic helix–loop–helix proteins E12 and E47, which are RNA splice variants (Quong et al., 2002). The two proteins were first identified as key regulators of B-cell functions (Henthorn et al., 1990). Later, it was demonstrated that both E12 and E47 act as repressors of E-cadherin expression and trigger EMT of mouse epithelial cells (Perez-Moreno et al., 2001). In the present study, we showed that E12/E47 mediated the potential of HBx to repress E-cadherin expression in human hepatocytes. E12 and E47 share identical N-terminal trans-activation domains but have distinct dimerization and DNA-binding motifs, having similar sizes (67.61 and 67.27 kDa, respectively) (Quong et al., 2002). As one of our antibodies and a small interfering RNA acted on both E12 and E47, it is unclear whether HBx upregulates levels of E12 and/or E47. Overexpression of E47 could repress E-cadherin expression in HepG2 cells, indicating that upregulation of E47 alone by HBx could repress E-cadherin expression. Interestingly, HBx appeared to elevate levels of E12/E47 by inhibiting their Ub-dependent proteasomal degradation without affecting their transcription. More extensive studies are required to elucidate the detailed mechanism for the regulation of E12/E47 by HBx. According to the present study, HBx induces EMT in human hepatocytes. In general, molecular markers for EMT include decreased expression of epithelial markers such as E-cadherin and plakoglobin and increased expression of mesenchymal markers including fibronectin, N-cadherin and vimentin. In particular, vimentin is the key
characteristic of the mesenchymal cell phenotype (Leonard et al., 2008), and an increase in the vimentin/E-cadherin ratio has been considered to be the first indicator of EMT (Kang & Massagué, 2004). The present study clearly showed alterations of these EMT markers by HBx using both HBx overexpression and HBV replicon systems. In addition, HBx induced phenotypic markers of EMT including an increased capacity for cell detachment and migration. In conclusion, HBx represses E-cadherin expression by upregulating levels of E12/E47 to facilitate cell detachment from the surrounding matrix and migration outside of the primary tumour site.

METHODS

Plasmids. The 1.2-mer WT replicon containing 1.2 units of the HBV genome (subtype ayw) and its HBx-null counterpart (1.2-mer HBx-null) have been described previously (Cha et al., 2009). The HBx expression plasmid pHA-HBX3 encoding HBx downstream of three copies of the influenza virus HA epitope has been described previously (Kwon & Jang, 2004). The E-cad-luc, which contains the promoter region of E-cadherin (Kwun & Jang, 2004). The E-cad-luc was constructed from E-cad-luc by mutating the 5'-CACCTG-3' sequence of three E-box motifs to 5'-AACCTA-3' via PCR-directed mutagenesis as described elsewhere (Batlle et al., 2000). The E2A promoter from -420 to +32 has been obtained from genomic DNA of HepG2 cells by PCR amplification using the primer set E2A-430 forward (5'-GCTCGAGAGGCCGAGCTA-3') and E2A-32 reverse (5'-TTCCCTGGGCACAAAGC-3') and then subcloned into a luciferase reporter vector, pGL3-basic (Promega), to create E2A-luc. The E2A small hairpin RNA (shRNA) plasmid and pcDNA hE47 encoding E47 were purchased from Santa Cruz Biotechnology and Addgene, respectively.

Cell culture and luciferase assay. HepG2 was obtained from the Korean Cell Line Bank (no. 580655). For transient expression, 2 × 10^4 cells per 60 mm dish were transfected with 1 μg appropriate plasmid(s) using WelFect-E PLUS (WelGENE) following the manufacturer's instructions. The HBV replicon cell lines were established by transfection with the 1.2-mer WT or 1.2-mer HBx-null replicon, followed by selection with 500 μg G418 (Gibco) ml⁻¹ as described previously (Sells et al., 1987). HBx-expressing stable cell lines were established by transfection of pHA-HBX3 as described elsewhere (Jung et al., 2007). For the luciferase assay, the reporter plasmids, Ecad-luc (Lee et al., 2005), E-cad-mE123-luc and E47 were co-transfected with an empty vector or an effector plasmid under the indicated conditions. pcH110 (0.1 μg; Pharmacia) containing the Escherichia coli lacZ gene under the control of the simian virus 40 promoter was included as an internal control. At 48 h after transfection, a luciferase assay was performed using a Luciferase Assay System (Promega) and the value obtained was normalized to the β-galactosidase activity measured in the corresponding cell extracts.

Western blot analysis. Cells were lysed in buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1 % SDS, 1 % NP-40] supplemented with protease inhibitors. Cell extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF; Amersham). Membranes were then incubated with antibodies to E2A (E12 and E47), E-cadherin, fibronectin, N-cadherin, NTCP, Snail, Slug, Twist, vimentin, Zeb1 and Zeb2 (Santa Cruz Biotechnology), plakoglobin (BD Biosciences), HBx (Millipore) and γ-tubulin (Sigma) and subsequently with an HRP-conjugated secondary antibody: anti-mouse, anti-goat, anti-human or anti-rabbit IgG(H + L)-HRP (Bio-Rad). An ECL kit (Amersham) was used to visualize protein bands using a ChemiDoc XRS imaging system (Bio-Rad).

PCR. For semi-quantitative RT-PCR, total RNA (3 μg) extracted using an RNasy Mini kit (QiaGen) was reverse transcribed with the corresponding reverse primer. One-quarter of the reverse-transcribed RNA was amplified with Taq DNA polymerase to detect E-cadherin, HBx and glyceraldehyde-3-phosphate dehydrogenase RNA levels as described elsewhere (Lee et al., 2005). PCR amplification for E47 was performed using forward primer 5'-ATGAAACCGCCGAGGGATG-3' and reverse primer 5'-CCGAGTCCCGTCCAGGAAT-3'. PCR conditions were set up for 30 cycles as follows: denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The products were resolved on a 1 % agarose gel containing ethidium bromide.

Methylation-specific PCR and bisulfite DNA sequencing. Genomic DNA (1 μg) denatured in 50 μl 0.2 M NaOH was modified by treatment with 30 μl 10 mM hydroquinone (Sigma) and 520 μl 3 M sodium bisulfite (pH 5.0; Sigma) at 50 °C for 16 h. For methylation-specific PCR, the modified DNA (100 ng) was amplified with Taq DNA polymerase using both methylated and unmethylated primer pairs of E-cadherin as described previously (Herman et al., 1996). For bisulfite DNA sequencing, modified genomic DNA was amplified by PCR using a primer pair to detect the GpC-rich region of the E-cadherin promoter (–179 to +30) as described elsewhere (Woo & Jang, 2012). The PCR products were subcloned into the pGEM-T Easy vector (Promega) and their nucleotide sequences were determined.

ChIP assay. A ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer’s instruction. The sheared chromatin was immunoprecipitated with an antibody against E2A (Santa Cruz Biotechnology) and a negative-control rabbit IgG (Santa Cruz Biotechnology). DNA released from the precipitated complexes was amplified to detect the E-cadherin promoter by PCR using the primer pair 5'-ACTCCAGGGCTAGGGTCACC-3' (forward) and 5'-CCGCGATGCTCAGGTGCATTGCAGTC-3' (reverse) as described previously (Lin et al., 2010).

Immunofluorescence analysis. Cells grown to confluence on coverslips were fixed in 4 % formaldehyde (15 min, at room temperature), permeabilized in 0.2 % Triton X-100 (5 min, at room temperature) and incubated with an appropriate primary antibody (overnight, 4 °C) and then an anti-mouse IgG–FITC (Santa Cruz Biotechnology; 1 h, at room temperature). Slides were prepared with UltraCruz mounting medium (Santa Cruz Biotechnology) and visualized with an AxioScope fluorescence microscope (Carl Zeiss).

Cell aggregation assay. A fast aggregation assay was performed as described previously (Kawanishi et al., 1995). Cells were dissociated with Hanks’ balanced salt solution (HBBS) supplemented with 0.01 % trypsin and 1 mM CaCl₂ and washed twice in Ca²⁺⁻free HBBS. The resulting cells (1 × 10^6) resuspended in 2 ml HBSS containing 1 mM CaCl₂ were incubated for 30 min at 37 °C on a gyratory shaker. After incubation, the total particle number (single cells plus cell clusters) in each cell suspension was counted. The cell aggregation degree was represented by the aggregation index (Nt/Ni), where N₀ is the total particle number before incubation and Nt is the total particle number after incubation for t min.

Wound-healing assay. To determine the cell migration rate, a scratch wound-healing assay was performed as described elsewhere (Rodriguez et al., 2005). Briefly, 1 × 10^5 cells were plated and cultured to create a confluent monolayer in a 60 mm dish. After gently scratching with a pipette tip to produce a wound, the cells were...
further incubated for 0 and 24 h. The wound-healing rate was measured by determining the closure rate of the wound.

**Statistical analysis.** Results are shown as means ± SD from at least three independent experiments. A two-tailed Student’s t-test was used for all statistical analyses. *P* < 0.05 was considered statistically significant.

**ACKNOWLEDGEMENTS**

We thank W.-S. Ryu for providing the HBV replicon system and pHBV-luc plasmid used in this study. We thank B. J. Park for providing human HCC samples. This research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry for Health & Welfare, Republic of Korea (grant number: H112C1315).

**REFERENCES**


