Hepatitis B virus X protein suppresses all-trans retinoic acid-induced apoptosis in human hepatocytes by repressing p14 expression via DNA methylation

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Abstract
All-trans retinoic acid (ATRA), the most biologically active metabolite of vitamin A, is known to activate p14 expression via promoter hypermethylation to induce p53-dependent apoptosis in human hepatocytes. In this study, we found that the oncogenic hepatitis B virus (HBV) X protein (HBx) of HBV, derived from both overexpression and 1.2-mer replicon systems, suppresses ATRA-induced apoptosis in p53-positive human hepatocytes. For this effect, HBx upregulated both protein and enzyme activity levels of DNA methyltransferase 1, 3a and 3b, in the presence of ATRA and thereby inhibited p14 expression via promoter hypermethylation, resulting in inactivation of the p14-mouse double minute 2 pathway and subsequent downregulation of p53 levels. As a result, HBx was able to impair the potential of ATRA to activate apoptosis-related molecules, including Bax, p53-upregulated modulator of apoptosis, caspase-9, caspase-3 and poly (ADP-ribose) polymerase. In conclusion, the present study provides a new oncogenic action mechanism of HBx, namely by suppressing the anticancer potential of ATRA to induce p53-dependent apoptosis in HBV-infected hepatocytes.

INTRODUCTION
Hepatitis B virus (HBV) is strongly associated with the development of hepatocellular carcinoma (HCC) [1, 2]. Although the precise molecular mechanism is not entirely understood, both in vivo and in vitro studies have accumulated considerable evidence that chronic inflammation and oxidative stress, liver regeneration and fibrosis, genetic instability and impaired DNA repair, as well as activities of viral proteins, contribute to HBV-related HCC development [2]. Among viral factors, HBV X protein (HBx) is known to play a pivotal role in both HBV replication and viral pathogenesis [1]. It is encoded by the smallest ORF of the HBV genome, which is the most frequently integrated viral sequence found in HCCs [3]. As a multifunctional regulatory protein, HBx can activate several transcription factors, including AP-1, ATF-2, CREB, Oct-1, Myc and TBP [2, 4, 5]. HBx also epigenetically silences several tumour suppressor genes, including p16, p21, E-cadherin and insulin-like growth factor binding protein-3 (IGFBP-3) [6–10]. HBx is also known to activate diverse cytoplasmic signalling pathways, which include Akt/PKB, JNK, MAPK, NF-κB, PI3K, Src and Wnt [11–17]. Moreover, HBx is able to induce HCC in transgenic mice [18]. Despite the accumulating evidence suggesting that HBx plays an important role during HBV-mediated hepatocarcinogenesis, the detailed mechanism of its action is still controversial.

All-trans retinoic acid (ATRA), the most biologically active metabolite of vitamin A, and its synthetic derivatives like acyclic retinoid (ACR) are being increasingly included in both chemopreventive and therapeutic regimens for managing various human cancers, including acute promyelocytic leukaemia (APL) and HCC [19, 20]. In general, ATRA exerts its anticancer effect via three mechanisms: inducing apoptosis, arresting the further growth of abnormal cells, and inducing the abnormal cells to dedifferentiate back to normal [19]. Among these, the ability of ATRA to induce apoptosis has attracted particular interest in both clinical and basic studies [21]. Activation of either intrinsic and/or extrinsic apoptosis pathways has been detected in this process. For example, ATRA upregulates the levels of pro-apoptotic caspase-9 and Bax in breast cancer cells [22] and downregulates the levels of anti-apoptotic Bcl-2 and...
survivin in myeloblastic leukemia cells [23]. ATRA also activates extrinsic apoptosis pathways through upregulation of tumor necrosis factor alpha, caspase-8 and the death receptor Fas [24, 25].

Earlier reports have demonstrated that HBx exerts its oncogenic effect, at least in part, by suppressing the anticancer potential of ATRA. For example, HBx suppresses the potential of ATRA to induce arrest of cell growth by downregulating the levels of retinoic acid receptor-β2 (RAR-β2), p16 and p21 [8, 26]. However, it is still unknown whether HBx suppresses ATRA-induced apoptosis in human hepatocytes.

Recently, we have demonstrated that ATRA upregulates p53 levels and thereby activates several apoptosis-related molecules, which include Bax, p53-upregulated modulator of apoptosis (PUMA), caspase-9, Bid, caspase-8, caspase-3 and poly (ADP-ribose) polymerase (PARP), inducing p53-dependent apoptosis in human hepatoma cell lines [27]. For this effect, ATRA downregulates the protein and enzyme activity levels of DNA methyltransferase 1 (DNMT1), 3a and 3b, thereby activating p14 expression via promoter hypomethylation [27]. In contrast, HBx is known to upregulate both the protein and enzyme activity levels of cellular DNMTs and silence tumour suppressor genes, including p16, p21, E-cadherin, IGFBP-3 and RAR-β2 via promoter hypermethylation [6–10, 26]. These observations prompted us to investigate whether HBx overcomes ATRA-induced apoptosis by antagonizing the potential of ATRA to activate p14 expression via promoter hypomethylation. In the present study, we first examined whether HBx overcomes ATRA-induced apoptosis in p53-positive human hepatic cell lines. Secondly, we investigated whether HBx represses p14 expression via promoter hypermethylation, thereby inactivating the p14-mouse double minute 2 (MDM2) pathway, resulting in p53 downregulation in the presence of ATRA. Thirdly, we investigated whether HBx prevents ATRA from activating the p53-dependent apoptotic pathway, thus neutralizing its ability to induce apoptosis in human hepatic cell lines. Finally, we attempted to confirm the ability of HBx to overcome ATRA-induced apoptosis in a 1.2-mer HBV replicon system that mimics the natural course of HBV infection.

RESULTS

HBx suppresses ATRA-induced apoptosis in human hepatocytes

We first examined whether HBx affects ATRA-induced apoptosis in a human hepatoma cell line, HepG2. According to data from the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, treatment with ATRA for 60 h decreased the viability of HepG2-vector cells in a dose-dependent manner, whereas the effect obtained at 5µM or higher concentrations of ATRA was significantly weaker in HepG2-HBx cells (Fig. 1a). ATRA increased caspase-3 and 7 activities (potential apoptotic markers) in HepG2-vector cells in a dose-dependent manner, whereas the effect was significantly lower than that in HepG2-HBx cells (Fig. 1b). The data from the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) assay clearly showed that the ability of ATRA to induce apoptosis in HepG2 cells was severely impaired in the presence of HBx (Fig. 1c, d). Similarly, HBx significantly lowered the potential of ATRA to induce apoptosis in another human hepatoma cell line, Huh-7 (Fig. 1e, f). From these various results, we concluded that HBx suppresses ATRA-induced apoptosis in human hepatocytes.

HBx attenuates the potential of ATRA to activate the p53-dependent apoptotic pathway in human hepatocytes

We next investigated the mechanism by which HBx suppresses ATRA-induced apoptosis in human hepatocytes. Consistent with a recent report [27], ATRA upregulated p53 levels, which led to the activation of apoptosis-related molecules, which include Bax, PUMA, caspase-9, caspase-3 and PARP in HepG2 and Huh-7 cells (Fig. 2a, b, compare lanes 1 and 2). HBx also upregulated p53 levels in HepG2 and Huh-7 cells in the absence of ATRA, resulting in activation of the p53-dependent apoptotic pathway (Fig. 2a, b, compare lanes 1 and 3), as demonstrated earlier [8]. However, HBx did not induce detectable apoptosis in these cells, as evidenced by data from the TUNEL assay (Fig. 1c, d). In spite of similar individual effects, ATRA and HBx in combination did not exhibit a synergistic or additive effect on the p53-dependent apoptotic pathway (Fig. 2a, b, lane 4). Instead, the ability of ATRA to activate the p53-dependent apoptotic pathway was severely impaired by the expression of HBx, suggesting that HBx attenuates the potential of ATRA to activate the p53-dependent apoptotic pathway.

To investigate whether ATRA and HBx required p53 for the regulation of apoptosis, we examined their effects in Hep3B cells, in which p53 was absent. Neither activation of the apoptotic pathway by ATRA, nor its suppression by HBx was observed in Hep3B cells (Fig. 2c, lanes 2 and 4). HBx alone did not affect the apoptotic pathway in these cells (Fig. 2c, lane 3). To confirm the p53-dependent regulation of apoptosis by ATRA and HBx, we examined their effects in Hep3B cells that express ectopic p53. Ectopic p53 slightly activated the apoptotic pathway in Hep3B cells (Fig. 2c, lane 6). ATRA upregulated ectopic p53 levels and thereby enhanced its effect on the apoptotic pathway (Fig. 2c, lane 7). The effects of ATRA almost completely disappeared in the presence of HBx (Fig. 2c, lane 8), indicating that HBx effectively prevents ATRA from activating the apoptotic pathway via the ectopic p53. In light of all these results, we concluded that HBx attenuates ATRA’s ability to activate the p53-dependent apoptotic pathway while it has intrinsic ability to activate the pathway in human hepatocytes.

HBx attenuates ATRA’s ability to block ubiquitin-dependent proteasomal degradation of p53

Next, we investigated how ATRA and HBx individually or in combination affect p53 levels in HepG2 cells. It is relatively well-established that p53 levels are negatively
regulated by an E3 ligase, MDM2, which induces ubiquitination and subsequent proteasomal degradation of p53 [28]. MDM2 levels are also regulated by another ubiquitin (Ub)-dependent proteasomal degradation pathway [28]. Therefore, we first investigated whether ATRA and HBx individually upregulate p53 levels by affecting its proteasomal degradation. Treatment with a proteasomal inhibitor, MG132, almost completely abolished the ability of ATRA and HBx to upregulate p53 levels in HepG2 cells (Fig. 3a, lanes 4 to 6). Treatment with MG132 also upregulated HBx levels (Fig. 3a, lane 6), as demonstrated earlier [29, 30]. The results of co-immunoprecipitation (co-IP) experiments showed that ATRA and HBx individually decreased the interaction between p53 and MDM2 (Fig. 3b, lanes 2 and 3). ATRA and HBx also increased the levels of the ubiquitinated MDM2, while they decreased those of p53 (Fig. 3c, lanes 2 and 3), without affecting the ubiquitination of most of the other proteins (Fig. 3c). The interaction between p53 and MDM2 and their ubiquitination were more dramatically affected by ATRA (Fig. 3b, c, lanes 2 and 3), which was
consistent with its higher ability to upregulate p53 levels in HepG2 cells (Fig. 3a, lanes 2 and 3).

The ability of ATRA to block the interaction between MDM2 and p53 was slightly diminished in the presence of HBx (Fig. 3b, lane 4). Consistently, HBx increased the levels of the ubiquitinated p53, but decreased those of MDM2 in the presence of ATRA (Fig. 3c, lane 4), resulting in p53 downregulation and MDM2 upregulation (Fig. 3c, lane 4). We therefore concluded that HBx attenuates the ability of ATRA to block Ub-dependent proteasomal degradation of p53 while it has the intrinsic ability to stabilize p53 by inhibiting the same pathway in the absence of ATRA.

**HBx inactivates the p14-MDM2 pathway to attenuate ATRA’s ability to upregulate p53 levels**

Next, we investigated the mechanism by which HBx attenuates ATRA’s ability to upregulate p53 levels. In general, the stability of p53 protein is altered in response to cell stress signals such as DNA damage and excess proliferation, which lead to activation of ataxia telangiectasia-mutated (ATM) protein and p14, respectively [31]. Therefore, we first investigated whether ATRA activates ATM protein to upregulate p53 levels and whether HBx inactivates ATM protein to reverse the effect of ATRA on p53. HBx induced phosphorylation of ATM protein at Ser-1981 residue without affecting the levels of total protein, both in the presence and absence of ATRA, which led to the phosphorylation of p53 at Ser-15 residue (Fig. 3d, lanes 3 and 4). The activated ATM protein also induced phosphorylation of checkpoint kinase 2 (CHK2) at Thr-68 residue, a specific marker for ATM protein activation, which led to phosphorylation of p53 at Ser-20 residue in HepG2-HBx cells. The ability of HBx to activate CHK2 via phosphorylation and upregulate p53 levels was almost completely abolished in the presence of

**Fig. 2.** HBx suppresses the potential of ATRA to activate the p53-dependent apoptotic pathway. HepG2 cells were transiently transfected for 24 h with either an empty vector or HBx expression plasmid and then either mock-treated or treated with 5 µM ATRA for an additional 48 h. Levels of p53, Bax, PUMA, HBx, γ-tubulin and active (cleaved) forms of caspase-9 (35 kDa), caspase-3 (20 kDa) and PARP (84 kDa) were measured by Western blotting. The protein bands of p53 and γ-tubulin were quantified using ImageJ image-analysis software (NIH, USA) to show the level of p53 relative to the loading control. The data graph shows the mean±SD from four independent experiments. (b) Huh-7 cells were prepared as in (a), followed by Western blotting. (c) Hep3B cells were prepared as in (a), followed by Western blotting. For lanes 5–8, the indicated amount of p53 expression plasmid was included in the transfection mixtures.
Fig. 3. HBx impairs ATRA’s ability to induce Ub-dependent proteasomal degradation of p53. (a) HepG2-vector and HepG2-HBx cells were either mock-treated or treated with 5 µM ATRA for 48 h. For lanes 5 and 6, cells were treated with 10 µM MG132 for 4 h before harvesting. Levels of p53 and γ-tubulin were measured by densitometry to show the level of p53 relative to the loading control (n=3). (b) HepG2-vector and HepG2-HBx cells were either mock-treated or treated with 5 µM ATRA for 48 h. Total p53 protein was immunoprecipitated with an anti-p53 antibody and then subjected to Western blotting using an anti-MDM2 antibody to measure MDM2 and IgG levels. The values indicate the level of MDM2 relative to the loading control (IgG). The input shows the total MDM2 levels in whole ccell extracts. (c) HepG2-vector and HepG2-HBx cells were transfected with pHA-Ub for 48 h in the presence or absence of 5 µM ATRA. For lane 5, the p14 expression plasmid was included in the transfection mixtures. Total MDM2 and p53 proteins were immunoprecipitated with either an anti-p53 antibody or an anti-MDM2 antibody and then subjected to Western blotting using an anti-haemagglutinin (HA) antibody, to detect Ub-complexed p53 and MDM2. Total ubiquitinated proteins in the cell extracts were detected by Western blotting using an anti-Ub antibody. The input shows the p53 and MDM2 levels in whole cell extracts. (d) Levels of the indicated proteins in HepG2-vector and HepG2-HBx cells prepared as in (b) were measured by Western blotting. For lanes 5 and 6, cells were treated with...
of an ATM inhibitor, KU-55933 (Fig. 3d, lane 5), indicating that HBx activates the ATM-CHK2 pathway to upregulate p53 levels in HepG2 cells. Unlike HBx, ATRA has little effect on the ATM-CHK2 pathway in both HepG2-vector and HepG2-HBx cells (Fig. 3d, lanes 2 and 4). In addition, ATRA could upregulate p53 levels in HepG2-HBx cells in the presence of KU-55933 (Fig. 3d, lane 6). Therefore, it was likely that neither the ATRA-induced p53 activation nor its suppression by HBx involves the ATM-CHK2 pathway.

According to a previous report [27], treatment with ATRA upregulates p14 levels in HepG2 cells, while downregulating those of MDM2, resulting in upregulation of p53. In addition, knock-down of p14 in HepG2 cells upregulates MDM2 levels and subsequently downregulates p53 levels, both in the presence and absence of ATRA [27], indicating that upregulation of p14 is critical for ATRA to stabilize p53 via activation of the p14-MDM2-p53 pathway. Therefore, we examined whether HBx inactivates p14 expression to reverse the effect of ATRA on p53. ATRA upregulated p14 and p53 levels in HepG2-vector cells, while downregulating MDM2 levels in these cells (Fig. 3e, lane 2), as previously reported [27]. In contrast, HBx downregulated p14 levels both in the presence and absence of ATRA (Fig. 3e, lanes 3 and 4). Despite p14 downregulation, lower levels of MDM2 and slightly higher p53 levels were detected in HepG2-HBx cells in the absence of ATRA (Fig. 3e, lane 3), compared to those in HepG2-vector cells (Fig. 3e, lane 1), probably due to activation of the ATM-CHK2 pathway by HBx (Fig. 3d, lane 3). However, higher MDM2 and lower p53 levels were detected in HepG2-HBx cells in the presence of ATRA (Fig. 3e, compare lanes 2 and 4), suggesting that HBx downregulates p53 levels in the presence of ATRA via downregulation of p14 levels.

To prove that HBx attenuates the potential of ATRA to upregulate p53 levels via downregulation of p14 levels, we ectopically complemented p14 in HepG2-HBx cells to the level in HepG2-vector cells (Fig. 3e, lane 5). As a result, the ubiquitination patterns of p53 and MDM2 in HepG2-HBx cells became similar to those in HepG2-vector cells in the presence of ATRA (Fig. 3c, lane 5), resulting in MDM2 downregulation and p53 upregulation in these cells (Fig. 3c, e, lane 5). These results suggested that ATRA activates p53 via upregulation of p14 levels, whereas HBx reverses it via downregulation of p14 levels.

**HBx attenuates ATRA’s ability to activate p14 expression via promoter hypomethylation**

Previous reports have demonstrated that ATRA activates p14 expression via promoter hypomethylation [27], whereas HBx inactivates p16 expression from the INK4a/ARF gene locus via promoter hypermethylation [8]. Consistent with these reports, ATRA lowered the protein levels of DNMT1, 3a and 3b (Fig. 4a, lane 2) and their enzyme activities (Fig. 4b, column 2), resulting in hypomethylation of p14 (Fig. 4c, lane 2) and upregulation of its protein levels in HepG2-vector cells (Fig. 4d, lane 2). In contrast, HBx elevated both protein (Fig. 4a, lane 3) and enzyme activity levels of DNMTs (Fig. 4b, column 3), resulting in p14 promoter hypermethylation (Fig. 4c, lane 3) and subsequent downregulation of its protein levels (Fig. 4d, lane 3). Moreover, a previous report has demonstrated that treatment with a universal DNMT inhibitor, 5-Aza-2’dexoycytidine (5-Aza-2’dC), does not alter the effect of ATRA on p14 expression in HepG2-vector cells [27]. In contrast, the present study clearly showed that 5-Aza-2’dC almost completely abolished the ability of HBx to downregulate p14 levels in HepG2-HBx cells (Fig. 4d, lane 5), indicating that ATRA and HBx oppositely regulate p14 expression via modulating a common mechanism, i.e. DNA methylation.

Next, we investigated whether ATRA and HBx antagonize each other in the regulation of p14 expression via DNA methylation. In the presence of HBx, ATRA lowered both protein (Fig. 4a, compare lanes 3 and 4) and enzyme activity levels (Fig. 4b, compare columns 3 and 4) of DNMTs, resulting in promoter hypomethylation of p14 (Fig. 4c, compare lanes 3 and 4) and upregulation of its protein levels (Fig. 4d, compare lanes 3 and 4). In contrast, in the presence of ATRA, HBx elevated both protein (Fig. 4a, compare lanes 2 and 4) and enzyme activity levels (Fig. 4b, compare columns 2 and 4) of DNMTs, resulting in promoter hypermethylation of p14 (Fig. 4c, compare lanes 2 and 4) and downregulation of its protein levels (Fig. 4d, compare lanes 2 and 4). In addition, treatment with 5-Aza-2’dC almost completely abolished the ability of HBx to downregulate p14 levels in the presence of ATRA (Fig. 4d, lane 6). Based on these results, we concluded that HBx attenuates the potential of ATRA to activate p14 expression via promoter hypomethylation.

**HBx attenuates the potential of ATRA to activate the p53-dependent apoptosis by inhibiting p14 expression via promoter hypermethylation**

According to a previous report [27], ectopic expression of DNMT1 almost completely abolished the potential of ATRA to induce p14 promoter hypomethylation, activate the p14-MDM2-p53 pathway and induce the p53-dependent apoptosis, indicating that activation of p14 expression via promoter hypomethylation is a primary event for the induction of apoptosis by ATRA in HepG2 cells. Therefore, we investigated whether HBx suppresses ATRA-induced apoptosis by downregulating p14 levels via promoter hypermethylation. The
The ability of HBx to suppress the potential of ATRA to activate the p53-dependent apoptotic pathway in HepG2 cells was severely impaired by either ectopic p14 expression (Fig. 3e, lane 5) or treatment with 5-Aza-2'dC (Fig. 4d, lane 6), which restored p14 levels in these cells. In addition, treatment with 5-Aza-2'dC almost completely rescued the ability of ATRA to increase caspase 3/7 activities (Fig. 4e, column 5) and its ability to induce apoptosis in HepG2-HBx cells (Fig. 4f, g), up to the levels observed in HepG2-vector cells. Based on these results, we concluded that HBx suppresses ATRA-induced apoptosis by inhibiting p14 expression via promoter hypermethylation.
HBx attenuates ATRA’s ability to induce p53-dependent apoptosis during HBV replication

Finally, we investigated whether HBx suppresses ATRA-induced apoptosis during HBV infection. For this purpose, we employed a 1.2-mer HBV replicon system, mimicking the natural course of HBV infection in human hepatic cell lines [32–34]. Transient transfection of the cells with a 1.2-mer HBV replicon, 1.2-mer WT, resulted in production of HBV proteins such as HBx and HBV surface antigen (HBs) and downregulation of p14 levels in HepG2 cells (Fig. 5a, lane 2), as demonstrated with ectopic HBx expression (Fig. 3e, lane 3). In contrast, transfection of the cells with a 1.2-mer HBx-null HBV replicon, 1.2-mer HBx-null, led to production of HBs but not HBx and did not affect p14 levels in HepG2 cells (Fig. 5a, lane 3). The ability of HBx expressed from 1.2-mer WT to downregulate p14 levels disappeared in the presence of 5-Aza-2’dC (Fig. 5a, lane 5), suggesting that HBx downregulates p14 levels via promoter hypermethylation during HBV replication.

Despite p14 downregulation, HBx derived from 1.2-mer WT upregulated p53 levels (Fig. 5a, lane 2), resulting in weak activation of the p53-dependent apoptotic pathway (Fig. 5b, lane 3). This was probably due to activation of the ATM-CHK2 pathway by HBx, as demonstrated by ectopic HBx expression (Fig. 3d, lane 3). This effect however could not induce detectable apoptosis in HepG2 cells (Fig. 5c, d), as demonstrated with ectopic HBx expression (Fig. 4f, g). In addition, HBx derived from 1.2-mer WT attenuated the potential of ATRA to activate the p53-dependent apoptotic pathway (Fig. 5b, lane 4) and induce apoptosis in HepG2

Fig. 5. HBx impairs the ability of ATRA to induce p53-dependent apoptosis during HBV replication. (a) HepG2 cells were transiently transfected for 48 h with an empty vector, a 1.2-mer WT HBV replicon or its HBx-null derivative and then subjected to Western blotting. For lanes 4–6, cells were treated with 5-Aza-2’dC for 24 h before harvesting. The graph shows the p14 levels relative to the loading control (n=4). (b) HepG2 cells were transfected with either an empty vector or a HBV replicon for 4 h and then either mock-treated or treated with ATRA for an additional 48 h, followed by Western blotting. The p14 and p53 levels relative to the loading control are shown. (c) HepG2 cells were transfected as in (b) and then either mock-treated or treated with ATRA for an additional 72 h, followed by the TUNEL assay. (d) The graph represents the percentage of TUNEL-positive cells shown in (c) (n=4).
cells (Fig. 5c, d). Neither the activation of p53 in the absence of ATRA nor the inhibition of the ATRA-induced apoptotic pathway was observed in cells transfected with 1.2-mer HBx-null (Fig. 5b, lanes 5 and 6). Accordingly, ATRA effectively induced apoptosis in these cells as in the control cells (Fig. 5c, d). These results indicated that HBx derived from the 1.2-mer HBV replicon is responsible for the effects against ATRA. Thus, it was possible to reproduce exactly the ability of HBx to suppress ATRA-induced apoptosis and understand the underlying mechanism in a replicon-based HBV replication system.

**DISCUSSION**

The effect of HBx on p53 remains controversial, despite several studies. Early studies have implicated HBx in the progression of HCC through its ability to block p53 from inhibiting cell cycle and inducing apoptosis [35, 36], as is the case with other viral oncoproteins of small DNA tumour viruses, including the adenovirus E1B 55 kDa protein [37], human papillomavirus E6 [38, 39] and simian virus 40 large T antigen [39]. For this effect, HBx binds to and partially sequesters p53 in the cytoplasm, inhibiting several p53 activities such as specific DNA binding and transcriptional transactivation. However, later studies have suggested a completely opposite role for HBx in the regulation of p53, wherein HBx upregulates p53 levels to activate its target genes such as p21, Siah-1 and several pro-apoptotic genes, including Bax, Fas and Nox, which ultimately result in growth inhibition and apoptotic death of the hepatocytes [40–42]. It is hard to reconcile the seemingly opposite effects of HBx on p53. However, several factors, including cell context [43], HBx variation [44], HBx levels and other experimental conditions may affect the interaction between HBx and p53. Indeed, the present study showed that HBx differentially regulates p53 levels depending on the presence or absence of ATRA (Fig. 2a, b). In the absence of ATRA, HBx upregulated p53 levels to activate Bax and PUMA expression in human hepatic cell lines. In the presence of ATRA, however, HBx downregulated p53 levels, resulting in the downregulation of Bax and PUMA levels. These effects were also reproduced exactly in a 1.2-mer HBV replicon system (Fig. 5b), mimicking the natural course of HBV infection [32–34]. Therefore, the present study may provide an experimental model for studies to understand the differential regulation of p53 by HBx in human hepatic cell lines.

Based on the present study and others, we propose a model for the antagonism between ATRA and HBx in the regulation of p53-dependent apoptosis in human hepatic cell lines (Fig. 6). According to this model, ATRA upregulates p53 levels by activating the p14-MDM2 pathway without affecting the ATM-CHK2 pathway (Fig. 3d, e), resulting in activation of the p53-dependent apoptosis in human hepatic cell lines (Fig. 4d–g), as previously demonstrated [27]. For this effect, ATRA downregulates both protein and enzyme activity levels of DNMTs and thereby activates p14 expression via promoter hypomethylation (Fig. 4a–d; [27, 45]). Inhibition of p14 expression via transfection of either p14 small hairpin RNA or DNMT1 expression plasmid almost completely abolishes the ability of ATRA to activate p53-dependent apoptosis [27], indicating that this effect is primarily responsible for the ability of ATRA to induce apoptosis. ATRA is also known to activate other tumour suppressor genes, including p16 and p21, via promoter hypomethylation, and exert its anticancer potential via inhibiting cell cycle progression [46]. It remains unknown how ATRA downregulates the levels of DNMTs to inhibit their enzyme activities. The potential of ATRA to downregulate protein levels of DNMTs appears to be mediated by RAR-β2, as demonstrated by experiments via both knockdown and complementation of RAR-β2 [45]. However, it is unknown whether ATRA in combination with RAR-β2 directly inhibits expression of DNMTs or executes its potential via modulation of other transcription factors such as E2F1 and AP1, which can activate DNMT1 expression [47, 48]. More extensive studies are thus required to clarify the epigenetic regulation of tumour suppressor genes by ATRA.

Unlike ATRA, HBx derived from both HBx overexpression and 1.2-mer HBV replicon systems inhibits p14 expression via promoter hypermethylation in HepG2 cells (Figs 4 and 5). For this effect, HBx upregulates both protein and enzyme activity levels of DNMTs (Fig. 4a, b; [7, 8, 26]). In addition, HBx is known to repress RAR-β2 expression via DNA methylation to directly counteract the effects of ATRA [26]. It has been shown that HBx activates the expression of DNMT1 via a regulatory circuit involving the p16-cyclin D1-CDK4/6-pRb-E2F1 pathway [49]. However, the mechanisms by which HBx upregulates DNMT3a and b levels remain unknown. Despite p14 downregulation, HBx upregulates the levels of p53 in the absence of ATRA through activation of the ATM-CHK2 pathway (Fig. 3d), a DNA damage signalling process. For this effect, HBx may alter the mitochondrial membrane potential to increase the production of reactive oxygen species [50]. HBx is also known to accumulate γ-H2AX, a marker of DNA double-strand breaks, and increase the levels of phosphorylated ATM protein [51] and CHK-2 [52], which was also observed in the present study (Fig. 3d). The ability of HBx to upregulate p53 levels was almost completely abolished in the presence of an ATM inhibitor, KU-55933 (Fig. 3d). Therefore, the negative regulation of the p14-MDM2 pathway by HBx appears to be masked when HBx activates the ATM-CHK2 pathway, resulting in upregulation of p53 levels in the absence of ATRA (Fig. 4d, e), but the effect does not appear to be strong enough to induce detectable apoptosis in human hepatic cell lines (Fig. 4d–g). In contrast, in the presence of ATRA, HBx derived from both HBx expression and 1.2-mer HBV replicon systems downregulates p53 levels in HepG2 cells (Figs 2a and 5b). For this effect, HBx suppresses the potential of ATRA to downregulate both protein and enzyme activity levels of DNMTs, thereby attenuating the ability of ATRA to activate p14 expression via promoter hypomethylation (Fig. 4a–d). Although HBx activates the ATM-CHK2 pathway in the presence of ATRA (Fig. 3d), this effect appears to be masked by the
inactivation of the p14-MDM2 pathway by HBx. Restoration of p14 levels by either ectopic expression or treatment with 5-Aza-2'dC almost completely abolished the effect of HBx on p53 levels in the presence of ATRA (Figs 3e and 4d), confirming the negative regulation of p53 levels by HBx in the presence of ATRA.

In conclusion, HBx and ATRA antagonize each other in the regulation of p53 levels and p53-dependent apoptosis. Under our experimental conditions, HBx appears to dominate ATRA in determining p53 levels through inactivation of the p14-MDM2 pathway. Considering the role of apoptosis in preventing the production of new virus particles and eliminating cells with abnormal phenotypes that may otherwise lead to the development of cancer, the ability of HBx to attenuate the potential of ATRA to induce apoptosis may play a critical role in both HBV replication and hepatocarcinogenesis. However, it is also possible to envision an opposite situation, wherein the anticancer potential of ATRA is dominant over the oncogenic ability of HBx. It was indeed possible to effectively induce apoptosis in HepG2-HBx cells as in the control cells by treatment with higher concentrations of ATRA (over 30 µM) (data not shown). The ability of ATRA to induce apoptosis in cells derived from various human cancers, including APL, hepatoma, breast cancer, lung cancer, and head and neck cancer has already been demonstrated [19, 21, 22, 53, 54]. Moreover, clinical studies have demonstrated that a synthetic retinoid, ACR, significantly reduces the incidence of post-therapeutic HCC recurrence and improved the survival rate of patients [55, 56]. Therefore, the antagonism between HBx and ATRA may provide a potential target for the development of chemopreventive and therapeutic drugs against HBV-associated HCC.

METHODS
Plasmids
The HBx expression plasmid, pCMV-3 ×HA1-HBX3 encoding the full-length HBx, downstream of the three copies of the influenza virus haemagglutinin (HA) epitope, was previously described [7]. The 1.2-mer WT replicon containing 1.2 units of the HBV genome and its HBx-null counterpart, 1.2-mer HBx-null, have also been described earlier [34]. The p14 expression plasmid, pEGFP-N1-p14, and the p53 expression plasmid, pCMV-p53-WT, were gifts from B. J. Park (Pusan National University, Korea) and C.W. Lee (Sungkyunkwan University, Korea), respectively. Plasmid pHA-Ub was kindly provided by Y. Xiong (University of North Carolina at Chapel Hill, USA).

Cell culture and transfection
HepG2 (KCLB No. 88065), Hep3B (KCLB No. 88064) and Huh-7 (KCLB No. 60104) cell lines were obtained from the Korean Cell Line Bank. For transient expression, 2 × 10^5 cells per 60 mm dish were transfected with 1 µg of appropriate
plasmid(s) using WelFect-EX PLUS (WelGENE) according to the manufacturer’s instructions. Stable cell lines, HepG2-vector and HepG2-HBx, were established by transfection with pCMV-3×HA1 and pCMV-3×HA1 HBx, respectively, followed by selection in the presence of 500 µg ml⁻¹ G418 (Gibco) [7]. Cells were treated with ATRA (Sigma), MG132 (Sigma), KU-55933 (Sigma) and 5-Aza-2′dC (Sigma) under the indicated conditions. For ATRA treatment, considering its low stability in cell cultures [57], cells were re-fed with new medium containing fresh ATRA at intervals of 24 h.

**Western blot analysis**

Cells were lysed in buffer (50 mM Tris-HCl, pH 7.5, 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 incubated with antibodies against HBs (Abcam), ATM protein, pSer-1981 ATM protein, p53, pSer-15 p53, pSer-20 p53 and pThr-68 CHK2, and p14 (Cell Signaling), Bax, caspase-3, caspase-9, Ub, PARP, PUMA, DNMT1, DNMT3a, DNMT3b and HA (Santa Cruz Biotechnology), HBx (Millipore) and γ-tubulin (Sigma). Primary antibodies were detected with the appropriate horseradish peroxidase (HRP) nti-goat IgG (H+L)-HRP (Bio-Rad) and anti-rabbit IgG (H ±L)-HRP (Bio-Rad). ECL kit (Amersham) was used to visualize protein bands via the ChemiDoc XRS imaging system (Bio-Rad).

**IP assay**

Cells plated in a 100 mm diameter plate at a density of 2×10⁶ cells were either mock-treated or treated with 5 µM ATRA for 60 h. IP assay was performed using Classic Magnetic IP/Co-IP assay kit (Pierce) according to the manufacturer’s specifications. Briefly, whole cell lysates (500 µg) were incubated overnight at 4 ºC with an appropriate IP antibody (2 µg). Pierce protein A/G magnetic beads (0.25 mg) were then added and incubated for an additional 1 h. The beads were then collected using a magnetic stand (Pierce) and the antigen/antibody complexes eluted from the beads were subjected to Western blotting.

**DNMT activity assay**

Cells plated in a 100 mm diameter plate at a density of 2×10⁶ cells were either mock-treated or treated with 5 µM ATRA for 60 h. DNMT activity in the cell lysates was measured using EpiQuick DNMT Activity/Inhibition Assay Ultra Kit (Epigentek), according to the manufacturer’s instructions.

**Methylation-specific PCR (MSP)**

Genomic DNA (1 µg) denatured in 50 µl of 0.2 N NaOH was modified by treatment with 30 µl of 10 mM hydroquinone (Sigma) and 520 µl of 3 M sodium bisulfite (pH 5.0; Sigma) at 50 ºC for 16 h. For MSP, the modified DNA (100 ng) was amplified with Taq polymerase using both methylated and unmethylated primer pairs of p14 under the conditions described before [58].

**Cell viability analysis**

For the determination of cell viability, MTT assay was performed as described before [26]. Briefly, 5×10⁵ cells per well in 96-well plates were treated with an increasing concentration of ATRA (Sigma) for 48 h. The cells were then treated with 10 µM MTT (Sigma) for 4 h at 37 ºC. The formazan compounds derived from MTT by mitochondrial dehydrogenases of living cells were dissolved in DMSO and quantified by measuring the absorbance at 550 nm.

**Caspase-3/7 activity assay**

Caspase-3 and 7 activities were measured using the Caspase-Glo 3/7 Assay (Promega). Briefly, 3×10⁵ cells seeded in 96-well plates, were treated with ATRA as above. After adding an equal volume of Caspase-Glo-3 reagent (Promega) to each well, the mixtures were incubated for an additional 1 h at 20 ºC, followed by measurement of the luminescence in LuBi Microplate Luminometer (Gobiz) as directed by the manufacturer.

**TUNEL and DAPI staining**

Apoptotic cells were detected by TUNEL assay using the DeadEnd Fluorometric TUNEL system (Promega), according to the manufacturer’s instructions. Cells grown on coverslips were fixed in 4% formaldehyde (Sigma) for 25 min at 4 ºC and then permeabilized with 0.2% Triton X-100 for 5 min. Cells were incubated with TdT reaction mix for 1 h at 37 ºC and then with 2×SSC for 15 min to stop the reaction. Slides were prepared by adding UltraCruz mounting medium (Santa Cruz Biotechnology) containing 4', 6-diamidino-2-phenylindole (DAPI) and then visualized with an Eclipse fluorescence microscope (Nikon). Over 200 cells per sample were counted for statistical evaluations.

**Statistical analysis**

Each experiment was repeated at least three times. The values represent means±S.D. The difference between the means of the treatment group and the control was assessed with the paired two-tailed t-test; the difference was considered to be significant if P<0.05 (*) or P<0.01 (**), while it was considered to be statistically non-significant (NS) if P>0.05.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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